scribed in previous studies was followed (Willms et al., 2004; Fernandez Presas et al., 2005; Willms et al., 2005; Willms and Robert, 2007). Briefly, 10 cysticerci were selected and inserted into the oral cavity in 1 ml of PBS with a needleless plastic syringe. Hamsters were immunosuppressed by injection of 20 mg/kg of methyl prednisolone acetate (Upjohn, Mexico City, Mexico) on the day of oral infection and treatment repeated every 2 wk until hamsters were killed 3 to 6 wk PI by i.p. injection of 440 mg/kg of sodium pentobarbital (Sedalphore). Adult *T. crassiceps* were recovered from the small intestine as previously described (Willms et al., 2004). Infectivity of hamsters was calculated by dividing the total number of strobilae recovered from each by the total number of larvae fed from a single mouse. The number of hamsters/lot varied according to availability. Animals were kept under controlled conditions and fed Purina Chow (Purina, Mexico City, Mexico) and water ad libitum.

**Taenia crassiceps** tapeworm grown in an experimentally infected dog

Two mongrel dogs donated by a local public health agency (Centro Antirabic”Dr. A. Angellini de la Garza.” Health Ministry, Mexico City (Mexico) in February 2004 were pretreated for 5 days with 30 mg/kg of albendazole (Vermisen, Novag, Mexico City, Mexico). Dogs were fed 8 days later with approximately 900 cysticerci recovered from 11th-generation WFU infrapopulations wrapped in strands of raw beef. Dogs were kept in a separate kennel and fed Purina Chow and water ad libitum. Starting on day 21 PI, dog feces were examined daily for gravid proglottids and eggs by the method of Faust et al. (1938). Released gravid proglottids were briefly rehydrated in cold PBS and examined via stereoscopy to confirm the presence of uterine branches. The presence of taeniid eggs released from gravid proglottids was confirmed by light microscopy.

At the end of the experiment, dogs were treated with a broad-spectrum anthelmintic by oral administration of 1 tablet/10 kg containing 150 mg of pyrantel pamoate, 50 mg of praziquantel, and 150 mg of sennbenzozol (Vermiplex Plus, Holland, Morelos, Mexico) and given up for adoption 30 days after 3 negative coproparasitoscopic examinations.

**Mouse infections with eggs from dog-derived tapeworm**

Six-week-old Balb/c female mice were inoculated either with fresh minced whole gravid proglottids from dog-derived tapeworms, administered orally in PBS with a pipette, or with a suspension of ≥350 eggs obtained by compressing proglottids in PBS and injecting them i.p. in 0.3 ml of PBS. Mice were maintained for 4–7 mo PI, killed, and subjected to a complete necropsy. Cysticerci recovered were examined via stereoscopy, and the number and location recorded.

Cysticerci from dog-derived ova were used to subinoculate naive mice with 10 i.p. parasites and were designated as dog-derived cysticerci (DDC). Mice were killed 2.5, 4.0, 5.0, 9.0, and 12.0 mo PI; cysticerci were counted and assayed for in vitro evagination properties and hamster infections.

To examine growth kinetics, fourth-generation DDC were examined at 2-wk intervals from the second week to the 20th week PI. Total cysticerci were recorded individually in 20 mice.

**Hamster infections with DDC**

Hamsters were inoculated with 8–10 cysticerci and killed 4 wk PI. Intestinal contents were examined and percentage of infections calculated by dividing the number of tapeworms by the number of cysticerci fed.

**In vitro evagination assays**

*Taenia crassiceps* cysticerci obtained from the peritoneal cavity of mice were rinsed in sterile PBS with 50 μg/ml gentamycin (Genkova, Laboratorios Quimica SON’S, Mexico City, Mexico). Thirty to 50 cysticerci were used for in vitro evagination at 37 C in 10 ml of RPMI 1640 (Sigma-Aldrich, St. Louis, Missouri) containing 10% fetal bovine serum (Gibco BRL, Life Technologies, Paisley, U.K.), 10 μg/ml trypsin (Gibco BRL), and 50 μg/ml gentamycin in an atmosphere of 95% air and 5% CO2. The number of free scolices exhibiting searching movements on an inverted compound microscope was recorded at 24 hr.

**Statistical analysis**

Two-sided Chi-square analysis was used to compare in vitro evagination assays and hamster infections using Graphpad Instat v.3.05 (Graphpad Software, San Diego, California). Significance was established at *P* < 0.05 and 95% confidence intervals.

**RESULTS**

**Mice inoculated with WFU and DDC cysticerci**

Subinoculations were carried out every 50–60 days. Infections were positive in all inoculated mice. The number of cysticerci recovered was variable, but increased over time, so that subpopulations in 9- and 12-mo-old mice were usually ±1,000/mouse.

Cysticerci from fourth-generation DDC inoculations recovered from 20 mice between 2 and 20 wk PI exhibited a very rapid growth pattern. Individual counts of total cysticerci from each mouse are shown in **Figure 1.** All cysticerci selected for subinoculations of WFU and DDC in mice and hamster inoculations exhibited normal scolices with a rostellum, hooks, and 4 suckers.

**Hamster infections with WFU and DDC cysticerci**

WFU cysticerci isolated from 10 consecutive mouse generations were used to infect 16 hamster lots comprising a total of 188 hamsters. Between December 1999 and June 2003, hamster infectivity expressed as the number of adult worms recovered from the small intestine of hamsters divided by the total number of cysticerci inoculated had declined from an initial 33% to almost 0% (Fig. 2).

Additionally, it was found that by plotting the age of WFU infrapopulations used for infection against the number of recovered strobilae in hamsters, the infrapopulations over 100 days PI were more infective for hamsters than those under 100 days PI (Fig. 3).

The number of individual tapeworms recovered from hamsters was highly variable in all experiments, so that results were plotted as the average percentage of infections in all experiments; a representative histogram of the individual number of
Figures 2, 3, and 4 are not included in the text as they are not mentioned in the document.

Infection of dogs with WFU cysticerci

The experimental infection of 2 dogs with WFU cysticerci from an eleventh-generation mouse infection resulted in 1 dog with a gravid tapeworm. The first gravid proglottid was passed in dog feces at day 37 PI; another 19 gravid proglottids were passed between days 38 and 70 PI. No gravid proglottids were found in the stool between days 71 and 97 PI. One strobila without a scolex or gravid proglottid was released on day 63 PI; this appeared to confirm the presence of a second tapeworm in the same dog, as the last gravid proglottid was released 7 days later on day 70 PI. Copro-parasitoscopic analysis was carried out on days 108, 109, and 110 PI after administration of a broad-spectrum antihelminthic and were negative in both dogs.

Table I. T. crassiceps cysticerci (DDC) recovered from Balb/c mice inoculated with gravid proglottids or ova from dog tapeworm.

<table>
<thead>
<tr>
<th>Mouse Inoculum</th>
<th>Months</th>
<th>PI</th>
<th>DDC</th>
<th>Tissue/cavity</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 egg i.p.</td>
<td>4</td>
<td>212</td>
<td>Pc</td>
<td></td>
</tr>
<tr>
<td>1 gp* oral</td>
<td>5</td>
<td>122</td>
<td>(100) Th; (22) Sc</td>
<td></td>
</tr>
<tr>
<td>2 gp oral</td>
<td>6</td>
<td>1</td>
<td>Sc</td>
<td></td>
</tr>
<tr>
<td>2 gp oral</td>
<td>6</td>
<td>135</td>
<td>Th</td>
<td></td>
</tr>
<tr>
<td>2 gp oral</td>
<td>7</td>
<td>1,099</td>
<td>(340) Pc; (499) Sc; (290) Th</td>
<td></td>
</tr>
<tr>
<td>300 eggs i.p.</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7-13</td>
<td>2 gp oral</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* gp, gravid proglottid; Pc, peritoneal cavity; Sc, subcutaneous; Th, intrathoracic.

Mouse infections with dog-derived tapeworm eggs

Eggs in gravid proglottids from the dog tapeworm were infective for 5 of 13 female Balb/c mice. The numbers and location of cysticerci recovered in necropsies are shown in Table I. Tapeworm eggs possessed the typical striated embryophore and 6 pairs of hooks in the oncosphere (Fig. 5). Failure to infect the remaining 8 mice was attributed to the loss of eggs in feces or to handling of gravid dog proglottids in vitro, which promoted the release of eggs into PBS.
worm in 30 hamsters (0.3%) with 12-mo-old infrapopulations (Figs. 6, 7).

Evagination values of 5-mo-old second-generation DDC infrapopulations were significantly higher (72%) ($P < 0.0001$) when compared with 15.2% evagination in 9-mo-old DDC infrapopulations (Fig. 6). Hamster infections with these infrapopulations produced 50 strobilae in 12 hamsters (41.7%) from 5-mo-old DDC and only 5 strobilae in 11 hamsters (4.6%) from 9-mo-old DDC (Figs. 6, 7).

The average evagination in fourth-generation DDC infrapopulations between 2 and 20 wk PI was 88%. Cysticerci from 2.5-mo-old infrapopulations produced 10 strobilae in 3 hamsters inoculated with 24 cysticerci (41.6%) and 8 strobilae in 8 hamsters inoculated with 64 cysticerci from 4-mo-old infrapopulations (12%) (Fig. 7).

Hamsters ($n = 16$) inoculated with first-generation DDC did not produce any adult $T. crassiceps$. Significant reactivation of hamster infectivity was found only with second- and fourth-generation DDC, which produced average infections in hamsters of almost 42% ($P < 0.0001$) and 41%, respectively (Fig. 7), closer to an infectivity of 33% observed in hamsters inoculated with third-generation WFU cysticerci (Figs. 2, 7).

**DISCUSSION**

The present study describes the loss of infective ability for experimental hamsters of $T. crassiceps$ WFU cysticerci and the recovery of infectivity by cysticerci after completing adult development in an experimental dog. The experiments report the gradual loss of infectivity after 10 generations of WFU infrapopulations were maintained in Balb/c mice by serial subinoculation for a period of 4 yr. The loss of cysticercus infectivity for hamsters was as marked as the loss of infectivity observed with increased budding periods of cysticerci in the individual mouse peritoneal cavity. Infrapopulations with budding periods of less than 100 days produced a significantly higher number of infections in hamsters than did infrapopulations with budding periods of over 100 days. It is noteworthy that in the present experiments, infectivity in hamsters was highly variable and the overall infectivity did not exceed 50%, although individual hamsters did exhibit occasional infectivity of 0 and 90%. Similar results have been reported by Sato and Kamiya (1989) in golden hamsters and in dogs infected with *Echinococcus granulosus* (Constantine et al., 1998), indicating that the definitive host has an important role in the variability.

It can be speculated that long-term asexual proliferation of the WFU strain in the rodent suppresses or shuts off the genes responsible for attaching to the intestinal wall of another rodent such as the hamster. In the experiments described here, gene expression could possibly be restored after cysticerci are released from the mouse peritoneum where the gene silencing factor is functional, or that contact with a canid definitive host may somehow reactivate the genes responsible for infectivity.

**Gene silencing or shutoff** (Kalinna and Brindley, 2007) is a recently developed experimental procedure in which double-stranded RNA (dsRNA) molecules have been used experimentally to turn off specific parasite genes. Examples include the
suppression of cathepsin B expression in the parasitic trematode Schistosoma mansoni (Skelly et al., 2003) and dsRNA interference for the experimental gene abrogation of myosin, which has been elegantly shown in the planarian platyhelminth Schmidtea mediterranea (Sanchez Alvarado and Newmark, 1999; Sanchez Alvarado et al., 2003). The fact that these procedures are experimental does not exclude the possibility of their functioning in vivo.

Freeman (1962) reported the failure to infect a dog with 90 cysticerci obtained from the ORF strain maintained for 20 generations, as well as the failure of further attempts to infect a red fox and 2 other dogs. Mount (1968) described significant scolex and rostellar anomalies in cysticerci of the ORF strain. Further studies of the ORF strain also confirmed that the abnormalities were the result of aposporous and that the strain lacked a homologous pair of chromosomes when compared with the normal KBS strain (Smith et al., 1972). Such changes can only be attributed to a mutation that was perpetuated by the artificial selection of the mutant strain now maintained indefinitely and widely used by continued transfer to the peritoneal cavity of susceptible mice strains. In the studies reported here, mutations as a cause for loss of infectivity in cysticerci are unlikely since the loss of infectivity is temporary; the cysticerci of the WFU and DDC strains exhibit no visible structural abnormalities. Moreover, WFU cysticerci produced a dog tapeworm that released 20 gravid proglottids from which eggs were obtained to infect naïve mice.

Since first-generation DDC cysticerci were noninfective for hamsters, it can be assumed that an adaptation period in the mouse peritoneum is required for the cysticerci to become infective, because hamster infections were only attained starting with the second generation of DDC.

Taenia crassiceps strains have been used extensively to examine immune responsiveness in mice and their cysticerci are known to modulate the mouse immune system (Terrazas et al., 1998; Spolski et al., 2000; Toenjes and Kuhn, 2003). In long-lasting infections, cysticerci promote a Th2 response, but do not lose their viability or the ability to multiply asexually (Spolski et al., 2000) or to reproduce very rapidly (Everhart et al., 2004). The present results illustrate that the DDC derived from a WFU infrapopulation would also grow very rapidly, so that loss of infectivity due to selective immune-mediated damage can be disregarded.

The ORF strain has also been found to induce feminization of male Balb/cAnN mice (Morales et al., 1996), providing evidence that infections with the ORF strain have important immune–endocrinological effects in that particular mouse strain. Recent experimental evidence has also shown that WFU-strain cysticerci metabolize steroid precursors in vitro, further evidence that this strain has biological attributes that may modulate the host response (Gomez et al., 2000; Jimenez et al., 2006).

A good evagination process is indispensable for the taenids to attach to the intestinal wall (Esch and Smyth, 1976; Cañedo et al., 1982) and the loss of this ability in vitro may be linked to the loss of cysticercus infectivity. Poor evagination and low infectivity in hamsters was particularly evident in 11th WFU and second DDC generation infrapopulations grown for over 9 mo in the peritoneal cavity of mice. Younger infrapopulations of DDC cysticerci exhibited significantly more evagination and greater infectivity in hamsters. We conclude that good evagination properties are somehow linked to fitness for infecting an experimental definitive host rodent, but only after long asexual reproduction periods in the mouse or as a result of several years of subinoculations.

ACKNOWLEDGMENTS

We thank Prof. Aline Aluja and Vet. Alicia Olivera A. of the Facultad de Medicina Veterinaria y Zootecnia, UNAM, for their help in providing the kennels and maintenance of dogs. We thank M.Sc. Antonio Ramírez B. for help in statistical analysis. This work was supported by DGAPA-PAPIIT Grant IN238602 and funds from the Facultad de Medicina, UNAM.

LITERATURE CITED


THE ROLE OF PHYLOGENY AND ECOLOGY IN EXPERIMENTAL HOST SPECIFICITY: INSIGHTS FROM A EUGREGARINE–HOST SYSTEM

J. Detwiler and J. Janovy, Jr.*
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. e-mail: jdetwile@purdue.edu

ABSTRACT: The degree to which parasites use hosts is fundamental to host–parasite coevolution studies, yet difficult to assess and interpret in an evolutionary manner. Previous assessments of parasitism in eugregarine–host systems suggest high degrees of host specificity to particular host stages and host species; however, rarely have the evolutionary constraints on host specificity been studied experimentally. A series of experimental infections were conducted to determine the extent of host stadium specificity (larval vs. adult stage) and host specificity among 6 tenebrionid host species and 5 eugregarine parasite species. Eugregarines from all host species infected both the larva and adult stages of the host, and each parasite taxa colonized several host species (Tribolium spp. and Palorus subdepressus). Parasite infections and host phylogeny are not a significant predictor of host–parasite interactions, suggesting that host specificity in this host–parasite system. Host specificity will be impossible in the absence of geographic or ecological constraints. However, the 2 host stages produced different numbers of parasite propagules, indicating that ecological factors may be important determinants of host specificity in this host–parasite system. While field infections reflect natural infection patterns of parasites, experimental infections can demonstrate potential host–parasite interactions, which aids in identifying factors that may be significant in shaping future host–parasite interactions.

All parasites exhibit some degree of specificity to their hosts during their life cycles (Rohde, 2002; Caira et al., 2003). For some parasite taxa, the extent of host specificity is even considered a taxonomic character (Levine, 1979). Despite being such a widely recognized and important aspect of parasitism, little is known about what determines a parasite’s host range (Perlman and Jaenike, 2003). The range of host species infected may be due to phylogenetic, ecological, physiological, and immunological factors. In turn, these factors influence current host range, as well as the potential host range of parasites.

A growing body of coevolutionary work has addressed host specificity from a phylogenetic perspective and has emphasized the use of both host and parasite phylogenies (Adamson and Caira, 1994; Caira et al., 2003; Poulin and Mouillot, 2003). This approach is important because certain parasite characteristics, such as host range, may be products of common ancestry, which only phylogenetic methodology can detect (Adamson and Caira, 1994). Once developed, the phylogenetic hypotheses place other potentially important factors affecting host specificity, such as adaptation in response to host ecology, into an evolutionary context that can suggest other potentially important influences on the evolution of host specificity.

Determining the causal mechanisms underlying host specificity will be impossible in the absence of information about host specificity (Brooks, 2003). In most cases, host specificity is inferred from previously published reports and surveys across a wide array of host and parasite taxa (Poulin, 1992; Poulin, 1997; Caira et al., 2003). However, data collected in this manner may not reflect host specificity appropriately (Brooks, 2003; Collins and Janovy, 2003). Alternatively, experimental approaches offer an opportunity to systematically study host specificity in the absence of geographic or ecological constraints.

Characteristics amenable to experimental host specificity research can be evaluated by the eugregarine–tenebrionid beetle model system. Eugregarines are apicomplexan parasites (Phylum Apicomplexa, Class Conoidasida, Order Eugregarinorida) of many invertebrate species and, like most apicomplexans, are often assumed to be very host specific (Perkins et al., 2000). However, with few exceptions, host specificity of most gregarines and other apicomplexans is unknown (Levine, 1988).

The present study defines host specificity as the number of host species a parasite can colonize and recognizes that specificity involves consideration of a parasite’s ability to establish, develop, and reproduce inside or on the body of the host. Using 6 tenebrionid host species (Tribolium spp. and Palorus subdepressus) and 5 eugregarine parasite species (Gregarina spp. and Avrygregarina billmani), 3 main objectives were addressed: (1) establish the extent of host stadium specificity (same species, but different host life cycle stage) with homologous cross infections among the gregarines and the tenebrionid species; (2) experimentally evaluate the extent of host specificity among these species of gregarines to determine whether they follow presumptions of strict host specificity suggested by the literature; and (3) determine whether phylogenetic constraints play a role in the observed host specificity in the eugregarine–tenebrionid model system.

METHODS AND MATERIALS

Host–parasite system


Flour beetles were acquired from research stocks and maintained in plastic jars (100 ml) at the University of Nebraska–Lincoln (see Janovy et al., 2007 for colony origins). The medium in each jar consisted of a 98%:1:1 mixture of whole wheat flour and wheat bran, 1% bakers’ yeast, and 1% commercial wheat germ. The stock colonies were kept in a moist incubator at 28 C.

Experimental design

Uninfected adult hosts were obtained by removing pupae from labor­atory colonies. Each pupa was washed with distilled water in a strain­er and gently blotted dry to remove any infective oocysts that may have been on the surface of the pupae. Individual pupae were then placed into sterilized glass vials (1 ml) containing 1 g of sterile medium (1:1 whole wheat flour and wheat bran). Vials were loosely capped to allow
air flow, secured within wooden holders (drilled boards), and stored in covered plastic storage boxes at room temperature. This method decreased the probability of oocyst contamination from the air.

Each experiment consisted of an experimental group ($T_{exp}$), 2 control groups ($T_0$ and $T_c$), and a positive control group. Prior to the start of the experiment, beetles from a time zero control group ($T_0$) were dissected to ensure individuals were uninfected. A second group of individuals from the same source as the $T_0$ control were placed in new sterilized vials with an uncontaminated piece of apple or potato. This group was then separated into 3 subgroups, i.e., a $T_c$ control group that was maintained throughout the experiment to detect any accidental infection, a positive control group (homologous infection) that was exposed to the parasite to ensure oocyst viability, and an experimental group (heterologous infection). Both the homologous and heterologous infections were obtained using 2 methods.

Infection methods

Infection method 1 (Using sporulated gametocysts): Gametocysts were collected from host larvae by isolating 20 individuals in the moat of a plastic container well 60 × 15 mm Falcon style 3010 organ tissue culture dish, hereafter called a well dish. Each well dish contained a crushed bran flake in the moat and a small section of wet paper towel in the center well. After 24 hr, well dishes were examined for presence of gametocysts within the feces from the larvae. With a moist single-haired paintbrush, gametocysts were removed from the frass and placed onto a moistened black construction paper disc (black dot) produced by a paper punch. One black dot was placed in the center well of each well dish, each outside moat was filled with −1 ml of water, and the well dishes were covered and set aside for 72 hr, after which the well dishes were observed for sporulated gametocysts. If oocyst chains were present, water was removed from the moat and the well dish was stored at room temperature.

The infective material for an experimental group ($T_{exp}$) was prepared by gently adding oocysts from 2–4 sporulated gametocysts to a 3 × 3 mm piece of apple or potato. The number of oocysts per gametocyst was unknown for this study, although gametocysts from a related species Gregarina niphandrodes contained approximately 3,500 oocysts (Schwank, 2004). Pieces of contaminated potato or apple were placed into a sterilized glass vial (1 ml) with 1 uninfected adult beetle.

Infection method 2 (Direct exposure to infected larvae): Inoculated larvae were directly removed from a stock culture, rinsed with distilled water to remove external oocysts, and placed in sterilized plastic containers (28 mm × 29 mm) with adults from the experimental and $T_c$ control groups. Control larvae were dissected from these stocks to ensure that the larvae placed in the plastic containers were infected. Similar experimental methodology was followed as in Infection method 1, except that control and experimental groups were maintained in plastic containers of a larger volume as opposed to the smaller glass vials.

For both infection methods, $T_c$ controls and $T_{exp}$ groups were stored in covered plastic storage boxes for 8 days at room temperature and then dissected. The adult head was removed and the intact gut was gently pulled from the body. The gut was placed into Tenebrio molitor muscle saline (Belton and Grundfest, 1962), teased apart, and examined using a ×100 compound microscope at a total magnification of 100×. If gregarines were observed, the slide was videotaped using a Nikon Alphaphot-2 compound microscope using 10× and 40× objectives, a MicroImagi Video Systems YC/NTSC 470-line horizontal resolution camera, and a Panasonic S-VHS recorder.

Infection patterns and host phylogeny

Results from the experimental infections were mapped onto a previously published host phylogeny (Meštrović et al., 2006) to understand the phylogenetic context of host stadium specificity and host specificity for this particular gregarine-tenebrionid model system.

Gametocyst shedding between adult and larval host stages

The colonizing dynamics of gregarines were contrasted amongst adults and larvae by comparing the number of gametocysts shed and mean abundance of parasite infection of larval and adult hosts. Twenty adults were placed into the moat of a well dish, and 20 larvae were placed into the moat of a well dish. Both adult and larval individuals were taken from stock colonies. In each of the well dishes, 1 bran flake was placed in the moat and a wet section of paper towel was placed in the center well. After 24 hr, each well dish was checked for presence of gametocysts in the host feces. The number of gametocysts from each well dish was recorded. After all gametocysts were removed, individuals from each well dish from both adult and larva groups were dissected following the methodology listed above.

Statistical analysis

Data from experimental infections (including both infection methods) were nonnormally distributed; however, homogeneity of variance was similar amongst groups (Sokal and Rohlf, 1981). Therefore, a Kruskal-Wallis test was performed to evaluate any potential differences between the $T_0$, $T_c$, and $T_{exp}$ groups. The Mann-Whitney U-test was used to determine whether there were differences among the adult and larval infections. All statistical analyses were executed using Statview 5.0.1 and were deemed significant at $P < 0.05$.

RESULTS

Forty-eight infection assays were performed. Twenty of 22 homologous experimental infections and 22 of 26 heterologous experimental infections were considered valid because the $T_0$ and $T_c$ controls were uninfected or the mean abundance of infection was extremely low. In addition, heterologous infections were only considered valid if the first condition was met, and a simultaneously run homologous infection (positive control) was infected.

There were no significant differences in parasite mean abundance between the infection methods. For example, the homologous infection G. palori:P. subdepressus did not differ between the first or second infection method (Mann-Whitney U-test: $z = -0.577$, $P = 0.5677$). Comparisons among the 3 groups in both homologous and heterologous infections are summarized in Table I. Of the total homologous experiments performed, statistically significant differences among the 3 groups occurred in at least 1 of the homologous infections for all parasite species ($P < 0.05$). Heterologous infection success varied, with statistically significant differences among the 3 groups occurring in assays where G. cloptoni infected Tr. confusum and Tr. castaneum, and G. palori infected Tr. confusum. Several heterologous experiments could not be statistically analyzed because mean abundance was equal to zero in all 3 groups. Notably, Tr. madens was not naturally infected by a parasite and was also not a suitable host for the parasite species in this study.

Extent of experimental host stadium specificity and host specificity

All homologous experimental infections resulted in gregarine infections of the target hosts (Table II). Therefore, no eugregarines were stastically specific to any host species in this study. Heterologous infections showed that some gregarine species could infect a range of host species (Table II). For example, Gregarina minuta infected 2 hosts, i.e., Tr. confusum and Tr. castaneum, whereas Awrygregarina billmani infected 4 hosts, namely, Tr. confusum, Tr. castaneum, P. subdepressus, and Tr. brevicornis.

Host phylogeny and resulting infection patterns

The host phylogeny used in this study was that of Meštrović et al. (2006) and was derived from combined segments of COI and 16S rDNA. The gregarine species parasitizing the ingroup...
(Tribolium spp.) were not host stadium specific; in all cases, parasites were from larvae infected adults of the same host species. In addition, we found that parasites could infect phylogenetically distinct host groups (Fig. 1). Gregarina minuta could infect Tr. confusum and Tr. castaneum, hosts that are members of 2 distinct species-groups and 2 separate clades. However, this species could not infect members of the brevicornis group (Tr. brevicornis) or the remaining member of the castaneum group (Tr. madens). Further comparisons between host phylogeny and host specificity demonstrated no clear patterns, which clearly indicated that the eugregarine parasites were infective to host species other than their natural ones (sources of parasites) (Fig. 1).

Gametocyst shedding between adult and larval host stages

After 24 hr, adults shed significantly fewer gametocysts than larvae (Mann–Whitney U-test, Tr. freemani, $z = -2.702, P = 0.0069$; P. subdepressus, $z = -2.340, P = 0.0012$; Tr. confusum, $z = -2.611, P = 0.0090$) (Table IIIa). In addition, Tr. castaneum adults tended to shed fewer gametocysts than larvae (Mann–Whitney U-test; $z = -1.928, P = 0.0539$) during this same time period.

Following the 24-hr shedding period, gregarines from Tr. freemani, P. subdepressus, and Tr. confusum larvae had a higher mean abundance than did their corresponding adults (Mann–

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Host</th>
<th>Hm/Ht</th>
<th>Mean abundance</th>
<th>Kruskal-Wallis</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. minuta and G. confusa</td>
<td>Tr. confusum</td>
<td>Hm</td>
<td>0.67</td>
<td>0.14</td>
</tr>
<tr>
<td>G. minuta and G. confusa</td>
<td>Tr. brevicornis</td>
<td>Hm</td>
<td>0.625</td>
<td>0.125</td>
</tr>
<tr>
<td>G. minuta</td>
<td>Tr. castaneum</td>
<td>Hm</td>
<td>0</td>
<td>0.64</td>
</tr>
<tr>
<td>G. minuta</td>
<td>Tr. brevicornis</td>
<td>Hm</td>
<td>0.9</td>
<td>6.6</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. brevicornis</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. madens</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. confusum</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. castaneum</td>
<td>Hm</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. billmani</td>
<td>Tr. palori</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. cloptoni</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>G. cloptoni</td>
<td>Tr. confusum</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. cloptoni</td>
<td>Tr. castaneum</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. cloptoni</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. cloptoni</td>
<td>Tr. madens</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. cloptoni</td>
<td>P. subdepressus</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. confusum</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. castaneum</td>
<td>Hm</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. madens</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>P. subdepressus</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. brevicornis</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. palori</td>
<td>Tr. freemani</td>
<td>Hm</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Indicates that the 3 groups are significantly different from each other ($P < 0.05$). A dash indicates that no statistical test was possible because of zeros in the dataset.
TABLE II. Results of experimental infections. “Yes” indicates that the cross resulted in the presence of trophonts and/or gamonts. “No” indicates that the cross resulted in no infection of either trophonts and/or gamonts. Parasite species (PS) are reported with the source host species (SH), which were the origin of the oocysts. Dashes (—) signify that experimental infections were not attempted.

<table>
<thead>
<tr>
<th>Target host species</th>
<th>Tr. confusum</th>
<th>Tr. castaneum</th>
<th>Tr. brevicornis*</th>
<th>Tr. freeman</th>
<th>P. subdepressus</th>
<th>Tr. madens</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS:</td>
<td>G. minuta and G. confusa</td>
<td>Yes</td>
<td>—</td>
<td>No</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SH:</td>
<td>Tr. confusum</td>
<td>—</td>
<td>Yes</td>
<td>No</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS:</td>
<td>G. minuta</td>
<td>—</td>
<td>Yes</td>
<td>No</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SH:</td>
<td>Tr. castaneum</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>PS:</td>
<td>A. bilimani</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SH:</td>
<td>Tr. brevicornis</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>PS:</td>
<td>G. cloptoni</td>
<td>Yes</td>
<td>—</td>
<td>No</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SH:</td>
<td>Tr. freeman</td>
<td>Yes</td>
<td>—</td>
<td>No</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS:</td>
<td>G. palori</td>
<td>Yes</td>
<td>—</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SH:</td>
<td>P. subdepressus</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Infections with Tr. madens were abandoned because their stock colonies were uninfected.

Whitney U-test: $z = -2.278, P = 0.0227; z = -2.694, P = 0.0071; z = -2.193, P = 0.0283$, respectively) (Table IIIb). After the shedding period, Tr. castaneum larvae tended to have higher infection levels than adults (Mann–Whitney U-test; $z = -1.776, P = 0.0758$).

DISCUSSION

The present study uses experimental and phylogenetic methodologies to understand the evolution of specificity in gregarines. This multifaceted approach emphasizes first, a methodical determination of host range among several parasite taxa and, second, the role phylogenetic constraints play in host specificity. For this study, experimentally derived data concerning host range were directly mapped onto a host species cladogram published by Mestrović et al. (2006). This phylogenetic analysis improves upon a previous molecular Tribolium phylogeny (Juan et al., 1993) by providing well-supported “castaneum” and “confusum” clades and resolving several relationships between species. However, the relationship of Tr. brevicornis to the other Tribolium clades remains unclear and should be resolved with future work.

Two levels of host specificity were examined in this study, including specificity at the host stage and species levels. Clopton et al. (1992) were among the first to address experimentally the extent of stadium specificity in a gregarine–tenebrionid model system. Their study indicated that 3 gregarine species (Gregarina steini, Gregarina cuneata, and Gregarina polymorpha) colonized only larval Te. molitor, and 1 gregarine species (G. niphandrodes) colonized only the adult stage. Consequently, the strict stadium specificity (not often observed in other host–parasite systems) was initially considered a signature characteristic of the gregarine–tenebrionid model system (Clopton et al., 1992). Recent molecular work by Leander et al. (2003) suggested that morphological characters may be suspect as taxonomic characters within species of Gregarina. However, in the study experimentally demonstrating strict host stadium specificity (Clopton et al., 1992), species were distinguished not only on trophont and gamont structure, but also on oocyst shapes and sizes. A second experimental study found neither stadium specificity nor host specificity between 2 hosts, Tr. confusum and Tr. castaneum, and the parasite Gregarina triboliorum (Watwood et al., 1997), thereby demonstrating that stadium specificity was not universal among the gregarines in this tenebrionid–host system.

The current study expands upon the previous studies by establishing the extent to which host stadium specificity occurs among a wider range of related hosts and their parasite taxa. Holometabolic insect hosts that have been used to experimentally determine host stadium specificity include 5 congeneric hosts (Tribolium spp.) and 7 confamilial host species (5 Tribolium spp., P. subdepressus, and Te. molitor). Furthermore, this study extends the number of gregarine taxa to 8 Gregarina spp., and 1 confamilial parasite, Awrygregarina.

Host stadium specificity represents a type of extreme restric-
tion, or a level of specificity beyond the species level and thus could have important evolutionary implications for gregarines. This is particularly true if each holometabolic insect species represents 2 distinct environments to be potentially colonized. Thus, consideration of host stadium specificity broadens the conceptual basis of host specificity in general. The 5 eugregarine species in this study were not host stadium specific since all the involved gregarine taxa infected both the larval and adult host stages of the host species they normally infect in the lab. Thus, the occurrence of host stadium specificity remains restricted to gregarines from *Te. molitor* as demonstrated experimentally by Clopton et al. (1992). There are no records of host stadium specificity occurring in any other septate gregarine taxa or any other gregarine taxa, including all other members of Subclass Gregarinasina. However, this observation may be misleading, since most gregarine taxa are not well studied beyond their species descriptions, so future reports of host stadium specificity among gregarine taxa are certainly plausible.

The present study also determined the extent of host specificity among host species for several eugregarine species. The approach was unique in that a range of host species and parasite taxa were studied experimentally. The gregarine taxa in this study could be classified as stenoxenous because the eugregarine species were found to infect numerous host species within a single host genus, a situation that is similar to that previously reported for other gregarine taxa (Perkins et al., 2000). At the host genus level, each of the 5 gregarine species infected 2 or 3 *Tribolium* spp., and 2 of the gregarine species also infected a noncongeneric host species (*P. subdepressus*). Therefore, the parasite species were similar in terms of the number of host species that each could colonize, but differed in the individual host species that they colonized. No attempt was made to experimentally infect other host families with the gregarine species, although such experiments would be an interesting test of evolutionary change. By comparing the host phylogeny with experimentally determined specificity of parasite taxa, the avenues for, and constraints on, evolutionary change can be postulated. In the present study, it was found that presumably closely related septate gregarine species did not experimentally infect their host species according to phylogenetic relationships. The experimental host specificity data showed that each host congener was colonized by a unique set of parasite species and this pattern did not follow phylogenetic relationships among the hosts. Given that the phylogenetic background of the host was not sufficient to explain the observed host specificity, the data concerning the colonizing dynamics of eugregarines suggest an alternative hypothesis.

In the present study, the difference in gametocyst shedding and level of infection between adult and larval stages is reported for the first time. Larval hosts had significantly different infection levels and different outputs of parasite propagules into the environment. Larvae of *Tr. freemani*, *Tr. confusum*, and *P. subdepressus* not only shed more gametocysts than adults but also were significantly more infected after the 24-hr shedding period. A fourth species, *Tr. castaneum*, exhibited the same trend, but the difference was not statistically significant, most likely due to the high variation levels that often characterize gregarine infection levels. These results suggest that the differential colonizing dynamics of the host stages may have important implications for parasite propagule dispersal (oocysts) and ultimately gregarine host specificity.

The immediate dispersion of oocysts is reliant on the distribution of the beetle stages within the environment. Once airborne, adult *Tr. castaneum* and *Tr. freemani* can fly well (Barnes and Kaloostian, 1940; Graham, 1962; Imura, 1987). In contrast, other species, including *Tr. confusum* and *Tr. brevicomis*, are not known to fly (Good, 1933; Mulder and Sokoloff, 1982). Interestingly, in our study, the adult stages that are more likely to colonize new populations than the larval stages were the least infected and had the slowest rate of parasite propagule (gametocyst) release. This observation suggests that the geographic dispersal of gregarine infections is probably most dependent on external transmission factors such as wind and water, or attachment to the external surface of the adult.

The observation that some gregarine species can mature and develop in a range of host species independent of phylogenetic relationships suggests that if oocysts are encountered and consumed they will mature and develop in numerous host species, and also suggests that when various species are not infected, the reason may be ecological instead of phylogenetic. *Tribolium* species may have similar physiological characteristics or resources for gregarine species because they are closely related host species, but the likelihood of infection may be more dependent upon the ecological interactions of the hosts.

Historically, *Tribolium* species were postulated to occur in geographically isolated species-groups, but now many members

---

**Table IIIa. Mean abundance of gametocysts shed for adults and larvae (n = 20 per well dish for each host stage).**

<table>
<thead>
<tr>
<th>Host species</th>
<th>Mean gametocysts shed by adults</th>
<th>Mean gametocysts shed by larvae</th>
<th>Number of well dishes examined</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tr. freemani</em></td>
<td>0.08</td>
<td>5.81</td>
<td>11</td>
</tr>
<tr>
<td><em>P. subdepressus</em></td>
<td>0.28</td>
<td>4.65</td>
<td>8</td>
</tr>
<tr>
<td><em>Tr. confusum</em></td>
<td>1.41</td>
<td>38.17</td>
<td>5</td>
</tr>
<tr>
<td><em>Tr. castaneum</em></td>
<td>0.00</td>
<td>32.82</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table IIIb. Numbers of gregarines (trophonts and/or gamonts) after 24-hr shedding period per well dish of adults (n = 20) and per well dish of larvae (n = 20).**

<table>
<thead>
<tr>
<th>Host species</th>
<th>Mean gregarine infection within adults</th>
<th>Mean gregarine infection within larvae</th>
<th>Number of well dishes examined</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tr. freemani</em></td>
<td>3.85</td>
<td>59.04</td>
<td>11</td>
</tr>
<tr>
<td><em>P. subdepressus</em></td>
<td>0.28</td>
<td>27.81</td>
<td>5</td>
</tr>
<tr>
<td><em>Tr. confusum</em></td>
<td>35.96</td>
<td>386.4</td>
<td>5</td>
</tr>
<tr>
<td><em>Tr. castaneum</em></td>
<td>21.22</td>
<td>407.84</td>
<td>5</td>
</tr>
</tbody>
</table>
of these species-groups are cosmopolitan in distribution as a result of human agriculture–related activities (Hinton, 1948). When mapped to host phylogeny, the pattern of host specificity determined in the present study may reflect the currently overlapping niches of these once geographically separate species-groups. Although no further investigation of colonizing dynamics beyond gametocyst shedding and mean abundance of infection was conducted in this study, overall, the colonizing dynamics of host species may have important implications for host specificity. An understanding of the mode of dispersal for oocysts in the environment, particularly in nature, may lead to important discoveries that provide more evidence for the constraints on and avenues for gregarine evolution in particular and apicomplexan evolution in general.

The present work contributes to the short list of studies that investigate the extent of host specificity among numerous parasite taxa using experimental and phylogenetic approaches. This scheme ensures that the results are not a product of the methods, as is often the case in studies that are ecological by design. More specifically, an experimental approach allows one to test all possible combinations of host–parasite interaction. By testing host specificity experimentally, the level of specificity resulting from such a study is not necessarily limited by ecological host–parasite interactions, but may ultimately reflect such interactions.

**ACKNOWLEDGMENTS**

We thank Dr. Gregory J. Sandland and Dr. Matthew Bolek for comments on an earlier version of this manuscript. This study was supported in part by the University of Nebraska–Lincoln Ashton C. Cuckler Fellowship.

**LITERATURE CITED**


SHORT-TERM SEASONAL CHANGES IN PARASITE COMMUNITY STRUCTURE IN NORTHERN LEOPARD FROGLETs (RANA PIPiens) INHABITING AGRICULTURAL WETLANDS

Kayla C. King*, André D. Gendron†, J. Daniel McLaughlin, Isabelle Giroux‡, Pauline Brousseau§, Daniel Cyr§, Sylvia M. Ruby, Michel Fournier§, and David J. Marcogliese†

Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. W., Montreal, Quebec, H3G 1M8, Canada.
e-mail: kingkc@indiana.edu

ABSTRACT: Parasite community structure can change seasonally with shifts in host habitat and in diet. However, anthropogenic activity may influence the natural changes in transmission dynamics of different parasite species. Effects of seasonal and agricultural activity on the parasite communities of newly metamorphosed northern leopard frogs (Rana pipiens) were investigated in July and September 2001 in 5 wetlands, 3 of which were exposed to pesticide runoff from surrounding agriculture. Nineteen parasite taxa were found. Component community richness was consistently high at the pristine reference wetland, whereas the communities at a managed reference wetland remained depauperate. Infracomunity richness increased throughout the season, but more so in frogs resident in agricultural wetlands. Digeneans using frogs as intermediate hosts dominated the communities, although many species were much lower in abundance in September, suggesting mortality of heavily infected frogs. Mean abundance of Haematoloechus spp. was positively related to that of odonate naiads in the frog diet, which appeared to reflect differential second intermediate host availability between reference and agricultural wetlands. Although virtually absent from wetlands in July just after frog metamorphosis, monoxenous nematodes were more prevalent and abundant at agricultural wetlands as the season progressed. Our results suggest that agricultural activity may further facilitate the transmission of monoxenous nematodes as frogs become more terrestrial.

Many factors can influence parasite recruitment, particularly during the initial stages of amphibian ontogeny. After metamorphosis, the frogs gradually become more terrestrial and their gape size increases with age. Thus, parasite species richness should increase as the season progresses (Gillilland and Muzzall, 1999). However, environmentally induced changes in immune function (Carey and Bryant, 1995; Voccia et al., 1999; Gilbertson et al., 2003) may further contribute to higher than usual parasite recruitment.

During much of their life cycle, leopard frogs that inhabit agricultural wetlands and landscapes are vulnerable to pesticide exposure, either from chemicals in the water or from fields where pesticides have been applied. A few studies have demonstrated that the prevalence of bacterial and fungal parasite infection can increase as a result of pesticide-induced immunosuppression (Carey, 1993; Taylor et al., 1999). Other investigations have discussed these implications for amphibian population declines (Carey and Bryant, 1995; Carey et al., 2003), but few have considered the responses of helminth parasites in hosts exposed to pesticides (Kiesecker, 2002; Christin et al., 2003; Gendron et al., 2003). The latter authors demonstrated that the monoxenous nematode Rhabdias ranae migrated faster to, and established in greater numbers in, the lungs of leopard froglets exposed to a pesticide cocktail than in control animals. In the field, the digeneans Ribeiroia ondatrae and Telorchis sp. have been shown to increase in prevalence in wood frog (Rana sylvatica) tadpoles exposed to agricultural runoff containing pesticides (Kiesecker, 2002). In complementary lab experiments, it was demonstrated that infection levels of these trematode species were higher in tadpoles exposed to 3 µg/L atrazine. The apparent cause was pesticide-induced immunosuppression as reflected in the eosinophil counts (Kiesecker, 2002).

Anthropogenic disturbances can have many consequences for the ecological interactions between hosts and parasites. Parasitism in fishes has been examined extensively in polluted aquatic habitats (Khan and Thulin, 1991; Poulin, 1992; Overstreet, 1993; MacKenzie et al., 1995), whereas in snails, parasite communities have been considered primarily in relation to perturbations influencing the distribution of bird definitive hosts (see examples in Lafferty, 1997). However, few studies to date have examined the parasite communities of amphibians inhabiting agricultural environments (Hamann et al., 2006; Kopriunikar et al., 2006; King et al., 2007; McKenzie, 2007). There are many links between anthropogenic disturbance and the emergence of particular parasites causing amphibian morbidity and mortality (Johnson et al., 2001; Kiesecker, 2002; Carey et al., 2003; Johnson and Chase, 2004). Therefore, the ecological consequences of habitat deterioration for parasite transmission require further exploration.

Those individuals found dying of infections are often newly metamorphosed frogs and later-stage tadpoles (Carey and Bryant, 1995), and there is increasing evidence that interactions between agricultural pollution and parasitism may play a substantial role in the global declines of many amphibian populations (Daszak et al., 1999). An investigation of parasite community composition in the first few months after frog metamorphosis is, therefore, merited, particularly in environments modified by agriculture. The present study describes the parasite communities of northern leopard frogs (Rana pipiens) from their natal wetlands in the St. Lawrence River basin in southern Quebec, Canada. Collections took place at the beginning (July) and the end (September) of the summer season to evaluate the
The effects of agricultural activity and seasonality on the parasite communities of newly metamorphosed northern leopard frogs.

**MATERIALS AND METHODS**

Five wetlands in the St. Lawrence River drainage basin in Quebec, Canada, were selected for study in 2001 (Fig. 1). Selection of these wetlands was based on long-term data on waterborne pesticides in rivers and water bodies in the area (Giroux, 1999, 2002) or on ongoing studies related to pesticide pollution and amphibians. All wetlands were verified to be a leopard frog breeding habitat. In July and September, young-of-the-year frogs were collected from 2 reference wetlands, i.e., Etang John-Sauro (Ref1) and Parc Le Rocher (Ref2), and 3 agricultural wetlands, i.e., Ruisseau Fairbanks (Ag1), Rivière Chibouet (Ag2), and Île de la Commune (Ag3). As opposed to the 2 reference wetlands, the agricultural wetlands were directly adjacent to agricultural land and were exposed to pesticide runoff mainly from nearby cornfields. In addition, Ref1 is a protected, relatively pristine, Ducks Unlimited, conservation wetland, whereas Ref2 is a wetland within a rural park, with managed landscape and regular human activity nearby.

Surface water samples were collected from 15 May to 25 July to monitor concentrations of pesticides at each wetland during the time of frog metamorphosis. Because waterborne concentrations of pesticides can fluctuate considerably with precipitation patterns and the times of application in surrounding crop fields, wetlands exposed to agricultural runoff were sampled repeatedly. Water samples were taken 1 time/week from Ag1, 3 times/week from Ag2, and 1 time/week from Ag3. Because of logistical and financial constraints, reference habitats were only sampled twice during the frog larval development period to confirm low pesticide levels. However, selected reference wetlands were neither adjacent to agricultural fields nor found to be pesticide-contaminated in previous years. Pesticide data presented for Ag1 and Ag2 were collected as part of a pesticides-monitoring program of St. Lawrence River tributaries operated by the Ministère du Développement durable, de l’Environnement et des Parcs du Québec. Water samples from these wetlands were collected by the latter department’s personnel and sent to the Centre d’expertise d’analyse environnementale du Québec (QC, Canada) to be analyzed for 13 neutral herbicides by National Laboratory for Environmental Testing at the National Water Research Institute in Burlington (ON, Canada). The surface water temperatures, surface pH, and conductivity were recorded once using a digital meter (YSI Model 63, Yellow Springs, Ohio) at the time of frog collections.

Between 20 and 32 young-of-the-year *R. pipiens*, with snout-vent length ≤45 mm, were collected from each wetland in mid July (soon after metamorphosis) and in mid September (before hibernation) with dip nets or by hand. Frogs were killed immediately in buffered 0.8% tricaine methane sulfonate (MS222), individually bagged, and subsequently stored at −80°C until examined. If the age of the leopard frog was in question, clippings of the frog’s longest toe phalanges were used to age the frogs (Leclair and Castanet, 1987). Frogs were thawed, weighed, measured from snout to vent, sexed, and then examined for parasites following Goater and Goater (2001). Identifications of the parasites were based on descriptions and surveys in the literature (Rau et al., 1978; Prudhoe and Bray, 1982; McAlpine, 1997; McAlpine and Burt, 1998; Gilliland and Muzzall, 1999). Once examined for parasites, stomach contents were fixed in 70% ethanol and sent to a commercial laboratory (Laboratoire SAB Inc. Longueuil, QC, Canada) for diet analyses. Prey items were sorted, counted, and identified to the lowest taxonomic level (Borror et al., 1989; Dindal, 1990).

Prevalence and mean abundance were calculated in accordance with the definition provided in Bush et al. (1997). As parametric assumptions were not met on raw or transformed data, the abundance values of individual species were ranked. One-way ANOVA and Tukey’s post hoc test were used to compare the abundance of parasite species among wetlands. Mann–Whitney U-test was used to detect seasonal differences in abundances of individual parasite species and total parasite abundance. Additionally, 2-way ANOVA was performed to determine whether season or agriculture affected the abundance of individual parasite species. Only parasites with an overall frequency >10% were included in this latter analysis. Both infra- and component community species richness were determined for each wetland in July and September. Component community richness is defined as the number of species present in a sample from a single wetland, whereas infracommunity richness is the number of parasite species per frog, including infected and uninfected frogs in a sample. The former measures were compared among wetlands through use of 1-way ANOVA, followed by Tukey’s post hoc test. Shannon–Wiener diversity index (Magurran, 1988) was also calculated for component communities. Moreover, Spearman’s rank correlations were used to determine relationships between the abundance of parasite species and that of corresponding invertebrate second intermediate hosts in diet contents across all wetlands in both months. All statistical tests were conducted using SPSS 13.0 (Chicago, Illinois).

**RESULTS**

Conductivity and pH tended to increase between July and September at each wetland (Table 1). Atrazine, widely used as a preemergence herbicide in corn crops, was detected in virtually all samples. However, the peak levels and mean concentrations of atrazine and metolachlor, another commonly used herbicide, over the sampling period were highest at Ag2 and Ag3. Only moderate levels were detected at Ag1. Trace levels of atrazine and a few other pesticides were measured at both reference wetlands.

In total, 19 species of helminth parasites were found infecting frogs in July and 18 in September. These included 3 adult digenocytes (*Glypheadmins quieta* in the intestine, *Gorgoderina attenuata* in the urinary bladder, and *Haematoloechus spp.*, including *H. medioplexus* and *H. varioplexus*, in the lungs), 8 metacercariae (*Apharyngostrigea picipitis* in the body cavity, *Clinostomum sp.* in the body muscle, *Diplostomum sp.* in the eye lens, *echinostomes in the kidney, another unknown echinozystid in the muscle, *Fibricola sp.* in the body muscle and cavity, an unknown gorgoderid in the body cavity, and an unknown Strigeidae in the muscle), 1 digenean mesocercaria (*Alaria sp.* in the muscle), 4 adult nematodes (*Osvaldocrania sp.*, *Cosmocercoides sp.*, and *Strongyloides sp.* in the intestine,
Richness of the component communities increased from July to September and was consistently lower at Ref2 and Ag2 and highest at Ref1 (Table V). In July, infracommunity richness was the lowest at Ref2 and Ag1 and remained low at the former locality in September. Species richness increased considerably, albeit not significantly, at the agricultural wetlands between sampling periods. With the exception of Ref1 and Ag2, Shannon–Wiener diversity index increased at most wetlands from July to September, but was consistently the lowest at Ag2.

Abundance of Haematoloechus spp. in the lungs was significantly and positively correlated with the abundance of anisop teran odonata naiads in stomach contents in September samples \( (r_s = 0.87; P = 0.0048) \) (Fig. 4). Odonates were absent in July, and so, similar analyses could not be conducted on those samples. Conversely, the abundance of Haematoloechus spp. was negatively associated with that of zygopteran odonates \( (r_s = -0.8944; P = 0.0405) \).

**DISCUSSION**

These results indicate that there are changes in parasite community structure in newly metamorphosed froglets over the first few months postemergence, and that those changes may be mediated by agricultural activities. Due to the limited number of wetlands sampled, however, our results should be interpreted with caution. Digeneans appear to be recruited first, for the most part, in agreement with the findings of McAlpine (1997) and Gillilland and Muzzall (1999), whereas nematodes are recruited later on in the season. This increase in the prevalence and abundance of monoxenous nematodes (Rhabdias ranae, Oswaldocruzia sp., and Strongyloides sp.) in September at all wetlands is likely because of the increased time spent on land by northern leopard froglets as they grow and, additionally, because of their increasing size and surface area. Yet, the largest seasonal increases in infection occurred in frogs from the agricultural wetlands in this study. This implicates agricultural disturbance as intensifying monoxenous nematode infections. Specifically, pesticide exposure has been demonstrated to alter components of the amphibian immune system, consequently af-
TABLE II. Prevalence (%) and mean abundance (Ab) (± 1 SD) of parasite species infecting *Rana pipiens* from reference and agricultural wetlands in July. Abbreviations for wetlands are as in Figure 1. Superscript letters indicate significant differences in abundance among wetlands (*P* < 0.05).

<table>
<thead>
<tr>
<th>Species</th>
<th>Ref1</th>
<th>Ref2</th>
<th>Ag1</th>
<th>Ag2</th>
<th>Ag3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Ab</td>
<td>%</td>
<td>Ab</td>
<td>%</td>
</tr>
<tr>
<td><strong>Digenea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alaria</em> sp.*</td>
<td>36</td>
<td>1.2&lt;sup&gt;ab&lt;/sup&gt; (2.1)</td>
<td>12</td>
<td>0.3&lt;sup&gt;b&lt;/sup&gt; (1.1)</td>
<td>35.5</td>
</tr>
<tr>
<td><em>Apharyngostriega pipientis</em></td>
<td>8.0</td>
<td>0.1 (0.4)</td>
<td>4.0</td>
<td>0.7 (3.4)</td>
<td>0</td>
</tr>
<tr>
<td><em>Clinostomum</em> sp.*</td>
<td>8.0</td>
<td>0.1 (0.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Diplostomum</em> spp.*</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
<td>0.04 (0.2)</td>
<td>25.8</td>
</tr>
<tr>
<td>Echinostomatidae gen. sp. 1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>96.0</td>
<td>93.6&lt;sup&gt;b&lt;/sup&gt; (47.5)</td>
<td>100.0</td>
<td>48.8&lt;sup&gt;c&lt;/sup&gt; (54.1)</td>
<td>71.0</td>
</tr>
<tr>
<td>Echinostomatidae gen. sp. 2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>8.0</td>
<td>0.2 (0.6)</td>
<td>9.7</td>
</tr>
<tr>
<td><em>Fibricola</em> sp.*</td>
<td>68.0</td>
<td>56.1 (123.6)</td>
<td>16.0</td>
<td>4.5&lt;sup&gt;c&lt;/sup&gt; (20.2)</td>
<td>25.8</td>
</tr>
<tr>
<td><em>Glypthelmins quieta†</em></td>
<td>40.0</td>
<td>1.8&lt;sup&gt;a&lt;/sup&gt; (4.7)</td>
<td>8.0</td>
<td>0.1&lt;sup&gt;b&lt;/sup&gt; (0.4)</td>
<td>3.2</td>
</tr>
<tr>
<td><em>Gorgoderina</em> attenuata†</td>
<td>4.0</td>
<td>0.04&lt;sup&gt;b&lt;/sup&gt; (0.2)</td>
<td>0</td>
<td>0</td>
<td>6.5</td>
</tr>
<tr>
<td>Gorgoderidae gen. sp.&lt;sup&gt;*&lt;/sup&gt;</td>
<td>76.0</td>
<td>1.8&lt;sup&gt;b&lt;/sup&gt; (3.7)</td>
<td>4.0</td>
<td>0.04&lt;sup&gt;c&lt;/sup&gt; (0.2)</td>
<td>16.1</td>
</tr>
<tr>
<td><em>Haematoloechus</em> sp.†</td>
<td>8.0</td>
<td>0.32 (1.1)</td>
<td>0</td>
<td>0</td>
<td>12.9</td>
</tr>
<tr>
<td>Strigeidae gen. sp.&lt;sup&gt;*&lt;/sup&gt;</td>
<td>24.0</td>
<td>0.5 (1.3)</td>
<td>12.0</td>
<td>0.2 (0.6)</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>Nematoda</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cosmocercoides</em> sp.&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
<td>0.2 (1.2)</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Oswaldocraziia</em> sp.&lt;sup&gt;†&lt;/sup&gt;</td>
<td>4.0</td>
<td>0.1 (0.4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Rhabdias ranae†‡</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Spiroxys</em> sp.&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>4.0</td>
<td>0.04 (0.2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Seuratoidea gen. sp.&lt;sup&gt;*&lt;/sup&gt;</td>
<td>44.0</td>
<td>5.84 (10.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Strongyloides</em> sp.&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.7</td>
</tr>
<tr>
<td><strong>Cestoda</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proteocephalus</em> sp.&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.0</td>
<td>0.04 (0.2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Larva/metacercaria.
† Adult.
‡ Immature.
### TABLE III. Prevalence (%) and mean abundance (Ab) (±1 SD) of parasite species infecting *Rana pipiens* from reference and agricultural wetlands in September 2001. Abbreviations for wetlands are as in Figure 1. Superscript letters indicate significant differences in abundance among wetlands ($P < 0.05$).

<table>
<thead>
<tr>
<th>Species</th>
<th>Ref1</th>
<th>Ref2</th>
<th>Ag1</th>
<th>Ag2</th>
<th>Ag3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Digenea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alaria sp.</em></td>
<td>9.5</td>
<td>0.3</td>
<td>16.7</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td><em>Apharyngostigea pipiens</em></td>
<td>19.0</td>
<td>0.3</td>
<td>10.0</td>
<td>0</td>
<td>9.7</td>
</tr>
<tr>
<td><em>Clinostomum sp.</em></td>
<td>19.0</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Diplostomum spp.</em></td>
<td>0</td>
<td>0.1</td>
<td>36.7</td>
<td>18.8</td>
<td>12.9</td>
</tr>
<tr>
<td><em>Echinostomatidae gen. sp. 1</em></td>
<td>95.2</td>
<td>80.0</td>
<td>56.7</td>
<td>90.6</td>
<td>77.4</td>
</tr>
<tr>
<td><em>Echinostomatidae gen. sp. 2</em></td>
<td>9.5</td>
<td>0.1</td>
<td>26.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Fibricola sp.</em></td>
<td>61.9</td>
<td>127.0</td>
<td>56.7</td>
<td>78.1</td>
<td>35.5</td>
</tr>
<tr>
<td><em>Glyphhelmin quieta†</em></td>
<td>10.0</td>
<td>0.1</td>
<td>6.7</td>
<td>18.8</td>
<td>0</td>
</tr>
<tr>
<td><em>Gorgoderina attenuata†</em></td>
<td>20.0</td>
<td>0.4</td>
<td>6.7</td>
<td>50.0</td>
<td>29.0</td>
</tr>
<tr>
<td><em>Gorgoderidae gen. sp.</em></td>
<td>33.3</td>
<td>5.0</td>
<td>23.3</td>
<td>25.0</td>
<td>80.6</td>
</tr>
<tr>
<td><em>Haematoloechus spp.†‡</em></td>
<td>28.6</td>
<td>1.3</td>
<td>30.0</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Strigeidae gen. sp.</em></td>
<td>42.9</td>
<td>0.7</td>
<td>6.7</td>
<td>6.5</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Nematoda</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cosmocercoide ssp.</em></td>
<td>5.0</td>
<td>0.1</td>
<td>0</td>
<td>9.4</td>
<td>3.2</td>
</tr>
<tr>
<td><em>Oswaldocraza sp.†</em></td>
<td>14.3</td>
<td>0.1</td>
<td>66.7</td>
<td>50.0</td>
<td>61.3</td>
</tr>
<tr>
<td><em>Rhabdiuss ranae†</em></td>
<td>28.6</td>
<td>0.5</td>
<td>66.7</td>
<td>37.5</td>
<td>96.8</td>
</tr>
<tr>
<td><em>Spiroxyx sp.</em></td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td><em>Seuratoidea gen. sp.</em></td>
<td>38.0</td>
<td>4.7</td>
<td>6.7</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td><em>Strongyloides sp.†</em></td>
<td>5.0</td>
<td>0.1</td>
<td>26.7</td>
<td>9.4</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* Larva/metacercaria.
† Adult.
‡ Immature.
fecting the establishment of helminth parasites (Kiesecker, 2002; Christin et al., 2003, 2004; Gendron et al., 2003). The infection patterns of *Rhabdias ranae*, *Strongyloides* sp., and *Oswaldocruzia* sp. in this study suggest that pesticides may be affecting the immune system of the young-of-the-year frogs. Gendron et al. (2003) found that when leopard froglets were exposed to a pesticide mixture containing ≥21 μg/L atrazine, the internal migration rate of *Rhabdias ranae* larvae and their abundance in the lungs were significantly enhanced. Lung-worms of this genus have been shown to be detrimental to juvenile anurans, reducing their growth and survival (Goater and Ward, 1992). In young toads, for instance, host ability to perform sustained physical activities was impaired by increasing infection with *Rhabdias bifonis*, presumably from interference of the parasite with the normal functioning of the lungs (Goater et al., 1993). Thus, an increase in *Rhabdias ranae* infection in September could reduce the ability of young-of-the-year leopard frogs to disperse and undertake fall migration toward hibernation areas or to escape predators. Additionally, *Strongyloides* sp. has been found to cause a thickening of the intestinal mucosa and eventually kill *Hyla chrysoscelis* (Patte-

**TABLE IV.** Significant changes in parasite species abundance and total parasite abundance between July and September, 2001. Results from Mann-Whitney *U*-test are shown, and the direction of change is indicated. Abbreviations for wetlands are as in Figure 1.

<table>
<thead>
<tr>
<th>Wetland</th>
<th>Parasite species</th>
<th>Direction of abundance change from July to September</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref1</td>
<td><em>Alaria</em> sp.</td>
<td>Down (<em>U</em> = 184, <em>P</em> = 0.046)</td>
</tr>
<tr>
<td></td>
<td>Echinostomatidae gen. sp. 1</td>
<td>Down (<em>U</em> = 212.5, <em>P</em> = 0.048)</td>
</tr>
<tr>
<td></td>
<td><em>Glypthelmins quieta</em></td>
<td>Down (<em>U</em> = 176.5, <em>P</em> = 0.03)</td>
</tr>
<tr>
<td></td>
<td><em>Haeematoloechus</em> spp.</td>
<td>Up (<em>U</em> = 194, <em>P</em> = 0.05)</td>
</tr>
<tr>
<td></td>
<td><em>Rhabdias ranae</em></td>
<td>Up (<em>U</em> = 200, <em>P</em> = 0.02)</td>
</tr>
<tr>
<td>Ref2</td>
<td>Echinostomatidae gen. sp. 1</td>
<td>Down (<em>U</em> = 152.5, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Rhabdias ranae</em></td>
<td>Up (<em>U</em> = 162.5, <em>P</em> = 0.002)</td>
</tr>
<tr>
<td></td>
<td><em>Total Parasites</em></td>
<td>Down (<em>U</em> = 91, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td>Ag1</td>
<td><em>Fibricola</em> sp.</td>
<td>Up (<em>U</em> = 328, <em>P</em> = 0.02)</td>
</tr>
<tr>
<td></td>
<td><em>Oswaldocruzia</em> sp.</td>
<td>Up (<em>U</em> = 155, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Rhabdias ranae</em></td>
<td>Up (<em>U</em> = 155, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td>Ag2</td>
<td>Echinostomatidae gen. sp. 2</td>
<td>Down (<em>U</em> = 206.5, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Gorgoderina attenuata</em></td>
<td>Up (<em>U</em> = 339.5, <em>P</em> = 0.03)</td>
</tr>
<tr>
<td></td>
<td><em>Strigeidae</em> gen. sp.</td>
<td>Down (<em>U</em> = 418.5, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Oswaldocruzia</em> sp.</td>
<td>Up (<em>U</em> = 232.5, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Rhabdias ranae</em></td>
<td>Up (<em>U</em> = 162.5, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Strongyloides</em> sp.</td>
<td>Up (<em>U</em> = 372, <em>P</em> = 0.01)</td>
</tr>
<tr>
<td>Ag3</td>
<td><em>Alaria</em> sp.</td>
<td>Down (<em>U</em> = 139.5, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Clinostomum</em> sp.</td>
<td>Up (<em>U</em> = 167.5, <em>P</em> = 0.002)</td>
</tr>
<tr>
<td></td>
<td><em>Fibricola</em> sp.</td>
<td>Down (<em>U</em> = 103, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Oswaldocruzia</em> sp.</td>
<td>Up (<em>U</em> = 90, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Rhabdias ranae</em></td>
<td>Up (<em>U</em> = 11, <em>P</em> &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td><em>Total Parasites</em></td>
<td>Down (<em>U</em> = 134, <em>P</em> = 0.001)</td>
</tr>
</tbody>
</table>

**FIGURE 3.** Results of 2-way ANOVA, with season (July = black bars, September = grey bars) and wetland type as independent variables. Mean abundance (+1 SD) of (a) *Oswaldocruzia* sp. (season: *F* = 63.90; *P* < 0.001; wetland type: *F* = 54.17; *P* < 0.001; season × wetland type: *F* = 61.24; *P* < 0.001); (b) *Rhabdias ranae* (season: *F* = 117.46; *P* < 0.001; wetland type: *F* = 30.55; *P* < 0.001; season × wetland type: *F* = 28.16; *P* < 0.001); and (c) *Strongyloides* sp. (season: *F* = 7.53; *P* = 0.007; wetland type: *F* = 13.87; *P* < 0.001; season × wetland type: *F* = 4.63; *P* = 0.032) were significantly affected by both variables and the interaction.
son-Kane et al., 2001), but that parasite has rarely been recorded in amphibians to date (Little, 1966; Schmidt and Whittaker, 1975; Patterson-Kane et al., 2001). Considered less harmful is Oswaldocruzia sp., although Hendrikx and van Moppes (1983) observed damage to the stomach and intestinal lining of Bufo bufo when infected with this nematode. Suppression of the immune system from agricultural contaminants may contribute to intense and, potentially, lethal infections of these parasites. Alternatively, nutrients in the soil may contribute to the increased success of nematodes with direct life cycles because of enhanced survival of their free-living stages, but that remains speculative at this point.

Parasites that increased in abundance over the season were those mainly acquired by froglets through their diet (Haematoloechus spp.) or movements onto land (R. ranae, Oswaldocruzia sp., and Strongyloides sp.). A striking observation, not limited to our agricultural wetlands, was the decrease in the abundance of many digenean metacercariae (Alaria sp., Clinostomum sp., the echinostomes, Fibricola sp., and the unknown strigeid), which are relatively long-lived parasites. Froglets with heavy metacercariae infections may be subject to parasite-induced host mortality or be more vulnerable to predation, although parasite-induced host mortality is difficult to verify from field samples (Kennedy, 1997a). However, given that all frogs were of the same age and that dispersal of newly metamorphosed frogs in or out of the habitats was unlikely, the potential for parasite-induced host mortality remains a possibility. Certainly, a heightened vulnerability to predation from intense parasitic infection has been demonstrated in numerous other host–parasite systems (Lafferty, 1999; Moore, 2002), and our data suggest it may be a possibility in frogs as well. Given that some of these parasites were more common in agricultural wetlands in July and that the decrease in abundance between July and September was more pronounced than at reference wetlands, that larval digeneans pose a threat to frog populations
in agricultural landscapes. In particular, echinostomes and *F. bricola* sp. decreased in abundance at Ag2 and Ag3, respectively, the 2 most contaminated wetlands. Considering that these parasites generally infect the host at the tadpole stage, a time when frogs are most vulnerable to the deleterious effects of pesticides (Carey and Bryant, 1995; Storrs and Kiesecker, 2004; Hayes, 2005), the probability of intense infections is likely enhanced. Echinostomes can be pathogenic when infections are heavy, inducing edema and eventually killing their hosts (Fried et al., 1997; Belden, 2006). In the wild, this parasite is thought to regulate amphibian population levels (Schotthoefer et al., 2003), but in populations that are immunocompromised, echinostomes may be contributing to population decline. Studies have documented changes in parasite communities associated with aquatic pollution and landscape disturbance. In fishes and snails, environmental stress is generally manifested as a decrease in parasite species richness (reviewed in Lafferty, 1997; Marcogliese, 2004, 2005). Results in July and September for the component communities are essentially consistent with the expectation of lower species richness in disturbed environments. Compared with the most pristine reference wetland (Ref1), component community richness at the agricultural wetlands was slightly reduced in both collection periods. The same tendency was not observed for infracommunity richness, which was relatively high in 2 of the 3 agricultural wetlands. In addition, infracommunity species richness remained constant in reference wetlands over the summer season but increased in agricultural wetlands mainly due to more nematode recruitment. Infracomponent community species richness were the lowest at Ref2 and Ag2, both wetlands surrounded by the largest areas of urbanization or agriculture. These types of landscape disturbances can limit the visitation of potential definitive hosts and consequently reduce parasite transmission to frogs (King et al., 2007). Similarly, landscape influenced the digenean fauna of grey tree frogs in Ontario ponds (Koprivnikar et al., 2006). These authors found that the prevalence of larval *Alaria* sp. was positively correlated with surrounding forest cover, an environmental factor thought to promote canid definitive host visitation to the area. Nevertheless, some have cautioned that measures of species richness and diversity may not always be good indicators of anthropogenic changes because different species respond differently to environmental stress (Kennedy, 1997b; Lafferty, 1997; Overstreet, 1997; Marcogliese, 2005). Indeed, different species and subsets or guilds of parasites comprising communities may be better indicators of environmental changes (Marcogliese, 2005), as was observed herein for digeneans and monoxenous nematodes. Certain parasite species differed in their abundance and prevalence between the agricultural and reference wetlands, potentially indicating that pesticide pollution is affecting invertebrate intermediate hosts. For instance, 2 species of adult *Haematoloechus* (*H. medioplexus* and *H. varioplexus*) were found to be more abundant in our samples from reference wetlands, a trend reflected by the occurrence of their second intermediate hosts, anisopteran odonates, in diet contents. Those parasite species only develop within that family of odonates as opposed to more generalist *Haematoloechus* species, which use zygopteran odonates and other arthropods as second intermediate hosts (Snyder and Janovy, 1996; Bolek and Janovy, 2007). This suggests that the availability of anisopteran odonate naiads is reduced at agricultural wetlands because of pesticide contamination, thus limiting the transmission of *H. medioplexus* and *H. varioplexus* in these habitats. Conversely, many larval digeneans and monoxenous nematodes were more abundant at the agricultural wetlands in July and September, respectively.

Our results suggest that agriculture likely influenced parasite community structure and seasonal recruitment in leopard frogs. However, samples from additional reference and agricultural wetlands would be required to verify infection patterns, especially the increase in monoxenous nematode abundance in agricultural wetlands. Indeed, the observed dynamics of parasite community structure in the first 3 mo after metamorphosis indicates potential negative effects on frog health and survival. Furthermore, examining the influence of anthropogenic disturbance on parasitism in frogs in their initial stages of development may aid in the conservation of frog populations at risk (Blaustein and Wake, 1990; Kiesecker et al., 2001; Green, 2005) as well as the wetland habitats in which those frogs develop.

**ACKNOWLEDGMENTS**

We are grateful to Stéphanie Barbeau, Lila Gagnon-Brambilla, Marie-Soleil Christin, Lucie Ménard, Roxane Péтел, Nathalie Pelchat, and Sylvie Baillargeon for assistance in the field collection and/or in the laboratory. François Boudreault kindly prepared the map, and Judith Price and Donald McAlpine provided voucher specimens, which greatly helped in our parasite identifications. Thanks also to John Sauro and Raymond Greffe who permitted us to sample on their lands, along with Danièle Chatillon of the Société des établissements de plein-air du Québec who facilitated access to Île de la Commune (Ag3). This work was supported by the Toxic Substances Research Initiative and the Pesticide Science Fund (Environment Canada), a Natural Sciences and Engineering Research Council of Canada (NSERC) Graduate Scholarship awarded to K.C.K., and an NSERC Discovery Grant to J.D.M.

**LITERATURE CITED**


**———, and C. J. Bryant.** 1995. Possible interrelationships among environmental toxicants, amphibian development, and decline of am-


Mc Alpine, D. F. 1997. Helminth communities in bullfrogs (Rana catesbeiana), green frogs (Rana clamitans), and leopard frogs (Rana pipiens) from New Brunswick, Canada. Canadian Journal of Zoology 75: 1883–1890.

— —. 1998. Helminths of bullfrogs (Rana catesbeiana), green frogs (Rana clamitans), and leopard frogs (Rana pipiens) in New Brunswick. Canadian Field-Naturalist 112: 50–68.


EVIDENCE OF PLASTICITY IN THE REPRODUCTION OF A TREMATODE PARASITE: THE EFFECT OF HOST REMOVAL

G. Loot*, S. Blanchet, M. Aldana†, and Sergio A. Navarrete†
Laboratoire Evolution et Diversité Biologique, Bâtiment IVR3, Université Paul Sabatier, 118 route de Narbonne, F-31062 Toulouse Cedex 4, France. e-mail: loot@cict.fr

ABSTRACT: The parasitic trematode Proctoeces lintoni requires 3 hosts (intertidal mussels, keyhole limpets, and clingfish) to complete its life cycle. The densities and size structure of host communities are modified by selective human harvesting. This study examined clutch and egg size of P. lintoni in 3 adjacent sites in rocky intertidal areas of central Chile demonstrating differences in the levels of human disturbance (i.e., from a fully protected marine reserve to free open-access areas). We found significant differences in parasite fecundity among sites. An increase in number of eggs was observed inside protected marine areas compared with open-access areas, suggesting a plastic response of the parasite reproductive strategies to the host community modification. These results show that host removal by humans in coastal ecosystems can strongly influence parasite life history traits.

Human harvesting is considered to be one of the major causes of biodiversity deterioration (Vitousek et al., 1997; Sale et al., 2005). Intense and uncontrolled harvesting removes individuals on the basis of traits that include size, behavior, and location. Selective harvesting could, therefore, negatively affect growth, maturation timing, density, and species biomass, leading to dramatic and irreversible effects such as local, or even global, extinction (Vermij, 1993; Coleman and Williams, 2002). Moreover, through the harvesting of a few target species, humans can cause numerous indirect cascading effects on the structure and dynamics of entire communities (Hockey, 1994; McClanahan, 1997; Diaz, 2001). Castilla (1999) illustrated such a scenario in the rocky intertidal food web of central Chile, where human predation on a keystone species, a carnivorous muricid gastropod (Concholepas concholepas), caused community-wide trophic cascade effects.

Despite the increasing number of studies focusing on marine food web structure and human effects, relatively few have incorporated parasites (Marcogliese and Cone, 1997; Marcogliese, 2002; Thompson et al., 2005). Recently, Loot et al. (2005) considered parasite responses to food web alterations in intertidal rocky ecosystems of central Chile and demonstrated indirect human effects on the parasitic trematode Proctoeces lintoni. This parasite requires 3 distinct hosts to complete its life cycle. The first intermediate host is the mussel Perumytilus purpuratus, in which the parasite produces cercariae. Cercariae leave the mussels to infect the gonads of the second intermediate host, the keyhole limpet Fissurella spp., where they develop into the metacercariae stage (George-Nascimento and Quiroga, 1983). The infected limpet is then ingested by the definitive host, the clingfish Sicyases sanguineus, wherein the parasite reaches sexual maturity and begins egg production (George-Nascimento et al., 1998; Oliva and Zegers, 1988). These hosts are all directly or indirectly influenced by human harvesting in positive or negative ways. For instance, the keyhole limpet and clingfish are strongly harvested by local fishermen (Oliva and Castilla, 1986; Durán et al., 1987), who represent size-selective predators by removing larger individuals first (Moreno et al., 1984; Castilla and Durán, 1985). Conversely, cover of mussels significantly decreases in nonharvested sites (see Loot et al. [2005] for a schematic illustration of the rocky intertidal food web). Therefore, host densities and size structure are modified, resulting in significant changes in parasite population dynamics (Loot et al., 2005).

Growing evidence demonstrates reproductive plasticity in all organisms, including mammals (e.g., Reilly et al., 2006), invertebrates (e.g., McGovern, 2003), fish (e.g., Aubin-Horth and Dodson, 2004), and plants (Barot et al., 2005). This sort of plasticity offers organisms the opportunity to produce phenotypes that are thought to confer high fitness in response to changes in environmental conditions during their lifetimes (Stearns, 1992). This could be particularly important for parasitic organisms that are restricted to the displacement of their hosts. In our view, a change in host community structure, as previously demonstrated in intertidal rocky habitats of central Chile (Loot et al., 2005), might also have the potential to induce changes in parasite reproductive strategies. Parasites are generally seen as highly prolific egg producers because of the massive losses suffered by infective stages during transmission (Arneberg et al., 1998). Poulin (1998) suggested that parasites with complex life cycles could be under greater pressure to evolve higher fecundity than sister clades with simpler life cycles because each egg has a very small probability of reaching the next host. Indeed, pressure from the host or the environment might constrain the evolution of egg production.

Our aim with this study was to quantify variability of egg production (clutch size and egg size) in the parasite P. lintoni in response to changes in host density and size structure induced by human harvesting. To do this, we compared parasite egg production of limpet parasites among 3 rocky intertidal sites varying in human harvesting intensity, ranging from a protected marine reserve to free open-access areas where harvesting of hosts is intense and largely unregulated (Castilla and Durán, 1985; Castilla and Bustamente, 1989; Durán and Castilla, 1989).

MATERIALS AND METHODS

Study sites

Samples were collected in 15 localities along a 1.5-km coastline at Las Cruces (33°31’S, 71°38’W) in central Chile. These localities are presented in Loot et al. (2005) and belong to 3 different adjacent sites, i.e., a protected marine area, (Estación Costera de Investigaciones Marinas [ECIM], which has been closed to fishermen and tourists since
Table I. (a) Abundance of host communities: mean percent cover (±SE) of mussels *Perna canaliculus* (%) and mean densities (±SE) of limpets *Fissurella crassa* (individuals per 0.25 m²) and mean densities (±SE) of clingshells *Sicyases sanguineus* (individuals per 100 m of shore per 30 min) at the 3 study sites. (b) Prevalence (%) of the parasite *Proctoeces lintoni* in the hosts (mussels, limpets, and clingshells) at the 3 study sites (see Loot et al. [2005] for more details).

<table>
<thead>
<tr>
<th>Study site</th>
<th>Mussels (% cover)</th>
<th>Limpets (ind. [0.25 m²]⁻¹)</th>
<th>Clingshells (ind. [100 m]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Host abundance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCN</td>
<td>24.04 ± 3.45</td>
<td>0.32 ± 0.11</td>
<td>0.65 ± 0.18</td>
</tr>
<tr>
<td>ECIM</td>
<td>1.02 ± 0.30</td>
<td>0.52 ± 0.11</td>
<td>6.30 ± 1.23</td>
</tr>
<tr>
<td>LCS</td>
<td>27.64 ± 4.51</td>
<td>0.81 ± 0.25</td>
<td>1.35 ± 0.64</td>
</tr>
<tr>
<td>b) Prevalence (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCN</td>
<td>2.2</td>
<td>25.0</td>
<td>54.5</td>
</tr>
<tr>
<td>ECIM</td>
<td>3.9</td>
<td>63.3</td>
<td>62.5</td>
</tr>
<tr>
<td>LCS</td>
<td>1.3</td>
<td>34.3</td>
<td>82.1</td>
</tr>
</tbody>
</table>

December 1982; Castilla and Durán, 1985; Durán et al., 1987] and 2 largely unregulated harvesting areas (Las Cruces South [LCS] and Las Cruces North [LCN]). These sites were directly exposed to wave and presented similar geological characteristics, thus limiting abiotic dissimilarities (Oliva and Castilla, 1986; Cornelius et al., 2001). To improve statistical power, the localities belonging to the same site were pooled. Significantly more people were recorded at LCN (x² = 13.1 ± 1.28 people per 100 m of coast per 30 min) than at LCS (3.52 ± 0.61 person [100 m coast]⁻¹ [30 min]⁻¹) (see Loot et al., 2005). Site replication was not possible for logistical reasons (particularly for the protected marine area, which is unique in Chile). Differences in abundance and size structure of host community (mussels, keyhole limpets, and clingshells) and parasite prevalence among the 3 study sites are detailed in Table I (see also Loot et al., 2005).

Biological material

The metacercariae in keyhole limpets provided an ideal system for such an investigation. Indeed, metacercariae of *P. lintoni* have been shown to become ovigerous (i.e., progenetic metacercariae) in invertebrate host *Fissurella spp.* (Balboa et al., 2001). Thus, parasite egg production is easily quantified by examining individual progenetic worms in limpet gonads, which contrasts with most previous studies in which fecal egg counts are divided by the number of worms in a host to estimate mean egg production. The ability to reach precocious maturity was already documented in the literature (see Discussion), we decided to analyze these 2 variables separately. We used 2 linear mixed-effects models to test for site effects (i.e., human disturbance and different host community structure on egg size and number). A mixed linear model is a generalization of standard linear models, with the generalization being that the sampling effort allows unbalanced design (Pinheiro and Bates, 2000). In our models, limpets were used as the random factors, with parasites nested within limpets. Site was considered a fixed factor. We included limpet size, parasite body size, and the total number of parasites (immature and mature metacercariae) within each limpet as covariates because parasite fecundity might be correlated with these variables (Poulin, 1998). The interactions between site and limpet size, site and parasite size, and site and total number of parasites were also evaluated. A model selection approach on the basis of Akaike Information Criteria (AIC; MacNally, 2000; Johnson and Omland, 2004) was used to assess the predictive power of the independent variables. In total, 26 models were assessed for each reproductive parameter (i.e., egg sizes and number of eggs), and models were compared and selected with likelihood ratio tests (LRT tests). Additionally, the relative explanatory power of each variable was assessed using hierarchical partitioning (Chevan and Sutherland, 1991; MacNally, 2000). In hierarchical partitioning, all possible models in a multiple regression setting are jointly considered to identify the most likely causal factor. This process involves computation of the increase of the fit of all models with a particular factor compared with an equivalent model without that factor (see Chevan and Sutherland, 1991; MacNally, 2000; Heikkinen et al., 2005, for more details). Hierarchical partitioning provided a relative explanatory power for each variable, which is segregated into independent and joint effects (i.e., caused jointly with other variables). Large, positive joint effects (relative to their independent effects) indicated highly colinear variables, whereas negative values indicated that a variable acted as a suppressor variable (Chevan and Sutherland, 1991). Statistical significances of the independent contributions of variables were tested by a randomization routine that yielded Z scores for the generated distribution and a measure of statistical significance based on an upper 0.95 confidence limit (Heikkinen et al., 2005).

To meet the assumption of normality, egg number was square root-transformed. Statistical analyses were performed with R 2.2.1 (lm function for the mixed models and hier.part function for the hierarchical partitioning; R Development Core Team 2005, http://www.r-project.org/).

RESULTS

We observed different reproductive strategies among study sites, with higher egg number and smaller egg size within the nonharvested area of ECIM compared with the 2 others sites (LCM and LCN) (Fig. 1). A single mixed model was selected to predict the variation observed in egg size (Table II).

This model included the 4 main effects and the 3 interactions (i.e., the more complete model). Limpet size had a significant effect on parasite egg size (Table II). However, site did not contribute significantly to the variation observed in egg size. Concerning egg number, the mixed model selected (Table II) included only the site effect. Multiple comparisons revealed that egg number was significantly higher within ECIM compared with the 2 other sites (contrast tests, ECIM vs. LCS, *P* < 0.05; ECIM vs. LCN, *P* < 0.05) and that there was no difference

(ProPlus 1.3 for Windows 95) were used to determine egg number and mean eggs size (μm) for each progenetic parasite.

Statistical analysis

Because the different parasite clutch and egg size relationships have already been documented in the literature (see Discussion), we decided to analyze these 2 variables separately. We used 2 linear mixed-effects models to test for site effects (i.e., human disturbance and different host community structure on egg size and number). A mixed linear model is a generalization of standard linear models, with the generalization being that the sampling effort allows unbalanced design (Pinheiro and Bates, 2000). In our models, limpets were used as the random factors, with parasites nested within limpets. Site was considered a fixed factor. We included limpet size, parasite body size, and the total number of parasites (immature and mature metacercariae) within each limpet as covariates because parasite fecundity might be correlated with these variables (Poulin, 1998). The interactions between site and limpet size, site and parasite size, and site and total number of parasites were also evaluated. A model selection approach on the basis of Akaike Information Criteria (AIC; MacNally, 2000; Johnson and Omland, 2004) was used to assess the predictive power of the independent variables. In total, 26 models were assessed for each reproductive parameter (i.e., egg sizes and number of eggs), and models were compared and selected with likelihood ratio tests (LRT tests). Additionally, the relative explanatory power of each variable was assessed using hierarchical partitioning (Chevan and Sutherland, 1991; MacNally, 2000). In hierarchical partitioning, all possible models in a multiple regression setting are jointly considered to identify the most likely causal factor. This process involves computation of the increase of the fit of all models with a particular factor compared with an equivalent model without that factor (see Chevan and Sutherland, 1991; MacNally, 2000; Heikkinen et al., 2005, for more details). Hierarchical partitioning provided a relative explanatory power for each variable, which is segregated into independent and joint effects (i.e., caused jointly with other variables). Large, positive joint effects (relative to their independent effects) indicated highly colinear variables, whereas negative values indicated that a variable acted as a suppressor variable (Chevan and Sutherland, 1991). Statistical significances of the independent contributions of variables were tested by a randomization routine that yielded Z scores for the generated distribution and a measure of statistical significance based on an upper 0.95 confidence limit (Heikkinen et al., 2005).

To meet the assumption of normality, egg number was square root-transformed. Statistical analyses were performed with R 2.2.1 (lm function for the mixed models and hier.part function for the hierarchical partitioning; R Development Core Team 2005, http://www.r-project.org/).
between the 2 harvested sites (contrast tests, LCS vs. LCN, \( P > 0.05 \)).

Hierarchical partitioning showed that the explanatory power was shared relatively evenly among the 4 main effects when considering egg size (Fig. 2A). Indeed, the 3 factors (i.e., site, limpet size, and parasite number) had significant independent effects, and overall joint effects between variables were relatively high. In contrast, when considering egg number, the independent effect of site explained 60% of the variation, whereas other variables had nonsignificant independent effects (Fig. 2B). Moreover, joint effects had a low contribution in explaining egg number variation.

### DISCUSSION

The establishment of a small marine reserve in a rocky intertidal zone of central Chile led to important changes in the prevalence and intensity of infection by *P. lintoni* (Loot et al., 2005). We compared clutch size and egg size of the progenetic *P. lintoni* metacercariae among physically similar environments showing differences in host community composition, and we showed that the parasite reproductive strategies varied at a small spatial scale. Specifically, we demonstrated that *P. lintoni* shifted from the production of many small eggs in the marine reserve to the production of few large ones in harvested areas. Although our analysis did not control for all confounding factors, our results suggest that harvesting of coastal marine communities could be a significant factor that shapes such a response.

Individual parasite reproductive output is partitioned between offspring quality and quantity. Our results show a clear difference between factors that influence egg size and number. For instance, we found that egg size is determined by limpet size,
parasite site, number of conspecifics, and site, whereas egg number is only determined by the site effect.

Several studies have shown that parasite reproductive strategy is affected by the general quality of the host, on the basis of its age, size, diet, or immune status (Ito et al., 1986; Poulin, 1996; Tsai et al., 2001; Rossin et al., 2005). In our study, we have demonstrated that parasites within limpets of large body size will produce bigger eggs rather than maximizing egg number. Because our sampling effort was not specifically designed to test for relationships between host size and parasite reproductive output, these latter results need to be considered with caution.

On the other hand, the positive relationship between parasite body size and fecundity has been largely documented in a range of free-living taxa (Stearns, 1992; Roff, 2002; Einum and Flemming, 2004). For parasites, Rossin et al. (2005) showed that both egg number and egg volume of the endoparasitic nematode Graphidiooides subterraneus were positively correlated with female body size. However, Lefebvre and Poulin (2005) demonstrated a nonsignificant relationship between parasite parasite size and the number of eggs for the progenetic trematode Coitocoeicum parvum. In parallel with the latter study, we did not find evidence for a positive relationship between parasite size and individual reproductive output. This can be explained by the parasite’s reproductive size within the limpet host, which is at the minimal size at which it can lay eggs (see Lefebvre and Poulin, 2005).

Changes in egg numbers and egg size could also be explained by the number of conspecifics sharing a host, which should influence the parasite’s per capita average fecundity (Anderson, 1993). For instance, experimental studies suggest that high intensity of infection has a negative effect on per capita egg production in many helminths (Jones et al., 1989). In our study, statistical analyses revealed that conspecific effects were relatively weak in explaining egg size and nonsignificant in explaining egg number.

The most striking finding of our study is that site represents a significant determinant to predict and explain parasite egg number. The differences between harvested and nonharvested areas could be related to the alteration of host communities by human harvesting. Alternatively, we could hypothesize that these patterns resulted from differences in abiotic factors. However, because the 2 human-harvested areas (LCS and LCN) were the most spatially distant (see Fig. 1 in Loot et al., 2005), they should exhibit larger differences in abiotic factors and thus in parasite egg number. On the contrary, these 2 harvested sites showed similar parasite fecundity, thus strengthening our first hypothesis.

In the marine reserve (ECIM) ground cover of the first intermediate host (P. purpuratus) was low compared with harvested areas (Loot et al., 2005). Thus, in ECIM, massive larval mortality could occur during the transmission of P. lintoni to P. purpuratus. It is generally assumed that the evolution of parasite reproductive strategies has been driven by the likelihood of survival and host infection during the transmission phases of the life cycle (Jennings and Calow, 1975; Calow, 1983). Therefore, we suggest that the local modification of clutch size in the marine reserve could be a response to the low percentage of cover of mussels and could promote the encounter between eggs/larvae and mussels. In marine systems, tidal flows can cause substantial transport of small-bodied animals, and thus, important gene flow of parasite and invertebrate is generally assumed (e.g., Vélix et al., 2004). Thus, the response we observed was more likely purely plastic. Some evidence of phenotypic plasticity in other parasite systems supports this line of argument. For instance, Poulin (2003) demonstrated that the parasitic trematode C. parvum can accelerate its development in its amphipod intermediate host in the absence of chemical cues emanating from its definitive host. He argued that C. parvum detects the physiological changes in its crustacean host associated with predator-induced responses. This led him to conclude that the trematode can use information about its opportunities of transmission to adjust its life history schedule. In this study, we hypothesize that P. lintoni responds to maximize encounter rate with mussel hosts, but it is unlikely that parasites are able to detect changes in population density of mussels by emanating cues from the mussels. However, limpets recruit into mussel beds (Castilla, 1999) and live in their proximity to seek refuge from predators. Differences in mussel abundance might, therefore, cause changes in limpet physiology or behavior that are detectable to reproductive parasites.

Our study illustrates a case in which changing environmental conditions (the removal of invertebrate hosts) can induce adjustments of parasite life history traits. Even if our study has considered relatively few study sites and our knowledge is still too limited to account for the specific mechanism behind the observed patterns, it seems essential to consider parasite life history strategies when predicting long-term population changes of human-exploited species.

ACKNOWLEDGMENTS

This study was supported by a Lavoisier Fellowship from the French Ministry to G.L. and funding by the Andrew Mellon Foundation to S.A.N., for which we are most grateful. Additional funding was provided by a grant Fondecyt-FONDAP 15001-0001 to the Center for Advanced Studies in Ecology and Biodiversity. We are grateful to David J. Paëz for his helpful comments on this manuscript and for correcting the English. We would like thank R. Poulin for his helpful comments.

LITERATURE CITED


NEW SPECIES OF Rhabdosynochus Mizelle and Blatz 1941 (Monogenoidea: Diplectanidae) from the Gills of Centropomid Fishes (Teleostei) Off the Pacific Coast of Mexico

Edgar F. Mendoza-Franco, Juan Violante-González, and Víctor M. Vidal-Martínez

Smithsonian Tropical Research Institute, Naos Island Laboratories, Apartado 0843-03092 Balboa, Ancon, Panama, Republic of Panama. e-mail: oberon.men@gmail.com

ABSTRACT: In the course of the investigations into the fish parasites in the Tres Palos Lagoon in the State of Guerrero off the Pacific coast of Mexico, the following diplectanid species (Monogenoidea) from the gills of centropomids were found: Rhabdosynochus alterinstitus n. sp. from Centropomus nigrescens; Rhabdosynochus lituparus n. sp., Rhabdosynochus volucris n. sp., and Rhabdosynochus liligauus n. sp. from Centropomus robalito (Centropomidae). The apparent synonymous ic character supporting a sister relationship of these diplectanids is a single, sheathlike accessory piece comprising 3 distal branches of the male copulatory organ. The origin of the present diplectanids on centropomids is discussed, and it is suggested that this may be the result of allopatric speciation as a result of the uplift of the Panamanian Isthmus, thereby separating the Pacific and Atlantic Oceans during Pleistocene (3–5 million yr ago).

Species of Centropomus Lacépède, 1802, the only genus within the Centropomidae, fishes commonly known as snooks or robalos, are widely distributed in the eastern Pacific and western Atlantic coastal waters, and associated estuaries and rivers (Rivas, 1986; Tringali et al., 1999). Within this latter geographical area centropomids (Centropomus undecimalis [Bloch, 1792] [wild and cultivated] and Centropomus ensiferus Poey, 1860, of Florida and Puerto Rico, respectively) are parasitized by species of Rhabdosynochus rhabdosynochus Mizelle and Blatz, 1941, Rhabdosynochus hargisi Kritsky, Boeger, and Robaldo, 2001, and Rhabdosynochus hudsoni Kritsky, Boeger, and Robaldo, 2001 (see Kritsky et al., 2001). In the Tres Palos Lagoon, situated on the Mexican coast of the Pacific Ocean, in the State of Guerrero, centropomids are commercially exploited as food fish (Secretaría de Pesca, 1994; Briones, 1998). However, the parasite fauna of these fishes in the Tres Palos Lagoon is insufficiently known; most information is limited to helminths from species of other host families, i.e., Dormitator latifrons (Richardson, 1844) (Eloietridae) and Ariopsis guatemalensis Günther, 1864 (Ariidae) (Garrido-Olvera et al., 2003; Pilo, 2004; Gopar-Merino et al., 2005). Until recently, only 1 monogeneoid species was described from the gills of Centropomus nigrescens Günther 1864 (see Mendoza-Franco et al., 2006). A subsequent examination of gill samples of centropomids collected in 2003 from that lagoon was made to generate further information regarding monogenean parasites of these hosts. Four new species of Rhabdosynochus (Diplectanidae) were found parasitizing gills of C. nigrescens and Centropomus robalito Jordan and Gilbert, 1882, during this research. The results of the systematic evaluation of the monogeneoids collected are described herein and a possible explanation about diversification of this parasite genus of tropical and subtropical centropomids is briefly discussed.

MATERIALS AND METHODS

Hosts were captured by hook-and-line and throw nets from August to November 2003 in the coastal lagoon of Tres Palos (16°47′N, 99°39′W) at the Pacific coast of the Guerrero state, Mexico (see Fig. 1 in Mendoza-Franco et al., 2006). The gills of each fish were removed and placed in fingers bowls containing 4–5% formalin solution to fix any of the ectoparasites that might be present. Subsequently, parasites were isolated and stained with Gomori’s triochrome and mounted in Canada balsam. In addition, some specimens were mounted unstained in gray and Wess’s medium to obtain measurements and line drawings of haptors structures and the copulatory complex. All other measurements were obtained from unflattened specimens stained with Gomori’s triochrome. Drawings were made with the aid of a drawing tube using an Olympus microscope with Nomarski interference contrast. Measurements, all in micrometers, represent straight-line distances between extreme points and are expressed as range and number (n) of structures measured in parentheses; body length includes that of the haptor. Numbering of hook pairs follows the scheme illustrated in Kritsky et al. (2001). Type specimens are deposited in the National Helminthological Collection of Mexico (CNHE), Institute of Biology, National Autonomous University of Mexico, Mexico; and the United States National Parasite Collection, Beltsville, Maryland (USNPC) as indicated in the respective descriptions. For comparative purposes, the following specimens were examined: 6 paratypes of R. hargisi (USNPC 89784).

DESCRIPTION

Rhabdosynochus alterinstitus n. sp.

(Figs. 1–8)

Description: Body 579 (500–650; n = 21) long, broad posteriorly; 118 (100–142; n = 23) wide at level of germinarium. Scales on body surface not observed. Cephalic margin broad; cephalic lobes moderately developed; cephalic glands at level of pharynx. Eyes 4; members of posterior pair larger and closer together than members of anterior pair; accessory granules usually absent or few in cephalic area. Pharynx sub-spherical, 32 (25–38; n = 20) in diameter; esophagus short. Peduncle broad, with narrow bilateral membranous frills; haptor 254 (217–282; n = 20) wide. Anchors dissimilar; ventral anchor 53 (50–55; n = 11) long, with elongate roots, curved shaft, recurved point extending to level of tip of superficial root; deep root channelled; anchor base 19 (17–23; n = 6) wide. Dorsal anchor 43 (40–46; n = 34) long, with triangular base, slightly curved shaft, point extending slightly past level of tip of superficial anchor root; anchor base 16 (15–18; n = 19) wide. Ventral bar 186 (177–200; n = 17) long, narrow, elongate, with delicate tapered ends; paired dorsal bar 79 (70–89; n = 43) long, medially expanded. Hook 14 (13–15; n = 35) long, with elongate slightly depressed thumb, delicate point, uniform shank; filamentous hooklet (FH) loop nearly shank length. Copulatory complex 50 (45–55; n = 15) long. Male copulatory organ (MCO) a loose coil of about ½ ring, frequently appearing inverted U-shaped; base elongate ovate, with basal opening...
directed posteriorly. Accessory piece comprising 3 subunits: 1 variable, proximally hook shaped; 1 tongue shaped, proximally bifurcate; 1 comprising delicate sheath enclosing the distal portion of the copulatory organ. Testis 25 (20–37; n = 9) wide, subterminal; course of vas deferens in relation to gut not observed; seminal vesicle a simple dilation of vas deferens, lying along body midline; prostatic reservoir pyriform, lying dorsal to seminal vesicle; prostatic glands (lungs) anteroventral to MCO, from base to the U-shaped shaft of the copulatory organ. Germarium 49 (33–68; n = 21) wide, looping right intestinal cecum; oviduct observed, ootype not observed; vagina sclerotized, funnel shaped, opening into small seminal receptacle near midline; vitellaria dense throughout trunk, except in regions of reproductive organs.

**Taxonomic summary**

*Type host:* Yellowfin snook *C. robalo* (Centropomidae).
*Site of infection:* Gill.
*Type locality/collection date:* Tres Palos lagoon, Pacific coast of Guerrero, Mexico, August 2003.
*Specimens deposited:* Holotype, CNHE (5796); 12 paratypes, CNHE (5797), and 7 paratypes, USNPC (99632).

*Prevalence and intensity of infections:* Twenty fish (average size 26 ± 5 cm of total length) infected of 49 examined (41%); mean intensity of infection 7 ± 4 worms per infected fish.

*Etymology:* The specific name is from Latin *altera* (= another, a second) and by the position and dimension of the vagina (on the left margin with diagonal aperture [33–40 wide] oriented to body midline vs. horizontal opening [13–18 wide], 6 paratypes USNPC 89784) on the middle portion of the body in *R. hargisi*. Prostatic cells surrounding copulatory organ were clearly stained in all studied specimens of *R. alterinotus* n. sp.

*Remarks* This species resembles *Rhabdosynochus volucris* n. sp. by the comparative morphology of the copulatory complex. In *R. lituparvus* n. sp., the vaginal tube opening into seminal receptacle is longer than that found in *R. volucris*.

*Rhabdosynochus volucris* n. sp. (Figs. 16–23)

*Description:* Body 318 (242–397; n = 31) long, rod-shaped; 59 (37–77; n = 31) wide near level of testis. Tegument smooth. Cephalic margin broad; cephalic lobes moderately developed; cephalic glands at level of pharynx. Eyes 4; members of anterior pair of eyes smaller and closer together than members of posterior pair; accessory granules usually absent or few in cephalic area. Pharynx subcylindrical, 20 (13–26; n = 33) in greatest width; esophagus short to nonexistent. Peduncle broad; haptor 94 (70–130; n = 18) wide. Anchors dissimilar; ventral anchor 31 (27–34; n = 30) long, with well-developed roots, evenly curved shaft, nonreversed point reaching level of tip of superficial anchor root; anchor base 11 (9–12; n = 16) wide. Dorsal anchor 30 (28–32; n = 40) long, with triangular base, straight shaft, nonreversed point extending past tip of superficial anchor root; anchor base 9 (8–10; n = 21) wide. Ventral bar 56 (52–60; n = 18) long, slender with tapered ends, slightly constricted midregion, ventral groove; paired dorsal bar 27 (25–31; n = 52) long, robust, straight with expanded medial end, subterminal lateral inflation. Hook 11 (11–12; n = 51) long, with elongate, slightly depressed thumb, delicate point, uniform shank; FH loop nearly shank length. Copulatory complex 36 (26–44; n = 36) long. MCO an ascendant sclerotized straight tube, twisted to form a poorly defined ring; basal opening directed posteriorly. Accessory piece comprising 3 distal branches, opposite E-shaped, elongate lateral branches like wings, medial branch enclosing distal end of the copulatory organ. Testis 27 (25–30; n = 4) in diameter, subcylindrical, course of vas deferens not observed; seminal vesicle a simple dilation of vas deferens, lying on left side of body midline, dorsal to vagina; prostatic reservoir not observed. Germarium 31 (25–48; n = 25) wide, elongate pyriform, diagonal, looping right intestinal cecum, slightly overlapping testis; oviduct, ootype not observed; vagina slightly sclerotized, corrugated bulb shaped, opening into small seminal receptacle at midline; vitellaria co-extensive with gut, absent in regions of reproductive organs.

*Taxonomic summary*  

*Type host:* Yellowfin snook *C. robalo* (Centropomidae).
*Site of infection:* Gill.
*Type locality/collection date:* Tres Palos lagoon, Pacific coast of Guerrero, Mexico, August 2003.
*Specimens deposited:* Holotype, CNHE (5800); 20 paratypes, CNHE (5801), and 15 paratypes, USNPC (99634).

*Prevalence and intensity of infections:* Twenty fish (average size 19 ± 2 cm of total length) infected of 69 examined (28%); mean intensity of infection 2 ± 1 worms per infected fish.
± 2 cm of total length) infected of 47 examined (42%); mean intensity of infection 8 ± 4 worms per infected fish.

**Etymology:** The specific name from Latin reflects the morphology of the lateral branches of the accessory piece (volucris = flying, winged).

**Remarks**

On the basis of comparative morphology of the copulatory complex, *R. volucris* n. sp. most closely resembles *R. lituparvus* n. sp. It differs from this species by having a robust and straight dorsal bar with expanded medial end and a subterminal lateral inflation (rod-shaped with spathulate medial end, subterminally arced in *R. lituparvus* n. sp.) and by having a dorsal anchor without a narrow deep root (dorsal anchor with a narrow deep root in *R. lituparvus* n. sp.).

**Rhobdosynochus siliquaus* n. sp.**

*(Figs. 24–30)*

**Description:** Body 339 (275–425; n = 18) long, fusiform, tapered anteriorly; 60 (45–80; n = 18) wide near body midlength. Cephalic margin broad; cephalic lobes moderately developed; cephalic glands at level of pharynx. Eyes 4; members of posterior pair of eyes larger and closer together than members of anterior pair; accessory grooves usually absent or few in cephalic area. Pharynx subcylindrical to ovate, 20 (17–23; n = 19) in greatest width; esophagus short to nonexistent. Peduncle narrow; haptor 144 (100–180; n = 10) wide. Anchors dissimilar; ventral anchor 50 (47–54; n = 23) long, with elongate roots (deep root longest), curved shaft and point extending slightly past level of tip of superficial root; anchor base 14 (13–15; n = 3) wide. Dorsal anchor 50 (46–53; n = 27) long, with triangular base, thimble-like deep root, evenly curved shaft, straight recurved point extending past tip of superficial root; anchor base 14 (12–15; n = 16) wide. Ventral bar 93 (80–118; n = 14) long, undulate, with tapered ends, ventral groove; paired dorsal bar 38 (34–46; n = 43) long, with postero-medial spinous projection at proximal end, subterminally arced. Hook 11 (10–12; n = 27) long, with elongate, slightly depressed thumb, delicate point, uniform shank; FH loop nearly shank length. Copulatory complex 27 (21–37; n = 23) long, with elongate, slightly depressed thumb, delicate point, uniform shank; FH loop nearly shank length. Copulatory complex 27 (21–37; n = 12) long. Anchors dissimilar; ventral anchor 50 (47–54; n = 23) long, with elongate roots (deep root longest), curved shaft and point extending slightly past level of tip of superficial root; anchor base 14 (13–15; n = 3) wide. Dorsal anchor 50 (46–53; n = 27) long, with triangular base, thimble-like deep root, evenly curved shaft, straight recurved point extending past tip of superficial root; anchor base 14 (12–15; n = 16) wide. Ventral bar 93 (80–118; n = 14) long, undulate, with tapered ends, ventral groove; paired dorsal bar 38 (34–46; n = 43) long, with postero-medial spinous projection at proximal end, subterminally arced. Hook 11 (10–12; n = 27) long, with elongate, slightly depressed thumb, delicate point, uniform shank; FH loop nearly shank length. Copulatory complex 27 (21–37; n = 12) long. MCO a loose coil of about 1 ring; base with delicate sclerotized basal margin; MCO opening directed posteriorly. Accessory piece comprising 3 subunits: 1 podlike, sigmoid; 1 with distal hook; 1 comprising delicate sheath enclosing the U-shaped shaft of the copulatory organ. Testis 20 (16–25; n = 16) in diameter, subcylindrical; vas deferens not observed; seminal vesicle a simple dilation of vas deferens, lying on left side of body midline, dorsal to vagina; prostatic reservoir not observed. Germarium 31 (25–48; n = 12) wide, looping right intestinal cecum; oviduct, ootype not observed; vagina slightly sclerotized, bulb shaped, opening into small seminal receptacle midline; vitellaria throughout trunk, except absent in regions of reproductive organs.

**Taxonomic summary**

**Type host:** Yellowfin snook *C. robalito* (Centropomidae).

**Site of infection:** Gill.

**Type locality/collection date:** Tres Palos lagoon, Pacific coast of Guerrero, Mexico, August 2003.

**Specimens deposited:** Holotype, CNHE (5802); 10 paratypes, CNHE (5803), and 3 paratypes, USNPC (99635).

**Prevalence and intensity of infections:** Seventeen fish (average size 19 ± 2 cm of total length) infected of 47 examined (36%); mean intensity of infection 5 ± 3 worms per infected fish.

**Etymology:** The specific name is from Latin (siliqua = pod, shell) and refers to the shape of the accessory piece.

**Remarks**

This species is easily differentiated from its congenic species by having a podlike accessory piece and a postero-medial spinule-like projection on the dorsal bar. A spinous projection at the proximal end has also been reported in other species of the Diplectanidae, i.e., *R. hudsoni* and *Prototamellodiscus semifalbus* Krisky, Jiménez-Ruiz, and Sey, 2000 (Kritsky et al., 2000, 2001).

**DISCUSSION**

This study represents the first finding of species of *Rhobdosynochus* (*R. alterinstitus* n. sp, *R. lituparvus* n. sp., *R. volucris* n. sp., and *R. siliquaus* n. sp.) on centropomids (*C. nigrescens* and *C. robalito) from the Pacific coast of Mexico. Results presented herein show that these species of *Rhobdosynochus* more closely resemble each other than the known species from the Atlantic (*R. rhobdosynochus*, *R. hargisi*, and *R. hudsoni* from *C. undecimalis* of Florida and *C. eniferus* of Puerto Rico [see Kritsky et al., 2001], suggesting that the Pacific forms have undergone allopatic speciation since isolation and subsequent divergence of a common ancestor within this latter geographical area, i.e., by allopatic separation initiated by the rise of Central American Isthmus (3 million yr ago [mya]) (Tringali et al., 1999). The apparent synapomorphic character supporting sister relationships of *Rhobdosynochus* from the Pacific is the sheathlike accessory piece comprising 3 distal branches (as an accessory piece comprising several subunits in species from the Atlantic) (Kritsky et al., 2001; present study).

In a phylogenetic hypothesis for Centropominae grounded on molecular data (Tringali et al., 1999), 4 major clades, each representing a total of 12 species of *Centropomus*, are present. On the basis of this hypothesis, a sister-species relationship is suggested between *C. robalito* (from the Pacific) basal lineage and the clade containing *C. undecimalis* (from the Atlantic). Analysis of this hypothesis indicates that the species from the Pacific appear to have diverged during the Pliocene rise of the isthmus (3 mya), while the *C. undecimalis* group diverged around the time of the late Miocene closure (24 mya) (Tringali et al., 1999). On the basis of this hypothesis and host specificity of *Rhobdosynochus*, the common ancestor for this parasite genus could have originated on an early marine centropomid form inhabiting both the Atlantic and Pacific (before formation of the Panamanian Isthmus) followed by its Neotropical forms closely tied to estuarine habitat in the Pacific waters. Furthermore, ecological diversification within *Centropomus* appears to be reflected in the size, i.e., centropomids from the Pacific are small-bodied species compared with those from the Atlantic (Tringali et al., 1999).

In this context, species of *Rhobdosynochus* from *C. undecimalis* and *C. eniferus* in the Atlantic waters probably have had more opportunity to specialize on predictable resources, i.e., those that are stable through time, thus minimizing extinction risks. For example, most *Lamellodiscus* (Diplectanidae) specialists that infect marine fish in the Mediterranean tend to use a larger host than generalist (Desdevises et al., 2002). So, if this applies for *Rhobdosynochus* spp., then probably determinants of host specificity and taxonomic diversification of this parasite genus are highly constrained by phylogeny, but also linked to host size.

This present paper represents the third report of diplectanids from the west coast of Mexico. *Heteroplectanum oliveri* Leon-Régagnon, Pérez Ponce de León, and García Prieto, 1997 (Diplectanidae) from *Kyphosus elegans* (Kyphosidae) and *Cormutahaptor nigrescens* Mendoza-Franco, Violante-González, and Vidal-Martínez, 2006 (Diplectanidae) from *C. nigrescens* (Centropomidae) were originally described from the Mexican coast of the Pacific Ocean (Chamela Bay, in the State of Jalisco and the Tres Palos Lagoon, in the State of Guerrero, respectively) (see Leon-Régagnon et al., 1997; Mendoza-Franco et al., 2006). Along the Pacific coast of Mexico only 2 (*C. nigrescens* and *C. robalito*) of 6 species of *Centropomus* have been studied for monogenoids (Mendoza-Franco et al., 2006; present study). It
is apparent that further survey of the *Rhabdosynochus* group infesting *Centropomus* spp. from the eastern Pacific (between Gulf of California and Peru) and the western Atlantic (between the Florida peninsula and Brazil) will be necessary to understand the evolutionary history of *Rhabdosynochus* spp. in the subtropical and tropical areas.

**ACKNOWLEDGMENTS**

The authors are grateful to Drs. Pat Pilitt and Eric Hoberg, USNPC, for loan of paratype specimens of *R. hargisi* (No. 89784). Delane C. Kritsky (Idaho State University) read an earlier draft of the manuscript and we thank him for his corrections and comments. The final preparation of this contribution was conducted during the postdoctoral stay of E.F.M.F. at the Smithsonian Tropical Research Institute, Republic of Panama.

**LITERATURE CITED**


**MENDOZA-FRANCO ET AL.—NEW SPECIES OF RHABDOSYNOCRUNS**


**PILO, T.** 2004. Evaluación de los parasitos metazoarios que atacan a *Mugil curema, Dormitator latifrons, Ariopsis guatemalensis, Eleotris pictus, Cichlasoma trimaculatum, Gobiomorus maculatus, Centropomus robalito* y *Centropomus nigrescens*, especies de mayor consumo local, que se capturan en la laguna de Tres palos Guerrero, México. M.S. Thesis. Universidad Autónoma de Guerrero, Guerrero, Mexico, 106 p.


ENDEMIC TOXOPLASMOSIS IN PIGS ON A FARM IN MARYLAND: ISOLATION AND GENETIC CHARACTERIZATION OF TOXOPLASMA GONDII


U.S. Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, Maryland 20705-2350. e-mail: jitender.dubey@ars.usda.gov

ABSTRACT: The prevalence of Toxoplasma gondii was investigated on a poorly managed pig farm in Maryland. Serum and tissue samples from 48 of the 100 pigs on the farm were available for T. gondii evaluation. Serological testing was performed using both ELISA and the modified agglutination test (MAT). Antibodies to T. gondii were detected by ELISA in 12 of 48 animals, while antibodies were detected in 34 of 48 pigs by MAT with titers of 1:10 in 3, 1:20 in 4, 1:40 in 7, 1:80 in 3, 1:160 in 8, 1:320 in 3, 1:640 in 4, and 1:1,280 in 4. Hearts of 16 pigs with MAT titers of >1:10 or higher were bioassayed for T. gondii in cats; 11 cats shed T. gondii oocysts. Hearts of 22 pigs were autolyzed and bioassayed only in mice; T. gondii was isolated from 3 of these 22 pigs. Genetic typing of the 14 T. gondii isolates using the SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico loci revealed 4 genotypes; 10 isolates belonged to type II lineage (genotypes 1 and 2), 3 belonged to genotype 3, and 1 belonged to genotype 4. Genotype 1 and 2 have type II alleles at all genetic loci, except the former has type II allele and the latter has a type I allele at locus Apico. Both genotypes 1 and 2 are considered to belong to the clonal type II lineages. Genotype 3 and 4 are nonclonal isolates. Results document high prevalence of T. gondii in pigs on a farm in Maryland.

The protozoan Toxoplasma gondii infects virtually all warm-blooded animals, including humans, livestock, and marine mammals (Dubey and Beattie, 1988; Dubey et al., 2003). In the United States, various surveys have found that 10–50% of the adult population has been exposed to this parasite (Dubey and Beattie, 1988; Jones et al., 2001, 2003, 2007). Toxoplasma gondii infection causes mental retardation, loss of vision, and other congenital health problems in humans. Toxoplasmosis is thought to be as high as $8.8 billion in the United States (Mead et al., 1999). In a recent nationwide study of the prevalence of T. gondii in retail meat, viable T. gondii was isolated from 7 of 2,094 pork samples, but not from 2,094 beef or 2,094 chicken meat samples (Dubey et al., 2005). Thus, while the scope of human infection resulting from meat sources remains undetermined, by exclusion, pigs may be considered the most important meat-borne source in the epidemiology of toxoplasmosis in the United States.

In the United States, infection was estimated in 23.9% of pigs in 1983–1984, with higher prevalence in breeders (42%) than in market pigs (23%) (Dubey et al., 1991). When pigs from these same areas were tested in 1992, prevalence had dropped to 20.8% of breeders and 3.1% of finisher pigs (Dubey, Weigel, Siegel et al., 1995). Prevalence of T. gondii was 20% in sows tested in the 1990 National Animal Health Monitoring System (NAHMS) swine survey and had fallen to 15.0% in sows and 3.2% in finisher pigs in the 1995 NAHMS swine survey (Patton et al., 1996; S. Patton, pers. comm.). The prevalence of T. gondii in pigs is influenced by management systems. In the northeastern United States, where pigs are managed largely in non-confinement systems, prevalence runs as high as 47.4% (Gamble et al., 1999). Viable T. gondii was isolated from 51 of 55 finisher pigs from a farm in Massachusetts (Dubey, Gamble et al., 2002).

Currently, there is no national identification system for individual pigs destined for human consumption, and pigs are not tested for T. gondii infection at slaughter. Therefore, the routes by which T. gondii-infected pigs from highly endemic areas enter the market and the role these pigs have in the overall epidemiology of T. gondii in humans remains unknown. In the present paper, we document high prevalence of T. gondii in pigs on a farm in Maryland. To identify these parasite isolates with high discriminative power, we performed genotyping using multilocus PCR-RFLP markers (Dubey et al., 2007). These data would allow us to compare parasite strains from different sources and to potentially identify the reservoirs that transmit T. gondii to humans.

Received 2 May 2007; revised 28 June 2007; accepted 1 July 2007.

* Maryland Department of Agriculture, Frederick Animal Health Laboratory, 1840 Rosemont Avenue, Frederick, Maryland 21702.
† Present address: Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology, 733 N. Broadway, 8th Floor, Baltimore, Maryland 21205-2196.
‡ Laboratoire de la Toxoplasmose, Institut de Puériculture, 26 Boulevard Brune, F-75014, Paris, France.
§ Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845.
TABLE I. Isolation of T. gondii from Maryland pigs by bioassay in cats (batch 1).

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Date killed</th>
<th>T. gondii serology in pig</th>
<th>Oocysts shed by cat no.*</th>
<th>Oocysts fed to mice</th>
<th>Sub passage of tachyzoites to mice</th>
<th>Strain designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>755948</td>
<td>18 July 2006</td>
<td>0.261 80</td>
<td>293 DK4,† DK4</td>
<td>K51, K51</td>
<td>TgPgUs1</td>
<td></td>
</tr>
<tr>
<td>755949</td>
<td>18 July 2006</td>
<td>0.224 40</td>
<td>307 DK5, DK5</td>
<td>K50, K50</td>
<td>TgPgUs2</td>
<td></td>
</tr>
<tr>
<td>755952</td>
<td>18 July 2006</td>
<td>0.594 160</td>
<td>313 DK5, DK5</td>
<td>K50, K50</td>
<td>TgPgUs3</td>
<td></td>
</tr>
<tr>
<td>755955</td>
<td>18 July 2006</td>
<td>0.604 160</td>
<td>308 K41, K41</td>
<td>K57, K57</td>
<td>TgPgUs4</td>
<td></td>
</tr>
<tr>
<td>755956</td>
<td>18 July 2006</td>
<td>0.617 160</td>
<td>305 DK4, DK4</td>
<td>K51, K51</td>
<td>TgPgUs5</td>
<td></td>
</tr>
<tr>
<td>755960</td>
<td>20 July 2006</td>
<td>0.251 80</td>
<td>303A DK5, DK5</td>
<td>K50, K50</td>
<td>TgPgUs6</td>
<td></td>
</tr>
<tr>
<td>755962</td>
<td>20 July 2006</td>
<td>0.351 80</td>
<td>296 DK4, DK4</td>
<td>K51, K51</td>
<td>TgPgUs7</td>
<td></td>
</tr>
<tr>
<td>755963</td>
<td>27 July 2006</td>
<td>0.311 40</td>
<td>303B DK4, DK4</td>
<td>K51, K51</td>
<td>TgPgUs8</td>
<td></td>
</tr>
<tr>
<td>755965</td>
<td>27 July 2006</td>
<td>0.824 160</td>
<td>294 DK4, DK4</td>
<td>K51, K51</td>
<td>TgPgUs9</td>
<td></td>
</tr>
<tr>
<td>06CP-4504</td>
<td>06 October 2006</td>
<td>0.221 160</td>
<td>339 DK4, DK4</td>
<td>K67, K67</td>
<td>TgPgUs10</td>
<td></td>
</tr>
<tr>
<td>06CP-4505</td>
<td>06 October 2006</td>
<td>0.056 20</td>
<td>429 DK4, DK4</td>
<td>K67, K67</td>
<td>TgPgUs11</td>
<td></td>
</tr>
</tbody>
</table>

* Fed pig tissues.
† K = killed in good health, DK = killed in comatose condition followed by the day PI.

MATERIALS AND METHODS

Naturally infected pigs

Serum and tissues were collected from 48 pigs (7 wk of age to adult, mixed sex) located on a farm in northwestern Maryland (39°32′31″N, 77°06′07″W). Approximately 100 pigs were allowed to roam freely on the farm and onto neighboring properties. The nutritional status of the pigs was low, and animals were observed to feed on the remains of pigs or other livestock that had died and whose carcasses were not properly discarded. Additional foodstuffs provided included ice cream waste contained in 19-L buckets and offal from the butcher operation on the farm. Other animals and birds, including sheep, cattle, llamas, and ducks, were observed to roam freely on the farm. Dead rats were also observed on the premises. The farm buildings and surrounding fences were in disrepair; abandoned vehicles, piles of trash, and garbage, including carcasses and bones, were evident. Ten pigs were humanely killed on the premises; blood (serum) and heart were collected from each animal. Thirty-eight escaped pigs were captured (five trapped) and subsequently killed; 22 of these carcasses were at room temperature for 3.5 days before they were available examination for T. gondii; only fluids from the autolyzed heart tissues was available for serological analysis.

Serological examination

At the time of slaughter, blood was collected from the first 26 pigs (batch 1) into serum separator tubes; serum was collected by centrifugation and stored frozen until used. Serum and tissue fluids were collected from 22 severely autolyzed pigs (batch 2) by centrifugation of heart tissue. Collected fluids were stored frozen until used.

Sera or tissue fluids of pigs were tested for T. gondii antibodies by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987) using 2-fold serum dilutions from 1:10 to 1:1,280. Duplicate serum samples were tested by ELISA for the presence of T. gondii antibodies using a commercial ELISA kit as recommended by the manufacturer (SafePath Laboratories, Carlsbad, California; Gamble et al., 2005). Sera were tested at a 1:50 dilution, and positive and negative control pig sera supplied by the manufacturer were included on each ELISA plate. ELISA values were reported as the mean of duplicate wells after subtraction of the optical density (OD) value for the negative control well. Optical densities that exceeded 0.2 after subtraction of the negative control OD value were considered positive.

Bioassay of pig tissues in cats

Twenty-six of the 48 pigs were killed between 18 July and 6 October 2006, and their tissues were in fairly good state of preservation. Hearts of 16 of these 26 pigs (batch 1) with a MAT titer of 1:10 or higher were bioassayed for T. gondii in cats as described (Dubey et al., 2005) (Table I). For this, the entire heart or 500 g of myocardium of each of the 16 pigs was fed separately to 16 T. gondii–free cats. Feces of cats were examined for shedding of T. gondii oocysts 3–14 days after feeding swine tissues as previously described (Dubey, 1995). Fecal floats were incubated in 2% sulfuric acid for 1 wk at room temperature on a shaker to allow sporulation of oocysts and were bioassayed by oral administration to mice (Dubey and Beattie, 1988). Four or 5 days after feeding oocysts, mesenteric lymph nodes of mice that died or were killed were removed and, after ascertaining the presence of tachyzoites, homogenates of lymph nodes were inoculated into new mice to exclude Hammondia hammondi infection (Dubey and Beattie, 1988). Tissue imprints of mice that died were examined for T. gondii tachyzoites or tissue cysts. Survivors were bled on day 40 postinoculation (PI) and a 1:25 dilution of serum from each mouse was tested for T. gondii antibodies using the MAT. Mice were killed 6 wk PI, and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with T. gondii when tachyzoites or tissue cysts were found in tissues.

Bioassay of pig tissues in mice

Carcasses of 22 pigs (batch 2) killed between 6 and 10 October 2006 were badly autolyzed and unfit for bioassay in cats. Blood or fluid was removed from each heart, and the myocardium was washed several times with running tap water to remove the smell. Tissues of all 22 pigs were bioassayed in mice, irrespective of antibody status. Fifty-gram portions of myocardium were homogenized in 0.85% NaCl (saline), digested in pepsin, centrifuged, neutralized, suspended in antibiotic saline, and inoculated subcutaneously into 4 outbred female Swiss Webster mice (Taconic Farms, Germantown, New York), as described (Dubey, 1995; Dubey, Graham, et al., 2002). Tissue impression smears of mice that died were examined for T. gondii tachyzoites or tissue cysts (Dubey, 1995; Dubey, Graham, et al., 2002). Survivors were bled 8 wk PI, and a 1:25 dilution of serum from each mouse was tested for T. gondii antibodies by using the MAT. Mice were killed 8–10 wk PI, and brain squashes from all mice were examined microscopically for tissue cysts as described previously (Dubey and Beattie, 1988). Mice were considered infected with T. gondii when tachyzoites or tissue cysts were demonstrable microscopically in tissue smears.

Genetic characterization

Toxoplasma gondii DNA was extracted from the tissues of infected mice and strain typing was performed using the genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, a new SAG2 and Apico with modification (Howe et al., 1997; Grigg et al., 2001; Khan, Su et al., 2005; Khan, Taylor et al., 2005; Su et al., 2006). In brief, the target DNA sequences were amplified by 2 groups of multiplex PCR using external primers. Group 1 included SAG1, SAG2,
TABLE III. Summary of genotyping of T. gondii isolates from Maryland pigs.

<table>
<thead>
<tr>
<th>T. gondii strain</th>
<th>SAG1</th>
<th>SAG2*</th>
<th>SAG2†</th>
<th>SAG3</th>
<th>BTUB</th>
<th>GRA6</th>
<th>c22-8</th>
<th>c29-2</th>
<th>L358</th>
<th>PK1</th>
<th>Apico</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgPgUs1</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs2</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs3</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs4</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs5</td>
<td>I</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>TgPgUs6</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs7</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs8</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs9</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs10</td>
<td>I</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>TgPgUs11</td>
<td>I</td>
<td>II</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs12</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs13</td>
<td>II or III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>TgPgUs14</td>
<td>I</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
</tr>
</tbody>
</table>

* SAG2 marker based on 5'- and 3'-ends of the gene sequence (Howe et al., 1997).
† A new SAG2 marker based on the 5'-end of the gene sequence (Su et al., 2006).
‡ nd = no data.

SAG3, BTUB, and GRA6 (Dubey et al., 2006), and group 2 included c22-8, c29-2, L358, PK1, the new SAG2, and Apico (Dubey et al., 2007). Multiplex PCR amplified products were then used for nested PCR with internal primers for each marker separately. Nested PCR products were treated with restriction enzymes and resolved in agarose gel by electrophoresis to reveal the RFLP patterns of the isolates. Allele types for all isolates were determined based on the RFLP patterns of 6 reference strains including RH88, PTG, CTG, COUGAR, MAS, and TgCatBr5 (Su et al., 2006). These reference strains allow us to capture most of all known alleles for each marker and to identify potential unique alleles in new samples.

RESULTS

Serology

MAT: Overall, antibodies to T. gondii were found in 34 of 48 pigs with MAT titers of 1:10 in 1, 1:20 in 4, 1:40 in 7, 1:80 in 3, 1:160 in 8, 1:320 in 3, 1:640 in 4, and 1:1,280 in 4. In batch 1, 16 of 26 (61.5%) were seropositive with MAT titers of 1:20 in 3, 1:40 in 2, 1:80 in 3, 1:160 in 2, 1:320 in 2, and 1:640 in 4. In batch 2, 18 of 22 (81.8%) were seropositive with MAT titers of 1:10 in 1, 1:20 in 1, 1:40 in 5, 1:160 in 1, 1:320 in 3, 1:640 in 3, and 1:1,280 in 4. Of the 14 pigs from whom T. gondii was isolated by cat or mouse bioassay, 1 had a MAT titer of <1:10, 1 had a titer of 1:20, and 12 had MAT titers of 1:40 or higher. Of the 14 pigs from which T. gondii was isolated by cat or mouse bioassay, 13 were also positive by MAT and 1 was negative.

ELISA: Antibodies were detected by ELISA in 12 of 48 pigs. Of the 14 pigs from which T. gondii was isolated by cat or mouse bioassay, 10 were also positive by ELISA and 4 were negative. The 10 ELISA-positive sera were from pigs in batch 1 (10 ELISA-positive of 11 cat bioassay positive pigs from batch 1); 3 ELISA-negative sera were from pigs in batch 2 (3 ELISA-negative of 3 mouse bioassay positive pigs from batch 2).

Isolation of T. gondii from batch 1 pigs

Toxoplasma gondii was isolated from the hearts of 11 pigs by bioassay in cats (Table I). Mice fed oocysts from each of these isolates became ill 4 days PI, and tachyzoites were found in their mesenteric lymph nodes. Mice inoculated with homogenates of lymph nodes became infected but remained healthy, and tissue cysts were found in their brains when killed 6 wk later (Table I).

Isolation of T. gondii from batch 2 pigs

Toxoplasma gondii was isolated from the hearts of 3 of the 22 pigs bioassayed in mice; none of the T. gondii–infected mice died of toxoplasmosis (Table II).

Toxoplasma gondii strain designation and genotyping

The 14 T. gondii isolates from pigs were designated TgPgUs 1–14 (Table III). Genetic analysis indicated that there were 4
genotypes (Table IV). Genotypes 1 and 2 have type II alleles at all genetic loci, except the former has type II allele and the latter has a type I allele at the Apico locus. Genotypes 1 and 2 are considered to belong to the clonal type II lineages. Genotypes 3 and 4 are nonclonal isolates. Of the 14 isolates, 10 isolates (TgPgUs1, 2, 3, 4, 6, 7, 8, 9, 12, 13) belong to type II lineage (genotypes 1 and 2) based on available genotyping data, 3 (TgPgUs5, 11, 14) belonged to genotype 3, and 1 (TgPgUs10) belonged to genotype 4. Because several isolates in type II lineages do not have data at locus Apico, all isolates of genotypes 1 and 2 are grouped together (Table IV).

**DISCUSSION**

**Antibody status and the parasite isolation**

*Batch 1 pigs*: Previous studies have demonstrated that the cat bioassay is a highly sensitive method for detection of *T. gondii* infection (Dubey and Frenkel, 1976; Dubey, 2001). It is considered the “gold standard” of detection methods, since cats become infected with very low numbers of bradyzoites. In the present study, viable *T. gondii* was isolated from the hearts of 13 MAT-seropositive and 1 MAT-seronegative pig, and 10 ELISA-seropositive and 4 ELISA-seronegative pigs. The antibody titer that should be considered specific for latent *T. gondii* infection has not been determined in any host, including humans, using the criteria of isolation of the viable parasite. Most of the work on validation of a serologic test for *T. gondii* has been done with the MAT of infected pigs (Dubey, Thulliez, Weigel et al., 1995. In a study of naturally infected sows, viable *T. gondii* was isolated from a total of 170 of 1,000 pigs. Of these 170 isolates, 141 pigs had MAT titers of 1:20 or higher (Dubey, Thulliez, and Powell, 1995). The remaining 29 isolates were from pigs with a MAT titer of <1:20; 17 of these pigs were found to have a MAT titer of 1:10. Thus, 12 infected pigs were seronegative at a 1:10 dilution. In another study involving market weight pigs, *T. gondii* was isolated from 51 of 55 pigs; 2 of the infected pigs were seronegative at a 1:10 dilution, and 1 had a titer of 1:10 (Dubey, Gamble et al., 2002). Therefore, even a low MAT titer of 1:10 maybe indicative of *T. gondii* infection in pigs.

The ELISA detected anti-*T. gondii* antibodies in 10 of 11 batch 1 pigs from whom isolates were derived by cat bioassay (TgPgUs 1–10) but not from the pig sources of isolate TgPgUs11 (also from batch 1) or TgPgUs 12–14, which were isolated by mouse bioassay using tissues from batch 2 pigs. Optical density values in the ELISA in serologically positive animals were low (6 of 10 were <0.40; 3 others were <0.62). Previous studies using ELISA to detect *T. gondii*–specific antibodies using serum obtained from experimentally infected pigs given large numbers of infective oocysts in a single dose resulted in large numbers of tissue cysts and high titers of anti-*T. gondii* antibodies in serum; consequently good results were obtained in terms of sensitivity and specificity (Waltman et al., 1984; Lind et al., 1997; Wingstrand et al., 1997). However, naturally infected pigs were shown to have lower ELISA OD values, as well as lower numbers of detectable tissue cysts (Hill et al., 2006); this observation is borne out in the current study.

*Batch 2 pigs*: The low number of isolations of *T. gondii* (3 of 20 pigs) was probably related to the status of the tissues sampled, in spite of the fact that 4 of these pigs had a high MAT titer of 1:1,280. The parasite was probably killed by autolysis. In addition, the sensitivity of the *T. gondii* ELISA using tissue fluids is considerably less than that obtained when testing serum samples (Hill et al., 2006). It is also likely that the autolysed condition of the tissue fluid from the TgPgUs 12–14 pig sources negatively impacted the conduct of the ELISA test.

**Pathogenicity of *T. gondii* isolates**

Toxoplasma gondii isolates differ markedly in their virulence to outbred mice. Virulence to mice depends on several factors, including the stage of the parasite, route, dose, types of mice used, host, and the strain of the parasite. Among the 3 infectious stages of *T. gondii*, oocysts are more virulent than tachyzoites or bradyzoites for nonfeline hosts (Dubey and Beattie, 1988). However, oocysts are not pathogenic for cats (Dubey, 2006). In the present study, mice fed oocysts of 10 of 11 isolates from pigs died (or had to be killed) of acute toxoplasmosis in the first wk PI, but tachyzoites and bradyzoites of all 14 isolates from pigs were not virulent for mice. Therefore, one must be cautious in calling the strains mouse pathogenic, based on limited data.

**Genotypes of *T. gondii* isolates**

The genotypes of *T. gondii* circulating in the domestic pig population in the United States may be epidemiologically important with respect to possible human infections. Of 170 isolates from Iowa sows (Dubey, Thulliez, and Powell, 1995), 43 were genotyped using SAG2. Most (36) of these isolates were type II, a few (7) were type III, and none was type I; 4 were thought to be recombinants of type I and III (Mondragon et al., 1998). Of the 25 of 51 isolates genotyped using SAG2 from market age pigs, 20 were type III and 5 were type II (Dubey, Gamble et al., 2002; Lehmann et al., 2003, 2004). In the present study using 11 markers, a higher genetic variability was revealed and 4 genotypes were identified. Genotypes 1 and 2 differ only at the Apico locus, and both genotypes belong to the type II lineage. Genotypes 3 and 4 are nonclonal types. In
total, 10 of the 14 isolates are either genotype 1 or 2 and are type II strains. Three isolates are genotype 3 and 1 is genotype 4. In conclusion, type II is the major lineage that infects pigs on the farm studied here. However, nonclonal lineages are also circulating on the same farm, though at a lower frequency. A previous study of human isolates from the United States indicated that most human patients (associated with AIDS and congenital toxoplasmosis) were infected with type II strains (Howe and Sibley, 1995). The bias of type II genotypes in human infection in the United States may be due to the predominance of type II strains found in food animals, which may serve as a major source of infection for humans.

The role of pigs in the epidemiology of T. gondii

Among the major food animals, T. gondii has been isolated more frequently from pigs than chickens or cattle. In a retrospective study of 131 mothers who had given birth to children infected with T. gondii, 50% recalled having eaten uncooked meat (Boyer et al., 2005). Toxoplasma gondii can persist in tissues in most edible cuts of pork (Dubey et al., 1986). A single T. gondii–infected pig can be a source of infection for many humans, since 1 market-weight hog (100 kg or more) can yield over 600 individual servings of meat.

The ingestion of infected, undercooked pork was thought to be a major source of T. gondii infection for humans in the United States (Mead et al., 1999). This is paradoxical because most consumers are aware since childhood of the danger of eating undercooked pork in the United States due to a fear of acquiring Trichinella spiralis infection. However, the decline in seroprevalence of T. gondii in humans in the U.S. has not paralleled the dramatic decline in the prevalence of T. gondii infection in pigs (Dubey, Thulliez, Weigel et al., 1995; Jones et al., 2001, 2003, 2007). In light of these inconsistencies, further study is required to determine the actual role of pork in human toxoplasmosis. Until definitive information is available, and because of the potential for T. gondii to lead to severe disease, particularly in pregnant women and immunosuppressed persons (many of whom are HIV positive), it is essential to cook all meat thoroughly to prevent infection with T. gondii (Dubey et al., 1990).

LITERATURE CITED


—. 2006. Comparative infectivity of oocysts and bradyzoites of Toxoplasma gondii for intermediate (mice) and definitive (cats) hosts. Veterinary Parasitology 140: 69–75.


ISOLATION AND GENETIC CHARACTERIZATION OF TOXOPLASMA GONDII FROM RACCOONS (PROCYON LOTOR), CATS (FELIS DOMESTICUS), STRIPED SKUNK (MEPHITIS MEPHITIS), BLACK BEAR (URSUS AMERICANUS), AND COUGAR (PUMA CONCOLOR) FROM CANADA

J. P. Dubey, T. Quirk*, J. A. Pitt†, N. Sundar, G. V. Velmurugan, O. C. H. Kwok, D. Leclaire‡, R. Hill§, and C. Su\pounds

United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, Maryland 20705-2350. e-mail: jitender.dubey@ars.usda.gov

ABSTRACT: Viable Toxoplasma gondii was isolated by bioassay in mice from tissues of 2 feral cats (Felis domesticus), 2 raccoons (Procyon lotor), a skunk (Mephitis mephitis) trapped in remote locations in Manitoba, Canada, and a black bear (Ursus americanus) from Kuujjuaq, northern Quebec, Canada. Genotyping of these T. gondii isolates using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and an apicoplast marker Apico revealed 4 genotypes. None of the isolates was clonal archetypal Types I, II, and III found in the United States. These results are in contrast with the Type II genotype that is widespread in domestic animals and humans throughout the United States and Europe. This is the first genotyping of T. gondii isolates from this part of North America.

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally by ingesting tissue cysts from undercooked meat or by consuming food and drink contaminated with oocysts. Only a small percentage of exposed adult humans develop clinical toxoplasmosis. Whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or other factors is largely unknown.

Toxoplasma gondii isolates have been classified in 3 genetic Types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997). Until recently most isolates of T. gondii were considered clonal, with little genetic diversity (Lehmann et al., 2006), although most of this information is derived from isolates from domestic animals and human patients. Little is known of the prevalence and distribution of genotypes of T. gondii in wildlife species in North America. There are a few reports of T. gondii genotypes in land (Dubey, Graham et al., 2004; Dubey, Parnell et al., 2004; Dubey, Sundar, Norden et al., 2007) and marine (Cole et al., 2000; Miller et al., 2004; Conrad et al., 2005) mammals in the United States. Little is known of genotypes of T. gondii circulating in humans or animals in Canada. There is only brief information on an isolate of T. gondii (called Cougar isolate) from the oocysts shed by a naturally infected cougar from Vancouver (Aramini et al., 1998; Lehmann et al., 2000; Su et al., 2006), and a human isolate (Lehmann et al., 2000) from an undefined location in Canada.

In the present paper we describe the genetic and biologic characteristics of isolates of T. gondii from striped skunk (Mephitis mephitis), raccoons (Procyon lotor), feral cats (Felis domesticus), and a black bear (Ursus americanus) from remote areas of Canada. We also properly designate cougar isolates from Canada and clarify the confusion about the origin of the isolates.

MATERIALS AND METHODS

Serological examination

Sera of carnivores were tested for T. gondii antibodies using 2-fold serum dilutions from 1:20 to 1:640 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Bioassay for T. gondii

For bioassay in mice, 50 g of brain, heart, and muscle were homogenized, digested by an acidic pepsin solution (Dubey, 1998), neutralized, and washed; the homogenate was inoculated subcutaneously (SC) into 5 outbred female Swiss Webster mice obtained from Taconic Farms, Germantown, New York, U.S.A., as previously described (Dubey et al., 2002) (Table 1). Imprints of lungs or brains of mice that died were examined for T. gondii tachyzoites or tissue cysts. Survivors were bled on day 40–42 post-inoculation (PI), and a 1/25 dilution of serum from each mouse was tested for T. gondii antibodies with the MAT. Mice were killed 6 wk PI, and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with T. gondii when tachyzoites or tissue cysts were found in tissues.

Toxoplasma gondii isolates from raccoons, cats, and skunk

As part of a larger study of wild carnivores in Manitoba, 3 raccoons, 2 cats, and 1 skunk were available for T. gondii isolation (Table 1). The cats and raccoons were collected near Minnedosa, Manitoba (50°10'N, 99°47'W), and the skunk was collected at Delta Beach along the south shore of Lake Manitoba (50°11’N, 99°19’W). The animals were killed with an intravenous inoculation of a euthanasia solution (T-61® 0.3 mg/kg; Hoechst Animal Health Benelux, Brussels, Belgium). Samples of blood, heart, brain, and skeletal muscle were collected and shipped cold to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland for T. gondii examination.

Toxoplasma gondii isolate from a black bear

A young (2- to 3-year-old) male black bear was killed near Kuujjuaq (58°5’N, 68°25’W), northern Quebec on 31 October 1999, and samples of blood, heart, and tongue were collected soon after the kill. The bear samples were stored in refrigerator (4°C) for 1 wk in the laboratory of the Nunavik Research Center in Kuujjuaq and were kept in a cooler for 1 wk during transit from Canada to the United States. The samples were foul smelling when received at the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland, on 15 November 1999. A serological examination revealed that the bear had antibodies to T. gondii (MAT titer 1:200). On November 20 (20 days from the kill) 50 g of heart and 35 g of tongue were homogenized separately and digested in

Received 6 June 2007; revised 9 July 2007; accepted 10 July 2007.
9 Department of Biology, University of Saskatchewan, Saskatoon, SK, S7N 5E2, Canada.
1 Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada.
2 Nunavik Research Center, Makivik Corporation, Kuujjuaq Quebec JOM 1MO, Canada. Present address: Food Safety Division, Canadian Food Inspection Agency, 159 Cleopatra Drive, Ottawa, ON K1A 0Y9, Canada.
§ Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845.
pepsin, before being inoculated into mice (5 for the tongue, and 10 for the heart). The mice inoculated with bear tissues were examined for *T. gondii* infection.

**Toxoplasma gondii** isolates from cougars

During an epidemiological study of a waterborne outbreak of toxoplasmosis in humans, *T. gondii* was isolated from feces (samples A and B) from 2 naturally infected cougars from Vancouver Island, British Columbia, Canada (Aramini et al., 1998). Sample A was obtained from the rectum of a 1.5-yr-old male that was killed; it had a serum MAT titer of 1:500 (Aramini et al., 1998). Mice orally inoculated with oocysts from both cougars developed toxoplasmosis, and the strains were cryopreserved in liquid nitrogen. These strains had no specific designation, until now. The strain from sample A is now designated as TgCgCa1 and the strain from sample B as TgCgCa2. For the present study both isolates were successfully revived after a 10-yr storage in liquid nitrogen.

There is a considerable amount of published information on the genetics of the TgCgCa1 isolate, because it was found to be different from the typical Types I, II, and III (Lehmann et al., 2000; Su et al., 2003, 2006; Fux et al., 2007); however, the isolate has been named Cougar2, Cougar, or COUG, but never clearly identified in these publications.

**Genetic characterization**

*Toxoplasma gondii* DNA was extracted from the tissues of infected mice from each group, and strain typing was performed using PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su et al., 2006; Dubey, Sundar, Gennari et al., 2007). Allele types for all isolates were determined based on the RFLP patterns of 6 reference strains including RH88, PTG, CTG, COUGAR (TgCgCa1), MAS, and TgCatBr5 (Su et al., 2006). These reference strains allow us to capture most known alleles for each marker and to identify potential unique alleles in new samples.

**RESULTS**

**Toxoplasma gondii** isolates from raccoons, cats, and skunks

*Toxoplasma gondii* was isolated from 2 cats, 2 raccoons, and 1 skunk (Table I). It is noteworthy that *T. gondii* was not isolated from the brain of any animal (Table I). The raccoon 1 isolate was obtained from the muscle of the animal; 1 of the 5 mice inoculated died of acute toxoplasmosis, and tachyzoites were found in its lungs. The *T. gondii* isolate from raccoon 1 was lost and was not available for DNA characterization.

**Toxoplasma gondii** isolate from a black bear

*Toxoplasma gondii* was isolated from the tongue but not from the heart of the bear. One of the 5 mice died on day 26 PI with tongue of the bear isolate. *Toxoplasma gondii* was not found in smears of the pulmonary lung because of autolysis; therefore, a homogenate of the lung of this mouse was inoculated SC into 2 mice. The recipient mice died of acute toxoplasmosis 11 and 14 days PI, and a homogenate of the lung containing tachyzoites was inoculated into 3 mice that were medicated with sulfadiazine sodium in drinking water (1 mg per 100 ml of drinking water) from day 7 to 12 PI. Two of these 3 mice died of toxoplasmosis in spite of therapy. Numerous tissue cysts were found in the brain of the mouse when killed 49 day PI. Bradyzoites released from the tissue cysts from this mouse were cryopreserved in liquid nitrogen. For genetic characterization, a vial containing the bear isolate frozen for 7 yr was thawed, and half of the contents were inoculated SC into 2 mice and the remainder half was processed for DNA extraction. The strain was revived successfully because the 2 mice inoculated with frozen bradyzoites died of acute toxoplasmosis on day 18 and 21 PI.

**Genetic characterization**

Genotyping was performed on 5 of the 6 *T. gondii* strains isolated in the present study (isolate from raccoon 1 was lost) and from the cougar from sample B of Aramini et al. (1998). Five different genotypic groups were recognized (Table II). None of the isolates belong to clonal archetypal Type I, II, III lineage. Two isolates (TgCatCa2 from cat 2 and TgSkCa1 from the skunk) were similar by all markers tested. The 5 genotypes had a combination of Type I and II, I and III, and I, II, and III (Table II).

---

**Table I. Isolation of Toxoplasma gondii from cats, raccoons, and a skunk in Manitoba, Canada.**

<table>
<thead>
<tr>
<th>Host</th>
<th>MAT titer</th>
<th>Brain</th>
<th>Heart</th>
<th>Muscle</th>
<th>T. gondii strain designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>4 (16)†</td>
<td>TgCatCa1</td>
</tr>
<tr>
<td>Cat 2</td>
<td>800</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>TgCatCa2</td>
</tr>
<tr>
<td>Raccoon 1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>1 (11)</td>
<td>TgReCa1</td>
</tr>
<tr>
<td>Raccoon 3</td>
<td>100</td>
<td>0</td>
<td>2 (43)</td>
<td>0</td>
<td>TgReCa2</td>
</tr>
<tr>
<td>Skunk</td>
<td>400</td>
<td>Not done</td>
<td>5</td>
<td>(13, 15)</td>
<td>TgSkCa1</td>
</tr>
</tbody>
</table>

* Five mice were inoculated with each tissue.
† Day of death of individual mouse.

---

**Table II. Genotype of *Toxoplasma gondii* isolates from wild animals from Canada.**

<table>
<thead>
<tr>
<th>Host</th>
<th>T. gondii isolate</th>
<th>SAG1</th>
<th>SAG2*</th>
<th>SAG2†</th>
<th>SAG3</th>
<th>BTUB</th>
<th>GRA6</th>
<th>c22-8</th>
<th>c29-2</th>
<th>L358</th>
<th>PK1</th>
<th>Apico</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 1</td>
<td>TgCatCa1</td>
<td>II or III</td>
<td>III</td>
<td>III</td>
<td>I</td>
<td>III</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>III</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Cat 2</td>
<td>TgCatCa2</td>
<td>u-1</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Raccoon 1</td>
<td>TgRaCa2</td>
<td>u-l</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Skunk</td>
<td>TgSkCa1</td>
<td>u-1</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Black bear</td>
<td>TgBBBeCa1</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>I</td>
<td>III</td>
</tr>
<tr>
<td>Cougar Remarks</td>
<td>TgCgCa2</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>u-1</td>
<td>I</td>
<td>u-2</td>
<td>I</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SAG2 marker based on 5'- and 3'-ends of the gene sequence (Howe et al., 1997).
† A new SAG2 marker based on the 5'-end of the gene sequence (Su et al., 2006).
‡ Sample B of Aramini et al. (1998).
DISCUSSION

The role of wildlife in the epidemiology of *T. gondii* infections in livestock or humans is unknown. *Toxoplasma gondii* infections are widely prevalent in white-tailed deer, raccoons, and black bears in the United States (reviewed in Dubey, Graham et al., 2004). Meat of many wildlife species is consumed by humans, and occasionally human cases of clinical toxoplasmosis have been linked to eating uncooked venison (Sacks et al., 1983; McDonald et al., 1990). A large outbreak of toxoplasmosis in humans in Vancouver, British Columbia, Canada, was epidemiologically linked to the contamination of a water reservoir by oocysts (Bowie et al., 1997); however, attempts to isolate *T. gondii* from water samples were unsuccessful (Isaac-Renten et al., 1998). It was suspected that feces of infected cats (domestic, or feral *Felis domesticus*, or cougars) entered the reservoir or 1 of its streams, resulting in contamination of the water supply with *T. gondii* oocysts (Aramini et al., 1998). Cougars are the only wild felid found on Vancouver Island. One cougar was known to be in the vicinity of the Victoria watershed, and sample A was derived from this cougar. Sample B was presumed to be of cougar origin (see Materials and Methods). Results of the present study indicate that *T. gondii* strains from sample A and B are genetically identical based on the results of 10 PCR-RFLP markers, and both might have come from the same cougar or derived from the ancestor strain. To our knowledge, there is no published information on the genetic makeup of the isolates of *T. gondii* from humans from the Vancouver outbreak. Results of the cougar isolate are documented here to exclude or incriminate the cougar with the human outbreak.

Recently we determined that 4 of the 5 isolates from raccoons and 1 skunk isolate from Wisconsin belonged to 5 *T. gondii* genotypes (Dubey, Sundar, Nolden et al., 2007); these genotypes are different from genotypes from the Canadian raccoon and the skunk in the present study. The 4 *T. gondii* genotypes identified in this study are also different from previously published results on isolates of chickens, dogs, and cats from Brazil (Su et al., 2006; Dubey, Gennari et al., 2007; Dubey, Sundar et al., 2007), dogs from Colombia (Dubey, Cortés Vecino et al., 2007), Vietnam (Dubey, Huang et al., 2007), and Sri Lanka (Dubey, Rajapakse et al., 2007), cats from Colombia (Dubey, Su et al., 2006) and China (Dubey, Zhu et al., 2007), and chickens from Chile (Dubey, Patitucci et al., 2006), Costa Rica (Dubey, Su, de Oliveira et al., 2006), and Nicaragua (Dubey, Sundar et al., 2006). However, the genotype of isolates TgCatCa2, TgRacCa2, and TgSkCa4 in this study was found in 5 isolates from the United States (white-tailed deer isolates WTD1 and WTD3 and 2 human isolates, AR1 and RAY, and an isolate from a wild turkey T61) initially genotyped as Type II–related strains by Howe and Sibley (1995), which suggests this particular genotype may be widespread in animals and in humans (C. Su, unpub. obs.). Currently there are no molecular markers to link the sources of *T. gondii* infection or severity of clinical toxoplasmosis. Circumstantial evidence suggests that certain genetic types of *T. gondii* may be associated with clinical ocular toxoplasmosis in humans (Khan et al., 2005, 2006). It has been suggested that Type I isolates or recombinants of Types I and III are more likely to result in clinical toxoplasmosis (see Khan et al., 2005, 2006, and references therein), but genetic characterization has been limited essentially to isolates from patients ill with toxoplasmosis. There is very little information regarding the genetic diversity of *T. gondii* isolates circulating in the general human population. Therefore, we must be cautious in claiming a linkage between parasite genotypes and disease presentations without the good knowledge of parasite biology in the human population and the environment.

ACKNOWLEDGMENTS

This study was supported in part by the Delta Waterfowl Research Foundation.

LITERATURE CITED


---, A. N. PATITUCCI, C. SU, N. SUNDAR, O. C. H. KWOK, AND S. K. SHEN. 2006. Characterization of *Toxoplasma gondii* isolates in...
free-range chickens from Chile, South America. Veterinary Parasitology 140: 76–82.


MORPHOLOGY AND 18S rDNA OF HENNEGUYA GURLEI (MYXOSPOREA) FROM AMEIURUS NEBULOSUS (SILURIFORMES) IN NORTH CAROLINA

Luke R. Iwanowicz*, Deborah D. Iwanowicz†, Linda M. Pote‡, Vicki S. Blazer, and William B. Schill

National Fish Health Research Laboratory, U.S. Geological Survey, Kearneysville, West Virginia 25430. e-mail: luke.iwanowicz@usgs.gov

ABSTRACT: Henneguya gurlei was isolated from Ameiurus nebulosus captured in North Carolina and redescribed using critical morphological features and 18S small-subunit ribosomal RNA (SSU rDNA) gene sequence. Plasmodia are white, spherical, or subpherical, occur in clusters, measure up to 1.8 mm in length, and are located on the dorsal, pectoral, and anal fins. Histologically, plasmodia are located in the dermis and subdermally, and the larger cysts disrupt the melanocyte pigment layer. The spore body is lanceolate, 18.2 ± 0.3 μm (range 15.7–20.3) in length, and 5.4 ± 0.1 μm (range 3.8–6.1) in width in valvular view. The caudal appendages are 41.1 ± 1.1 μm (range 34.0–49.7) in length. Polar capsules are pyriform and of unequal size. The longer polar capsule measures 6.2 ± 0.1 μm (range 5.48–7.06), while the shorter is 5.7 ± 0.1 μm (range 4.8–6.4) in length. Polar capsule width is 1.2 ± 0.03 μm (range 1.0–1.54). The total length of the spore is 60.9 ± 1.2 μm (range 48.7–68.5). Morphologically, this species is similar to other species of Henneguya that are known to infect ictalurids. Based on SSU rDNA sequences, this species is most closely related to H. exilis and H. ictaluri, which infect Ictalurus punctatus.

Henneguya Thélohan, 1892 (Myxozoa) contains more than 146 species and is one of the largest genera in the Myxobolidae, second only to Myxobolus (Eiras, 2002). Species of Henneguya have a cosmopolitan distribution and parasitize freshwater, estuarine, and marine fishes (Lom and Dyková, 1992; Hallett and Diamant, 2001); they typically exhibit tropism for gill tissue. Henneguya spp. infections are generally of little clinical significance, although a few species are of considerable economical impact in ictalurid aquaculture worldwide (Pote et al., 2000; El-Mansy and Bashtar, 2002). Currently, 21 species of Henneguya have been described that infect ictalurids (Table I). These species designations are based primarily on morphological characteristics of mature spores, site of infection (microhabitat), host species, and, in a few instances, 18S small-subunit ribosomal RNA gene (SSU rDNA) sequence. Extensive research has primarily focused on species of myxozoans that impact intensive aquaculture. It has been found that identification of these zoospores using morphological characteristics alone is often insufficient for an accurate diagnosis (Ali, 1999; Pote et al., 2000). Incorporation of molecular methods, namely utilization of SSU rDNA, in addition to morphological identifiers, has effectively allowed reliable life-stage–independent discrimination between species (Lin et al., 1999; Pote et al., 2000; Hanson et al., 2001).

At least 7 species of Henneguya are known to infect Ictalurus punctatus (Kudo, 1929; Guilford, 1965; Minchew, 1977; Current, 1979; Pote et al., 2000). Of these species, H. exilis and H. limatula are also known to infect bullheads of the genus Ameiurus (Kudo, 1929; Guilford, 1965). Only 2 species of Henneguya (H. ameiurensis and H. gurlei) have been described that exclusively infect species of Ameiurus. Henneguya ameiurensis, which was thoroughly characterized by Nigrelli and Smith (1940), was isolated from a single Ameiurus nebulosus from a lake in New Hampshire during the summer of 1938. Henneguya gurlei was isolated from black bullheads Ameiurus melas in Storm Lake, Iowa (Kudo, 1920). Spore characteristics, including body length, width, and thickness, were described; however, other critical morphological characteristics necessary for accurate species identification of Henneguya sp. are absent from this report. Both species of parasites develop macroscopically visible plasmodia within the skin, but the specific microhabitat (barbels versus fin bases) differs. Morphology of the mature spores is similar between the 2 species but different enough to warrant designation as different species (Nigrelli and Smith, 1940).

The present study is a redescription of Henneguya gurlei and is, to our knowledge, the first isolation of this species from Ameiurus nebulosus. The amended species description accounts for the new host species, and the description includes morphological features, tissue microhabitat, and SSU rDNA sequences not reported in the original description (Kudo, 1920). Characteristics necessary to describe myxosporean species as recommended by Lom and Arthur (1989) are included and complemented with SSU rDNA gene sequences to facilitate accurate future identification.

MATERIALS AND METHODS

Fish maintenance

During the summer months of 2004, brown bullheads, A. nebulosus, were collected from South Creek in Aurora, North Carolina (35°21′34″N, 76°42′59″W). During late September of 2004, ~500 of these fish (12–25 cm total length) were transported to the National Fish Health Research Laboratory in Leetown, West Virginia. Fish were held in a 2,000-L recirculating system with approximately 5–10% water replacement daily. Make-up water originated from an underground spring (12–16 °C). Oligochaete habitat was not present in the recirculating system. Target water temperature was 22 °C, but it ranged from 16 to 24°C during the 6 mo of captivity. Fish experienced a spontaneous Edwardsiella ictaluri outbreak during October 2004 (Iwanowicz et al., 2006). Survivors of this bacterial disease were sampled for the present research during May 2005 when notable, fin-associated cysts were observed.
**Forward primer**

![Map of primers and approximate annealing sites on the 18S small-subunit rRNA gene.](image)

Figure 1. Map of primers and approximate annealing sites on the 18S small-subunit rRNA gene. The entropy (Hx) plot depicts sites of nucleotide variation (Hx greater than 1) within the SSU rDNA sequence between all known species of *Henneguya*. Variable regions (DVR1, DVR2, and DVR3) useful to distinguish like species of *Henneguya* are noted. PCR product sizes of *H. gurlei* are also shown.

---

**Wet mount preparation and morphometrics**

Plasmodia were removed from the fins, placed on microscope slides, and disrupted with mechanical pressure to release the contents. The exudate was diluted with dH2O, and slides were mounted and viewed with a Nikon Eclipse E600 microscope. Images of spores (n = 100) were captured with a Hitachi HV-C20 CCD camera and analyzed with Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, Maryland) image analysis software.

**Histology**

Clippings of infected caudal fins were fixed in 10% Z-Fix (Anatech Ltd., Hayward, California). Tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Additional sections were stained with Geimsa to enhance spore staining.

**DNA sequencing and analysis**

Intact plasmodia were removed from the fins and stored in 70% ethanol. DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, California) according to the manufacturer’s protocol. The 18S SSU rRNA gene cluster was targeted for amplification using polymerase chain reaction (PCR). Primers used for the PCR amplifications targeted myxosporidian sequences and excluded host sequence (Table II; Fig. 1). Conditions for PCRs differed slightly for the different primer sets as follows. Sample DNA amplified with the H9-H2 primer set was denatured at 92°C for 4 min. This was followed by 30 cycles at 92°C for 1 min, 60.7°C for 1 min, and 72°C for 1 min. Amplification using the MYXF-MYXR and HENF-HENR primer sets commenced with denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 20 sec, 58°C for 40 sec, and 72°C for 40 sec. Products were subjected to a final extension at 72°C for 10 min and held at 4°C to prevent possible genomic amplification. Both forward and reverse sequences from the products produced with the H9-H2 primer set. Forward and reverse PCR primers were used as sequencing primers for the other respective reactions. Both forward and reverse sequences of the PCR products were generated using the Standard ABI Prism Big Dye Terminator Cycle Sequencing protocol (Applied Biosystems, Foster City, California).

Myxozoan SSU rDNA sequences were acquired from the NCBI nucleotide database, aligned with ClustalX v1.83 (Thompson et al., 1997), and cropped to yield sequences of equal length (1694 bp including gaps). Sequences (n = 22) used for the analysis were as follows: *Chloromyxa cyprini* (AY604198), *C. truttae* (AJ581916), *Ceratomyxa labracis* (AF411472), *C. sparsauratii* (AF411471), *Myxobolus bizerti* (AY129318), *M. bramae* (AF507968), *M. cerebralis* (AF115254), *M. culcis* (AB121146), *M. episquamalis* (AY129312), *M. lentissuturalis* (AY119688), *M. longisporus* (AY364637), *M. spina cu rvalura* (AF378337), *Henneguya doori* (U37549), *H. exilis* (AF021881), *H. ictaluri* (AF195510), *H. lateolabracis* (AB183747), *H. pagri*
Table 1. Morphological characteristics of *Henneguya* spp. isolated from ictalurids. LSB, length of spore body; WSB, width of spore body; TSB, thickness of spore body; TSL, total spore length; LPC, length of polar capsules; WPC, width of polar capsules; CAL, caudal appendage length; CD, cyst diameter; and SI, site of infection. All measurements are provided in microns (μm) except for the cyst dimension (mm). Values in parentheses denote size range.

<table>
<thead>
<tr>
<th>Species</th>
<th>LSB</th>
<th>WSB</th>
<th>TSB</th>
<th>TSL</th>
<th>LPC</th>
<th>WPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. gurlei</em> (case isolate)</td>
<td>18.2 (15.7–20.3)</td>
<td>5.4 (3.8–6.1)</td>
<td>3.2 (2.8–3.5)</td>
<td>60.9 (48.7–68.5)</td>
<td>5.9 (4.8–7.1)</td>
<td>1.2 (1.0–1.5)</td>
</tr>
<tr>
<td><em>H. gurlei</em> (previous description)</td>
<td>19</td>
<td>(5–6)</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. ameiurensis</em></td>
<td>23.3</td>
<td>4.1</td>
<td>3.0</td>
<td></td>
<td>5.4</td>
<td>1.6</td>
</tr>
<tr>
<td><em>H. exilis</em></td>
<td>(18–20)</td>
<td>(4–5)</td>
<td>(3–3.5)</td>
<td>(60–70)</td>
<td>(8–9)</td>
<td>(1–1.5)</td>
</tr>
<tr>
<td><em>H. limatula</em></td>
<td>13.7</td>
<td></td>
<td>(5–6)</td>
<td></td>
<td>(6.5–8)</td>
<td>(1.5–2)</td>
</tr>
<tr>
<td><em>H. adiposa</em></td>
<td>16.3 (12–19)</td>
<td>4.0 (3.5–5.0)</td>
<td>3.0 (2.5–3.5)</td>
<td>61.0 (45–75)</td>
<td>7.7 (6.2–9.0)</td>
<td>1.5 (1.0–2.0)</td>
</tr>
<tr>
<td><em>H. diversis</em></td>
<td>14.8 (13.5–16.5)</td>
<td>4.0 (3.2–5.0)</td>
<td>3.9 (3.0–4.5)</td>
<td>49.5 (40–62)</td>
<td>6.2 (6.0–7.5)</td>
<td>1.5 (1.0–2.0)</td>
</tr>
<tr>
<td><em>H. ictaluri</em></td>
<td>23.9 (20.8–26.1)</td>
<td>6.0 (4.5–6.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. longicauda</em></td>
<td>16.2 (14–17.5)</td>
<td>4.0 (3.5–4.5)</td>
<td>4.0 (3.5–4.0)</td>
<td>108.3 (91–127)</td>
<td>7.7 (7.0–8.5)</td>
<td>1.8 (1.5–2.0)</td>
</tr>
<tr>
<td><em>H. postexilis</em></td>
<td>15.0 (13.5–15)</td>
<td>3.4 (3.5–4.0)</td>
<td>3.0 (3.5–4.0)</td>
<td>52.0 (42–62)</td>
<td>6.6 (5.9–7.2)</td>
<td>1.5 (1.0–2.0)</td>
</tr>
<tr>
<td><em>H. pellis</em></td>
<td>13.0 (11.0–14.5)</td>
<td>5.0 (4.5–5.2)</td>
<td>4.8 (4–5)</td>
<td>100.4 (79–124)</td>
<td>6.9 (5.5–8.5)</td>
<td>1.8 (1.5–2.0)</td>
</tr>
<tr>
<td><em>H. fusiformis</em></td>
<td>(29–33)</td>
<td>(5–7)</td>
<td></td>
<td></td>
<td>(5–6)</td>
<td>(3–4)</td>
</tr>
<tr>
<td><em>H. laterocapsulata</em></td>
<td>14.7 (13.8–16)</td>
<td>4.3 (3.7–5.3)</td>
<td>3.8 (3.3–4.3)</td>
<td>32.7 (29.0–36.2)</td>
<td>4.8 (4.1–5.3)</td>
<td>2.6 (2.2–3.0)</td>
</tr>
<tr>
<td><em>H. branchialis</em></td>
<td>(12.5–17.5)</td>
<td>(4.5–6.5)</td>
<td></td>
<td></td>
<td>(28–41)</td>
<td>(6.8–5.3)</td>
</tr>
<tr>
<td><em>H. clariae</em></td>
<td>(17.5–28.5)</td>
<td>(5.5–8.5)</td>
<td></td>
<td>(45–107)</td>
<td>(5–13.5)</td>
<td>(2.5–3.5)</td>
</tr>
<tr>
<td><em>H. suprabranchiae</em></td>
<td>13.5 (12.2–14.3)</td>
<td>6.4 (5.6–6.9)</td>
<td>4.4 (3.9–4.9)</td>
<td>37.5 (30.7–43.3)</td>
<td>7.6 (7.0–8.1)</td>
<td>2.1 (1.8–2.3)</td>
</tr>
<tr>
<td><em>H. chrysiichthyi</em></td>
<td>(13.5–16)</td>
<td>(4.5–6.5)</td>
<td></td>
<td>(27–32)</td>
<td>(4–5.5)</td>
<td>(1–2)</td>
</tr>
<tr>
<td><em>H. linearis</em></td>
<td>3–4× breadth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. camerounensis</em></td>
<td>9</td>
<td>(4–5.5)</td>
<td>(13.5–21.5)</td>
<td>(4.5–6.5)</td>
<td>(1–2)</td>
<td></td>
</tr>
<tr>
<td><em>H. strongylia</em></td>
<td>9</td>
<td>5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(AB183748), *Henneguya* sp. (U13826), Kudoa meggacapsula (AB188529), *K. thyrsites* (AY152747), *Tetracapsuloides bryosalmonae* (U70623), and the case isolate *Henneguya gurlei* (DQ73465). *Tetracapsuloides bryosalmonae* was used as an out-group for phylogenetic analysis. Maximum parsimony and maximum likelihood phylogenetic analyses were performed using PAUP*4.0b1 and Treefinder v. May 2006, respectively (Swoford, 1998; Jubb et al., 2004). Bayesian analysis was conducted using Mr. Bayes 3.1.1 software (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). Gaps were treated as missing data in all analyses. Maximum parsimony analysis utilized the Goloboff fit criterion (kappa = 2) using the heuristic search algorithm with 100 random additions of sequences and tree bisection-reconnection (TBR) branch swapping. Bootstrap values were calculated with 1,000 replicates using the same heuristic search. Maximum likelihood analysis used the default analysis settings in Treefinder with the appropriate substitution model and bootstrap values calculated from 1,000 replicates. Maximum likelihood analysis and Bayesian inference of phylogeny were conducted using the general time reversible (GTR + I + G) model based on the most appropriate DNA substitution model as calculated by Mr. Modelttest v.2.2 (Posada and Crandall, 2001; Nylander, 2004). Parameters for the Bayesian analysis included 1,000,000 generations of Markov Chain Monte Carlo sampling that were sampled once every 50 generations. A consensus phylogram was constructed in Treefinder based on the trees sampled in the asymptotic phase of the Bayesian analysis (burnin = 101 trees used for consensus = 9,000).

A sequence identity matrix was calculated for the bullhead isolate with the 5 best *Henneguya* spp. sequence matches identified using a low complexity, E = 0.01 NCBI BLAST search. Sequences were aligned with ClustalX v1.83 and cropped to yield aligned sequences of equal length (1,920 bases including gaps). The resulting sequences were degapped and realigned in a pair-wise fashion for all possible combinations again using ClustalX v1.83. Sequence identities were determined for these pair-wise alignments with the BioEdit Sequence Alignment Editor v.7.0.1 (Hall, 1999). Degapped lengths of sequences used for the analysis were *H. gurlei* (1,892), *H. exilis* (1,885), *H. ictaluri* (1885), *H. doori* (1,875), *H. limatula* (1,883), *H. laterocapsulata* (1,880), and *H. pellis* (1,875).

**REDESCRIPTION**

*Henneguya gurlei*

**General characters**

*Henneguya gurlei* is plasmodia polysporyc, up to 1.8 mm in diameter, with a well-defined, 3–8-μm-thick layer of connective tissue located on the anal dorsal and pectoral fins, not restricted to the fin bases; it appears as clusters, 1–5 mm in diameter, spherical or subspherical, and is milky-white in color (Fig. 2). Sporogenesis is asynchronous, with less mature, pansporoblast stages at the periphery, and mature spores in a central zone of plasmodium; ectoplasm is located at the periphery of plasmodium-containing germ cells and immature spores (Fig. 3). The spore body is lanceolate and is flattened parallel to the suture line; it is 18.2 ± 0.3 μm (15.7–20.3) long, 5.4 ± 0.1 μm (3.8–6.1) wide in valvar view, and 3.2 ± 0.1 μm (2.8–3.5) thick in sutral view. There are 2 caudal appendages, each of which is a continuation of 1 valve, 41.1 ± 1.1 μm (34.0–49.7) long. Total spore length is 60.9 ± 1.2 μm (48.7–68.5). There are 2 valves and 2 polar capsules. Polar capsules are pyriform and of unequal size, oriented in plane with the sutural ridge. The valve is lanceolate and is flattened parallel to the suture line; it is 18.2 ± 0.3 μm (15.7–20.3) long, 5.4 ± 0.1 μm (3.8–6.1) wide in valvar view, and 3.2 ± 0.1 μm (2.8–3.5) thick in sutral view. There are 2 caudal appendages, each of which is a continuation of 1 valve, 41.1 ± 1.1 μm (34.0–49.7) long. Total spore length is 60.9 ± 1.2 μm (48.7–68.5). There are 2 valves and 2 polar capsules. Polar capsules are pyriform and of unequal size, oriented in plane with the sutural ridge. The longer polar capsule measures 6.2 ± 0.1 μm (range 5.48–7.06), while the shorter is 5.7 ± 0.1 μm (range 4.8–6.4) in length; the width is 1.2 ± 0.3 μm (1.0–1.54). Three polar capsules are occasionally observed.
but this is rare (Fig. 4). The number of turns in the polar filament coil is \(-9\); sporoplasm is binucleate (Figs. 5, 6).

**Taxonomic summary**

_Type host:_ Adult Brown bullhead, _Ameiurus nebulosus_ (Lesueur, 1819) (Siluriformes: Ictaluridae).

_Other hosts:_ _Ameiurus melas_ (Rafinesque, 1820) (Siluriformes: Ictaluridae).

_Type locality:_ South Creek, Aurora, North Carolina (35°21'34"N, 76°42'59"W).

_Other locations:_ Storm Lake, Iowa.

**Site of infection:** Caudal, pectoral, and anal fins (dermal, subdermal).

_Type material:_ Histological sections of an infected caudal fin stained with H&E or Geimsa; deposited in the U.S. National Parasite Collection (USNPC), Beltsville, Maryland; Voucher accession number USNPC 98810.

**Prevalence of infection:** Less than 1% of the bullheads collected from South Creek developed macroscopically visible plasmodia. Vegetative stages of the parasite were visible 6 mo following capture from the wild.

**Remarks**

Spores of _H. gurlei_ are similar (overlap in measured ranges of 4 or more of 7 diagnostic features, see Table I) to those of _H. adiposa, H. ameiurensis, H. diversis, H. exilis, and H. postexilis_, all of which parasitize ictalurids in North America. Mean caudal appendage length (CAL) and mean length of polar capsule (LPC) differ between _H. gurlei_ and these 5 species and appear to be the best diagnostic morphological

### Table I. Extended.

<table>
<thead>
<tr>
<th>CAL</th>
<th>CD</th>
<th>SI</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.1 (34.0–49.9)</td>
<td>Up to 1.8</td>
<td>Dorsal, pectoral, and anal fins</td>
<td><em>Ameiurus nebulosus</em></td>
<td>This paper</td>
</tr>
<tr>
<td>(15 – 41.5)</td>
<td>(0.19 × 0.34–0.76 × 1.2)</td>
<td>Base of spines of 2nd dorsal fin</td>
<td><em>Ameiurus melas</em></td>
<td>Kudo (1920)</td>
</tr>
<tr>
<td></td>
<td>(0.5–2)</td>
<td>Barbels</td>
<td><em>Ameiurus nebulosus</em></td>
<td>Nigrelli and Smith (1940)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td><em>Ictalurus punctatus, Ameiurus melas, Ameiurus nebulosus</em></td>
<td>Kudo (1929)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gall bladder</td>
<td><em>Ictalurus punctatus</em></td>
<td>Guilford (1965)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipose fin</td>
<td><em>Ictalurus punctatus</em></td>
<td>Current (1979)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Base of barbels, pectoral fins, and along isthmus, liver &amp; kidney</td>
<td><em>Ictalurus punctatus</em></td>
<td>Minchew (1977)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td><em>Clarias anguilaris</em></td>
<td>Kostoingue et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63 (48.1–80.2)</td>
<td><em>0.12 × 0.29–0.5 × 0.15</em></td>
<td>Gall bladder</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90.5 (75–110)</td>
<td>(0.13–0.37 × 0.11–0.12)</td>
<td>Ictalurus punctatus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.0 (28–49)</td>
<td>(0.012 × 0.012–0.08 × 0.07)</td>
<td>Ictalurus punctatus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87.8 (66–112)</td>
<td>(1–2)</td>
<td>Ictalurus punctatus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 (15.2–20.2)</td>
<td>(0.5–0.7)</td>
<td>Membrane lining branchial cavity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.0 (18.5–29.0)</td>
<td>Up to 1</td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane lining branchial cavity</td>
<td><em>Clarias lazera, H. bidorsalis hybrid</em></td>
<td>Landsberg (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gills, Intestine</td>
<td><em>Clarias lazera</em></td>
<td>Ashmawy et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td><em>Clarias lazera</em></td>
<td>Kostoingue et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td><em>Chrysichthys nigrodotatus</em></td>
<td>Fomena and Bouix (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gills</td>
<td><em>Chrysichthys nigrodotatus</em></td>
<td>Obiekezie and Enyenhi (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane lining branchial cavity</td>
<td><em>Rhamda sebae</em></td>
<td>Kudo (1920)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane lining branchial cavity</td>
<td><em>Synodontis batesi, Eutropius multitaeniatus</em></td>
<td>Fomena and Bouix (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Membrane lining branchial cavity</td>
<td><em>Synodontis shall</em></td>
<td>Kudo (1920)</td>
</tr>
</tbody>
</table>

### Table II. Primers used for the amplification and sequencing (*) of _Henneguya gurlei_.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H9</td>
<td>TTA CCT GGT CCG GAC ATC AA</td>
<td>Hanson et al. (2001)</td>
</tr>
<tr>
<td>H2</td>
<td>CGA CTT TTA CTT CCT CGA AAT TGC</td>
<td>Hanson et al. (2001)</td>
</tr>
<tr>
<td>Pr6*</td>
<td>GCA TGG CGG TCC TTA GTT</td>
<td>This paper</td>
</tr>
<tr>
<td>Pr10*</td>
<td>ATC TTT CGA TCC AGA GAC</td>
<td>This paper</td>
</tr>
<tr>
<td>MXYF*</td>
<td>ACC GTG GGA AAT CTA GAG CTA A</td>
<td>This paper</td>
</tr>
<tr>
<td>MYXR*</td>
<td>GTT CCA TGC TAT YAA CAT TCA A</td>
<td>This paper</td>
</tr>
<tr>
<td>HENF*</td>
<td>TCT CAC GGA GTG TGC CTT GAG TAA A</td>
<td>This paper</td>
</tr>
<tr>
<td>HENR*</td>
<td>AGA TCA CTC CAC GAA CTA AGA ACG GC</td>
<td>This paper</td>
</tr>
</tbody>
</table>

**Prevalence of infection:** Less than 1% of the bullheads collected from South Creek developed macroscopically visible plasmodia. Vegetative stages of the parasite were visible 6 mo following capture from the wild.
characteristic. Additionally, *H. gurlei* has polar capsules of unequal size, which clearly distinguishes it from other *Henneguya* species that infect ictalurids (Table III).

Plasmodia containing both immature stages and mature spores were found in the dermis of the fins. The large size of the cysts in some instances caused disruption of the dermal pigment layer and the epidermis (Fig. 7). In other areas, hyperplastic epithelial cells and particularly alarm substance cells were noted (Fig. 8). The plasmodia were covered with a well-vascularized layer of host cells and were composed of a single-layer, hyaline wall surrounding the ectoplasm that consisted of a layer containing generative cells and a center filled with mature spores (Figs. 3, 8).

While the inflammatory response to the plasmodium was minimal in many areas, phagocytic cells resembling macrophages, as well as an increased number of lymphocytes, were evident in the surrounding epidermis and subdermal connective tissue (Fig. 8). In some areas, marked inflammation, congestion, or hemorrhage was noted. These changes were likely caused by, or were a response to, damaged host tissue as the plasmodium expanded.

The combination of the 3 primer sets enabled sequencing in both directions of 1,912 bp of SSU rDNA of *H. gurlei* (DQ673465). Primer sets H9-H2, MYXF-MYXR, and HENF-HENR amplified a single product per amplification of 716, 736, and 631 bp, respectively (Fig. 1). Multiple alignment revealed 3 variable regions within the 18S rDNA locus among species of *Henneguya* (Fig. 9). Multiple bases differed in these regions between *H. gurlei* and the most closely related *Henneguya* species for which sequence data are available. These 3 regions are diagnostic for species discrimination in *Henneguya*. Pair-wise comparison of SSU rDNA revealed that the isolate is most similar to the *Henneguya* spp. known to infect *Ictalurus punctatus* (Table IV). Phylogenetic analysis placed *H. gurlei* sister to the clade containing the *I. punctatus* parasites *H. ictaluri* and *H. exilis* (posterior probability = 1.00 by Bayesian analysis; 100% bootstrap support by MP and 98.2% by ML analysis). *Henneguya* spp. were recovered as paraphyletic and *Myxobolus* spp. as polyphyletic, although it has been shown previously that both groups are polyphyletic (Yokoyama et al., 2005). Other phylogenetic methods strongly supported the relationships determined by Bayesian analysis, although there were minor differences in relationships that are irrelevant to this study (Fig. 10).

**DISCUSSION**

*Henneguya* Thélohan, 1892 (phylum Myxozoa) is one of the largest genera in the Myxobolidae, and it contains pathogens

---

**Figure 3.** Histological section of a plasmodium containing sporoblasts (Sb), germ cells (Gc), and immature spores. A well-defined hyaline wall (Hw) externally lined with fibroblasts (F) of host origin internally borders a diffuse layer of ectoplasm (E). H&E staining.

**Table III.** Morphological characteristics of mature *H. gurlei* spores (n = 100). All measurements are provided in microns (μm).

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length</td>
<td>60.9 ± 1.2</td>
<td>48.7</td>
<td>68.5</td>
</tr>
<tr>
<td>Polar capsules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (long)</td>
<td>6.2 ± 0.1</td>
<td>5.48</td>
<td>7.06</td>
</tr>
<tr>
<td>Length (short)</td>
<td>5.7 ± 0.1</td>
<td>4.8</td>
<td>6.4</td>
</tr>
<tr>
<td>Width</td>
<td>1.2 ± 0.03</td>
<td>1.0</td>
<td>1.54</td>
</tr>
<tr>
<td>Spore body</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>18.2 ± 0.3</td>
<td>15.7</td>
<td>20.3</td>
</tr>
<tr>
<td>Width</td>
<td>5.4 ± 0.1</td>
<td>3.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Thickness</td>
<td>3.2 ± 0.1</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Caudal appendage length</td>
<td>41.1 ± 1.1</td>
<td>34.0</td>
<td>49.7</td>
</tr>
</tbody>
</table>
of significance in settings of intensive aquaculture. Complete life cycles have only been described for a few species of *Henneguya* (Lin et al., 1999; Pote et al., 2000; Kallert et al., 2005) and, based on these studies, it appears that species of *Henneguya* utilize a multihost (fish and aquatic oligochaete) life cycle similar to that of *Myxobolus* (Kent et al., 2001). To date, an actinospore stage of *H. gurlei* has not been identified. As such, the *A. nebulosus* discussed in this research were not purposefully infected. Moreover, they were not cultured in tanks containing an oligochaete population that may have been infected with this parasite. These fish were captured from the wild prior to tank rearing and were most likely infected with this parasite at the time of capture.

*Ameiurus nebulosus* and *A. melas* have been utilized as sentinel species of environmental health for the past 25 yr. Much of this work has focused on the frequency of neoplastic lesions,
including skin papillomas (Baumann, 1984; Smith et al., 1989; Bowser et al., 1991; Baumann and Harshbarger, 1998; Pyron et al., 2001; Pinkney et al., 2004). Despite frequent sampling of these fish and rigorous histological evaluation, especially of skin abnormalities, cutaneous species of *Henneguya* are not commonly observed in ameiurids. Following this trend, isolates of *H. gurlei* and *H. ameiurenensis* were found in a limited number of fish, and attempts by Nigrelli and Smith (1940) to isolate *H. ameiurenensis* from other bullheads were unsuccessful. In an effort to conduct molecular comparisons of the current isolate with other species of *Henneguya* species from the skin of ameiurid or ictalurid catfishes, we attempted to obtain historical samples from investigators active in ameiurid histological surveys or *Henneguya* spp. diseases of cultured ictalurids. Sequences (currently unpublished and kindly provided by Matt Griffin, Mississippi State, pers. comm.) of SSU rDNA from 2 isolates (a novel species and a probable, but unconfirmed, isolate of *H. adiposa*) of *Henneguya* spp. that have a cutaneous presentation in *I. punctatus* were genetically different than *H. gurlei*. Acquisition of historical samples from ameiurids was unsuccessful. Even the Registry of Tumors in Lower Animals, which archives more than 1,000 samples of neoplastic and non-neoplastic lesions from species of *Ameiurus*, does not have a single diagnosis of a cutaneous *Henneguya* sp. infection (Wolf and Wolfe, 1965–2006). The infrequency of observed cutaneous *Henneguya* sp. in this intensively studied host is curious. In the case of the present isolate, manifestation of this disease may be the result of culture stress from rearing these bullheads in a recirculating system at a moderate density. Perhaps such stressors are required for the clinical presentation of the parasite reported here.

Spore morphology has traditionally been used to identify species of *Henneguya* and other myxosporeans. However, it is clear that other features such as host- and tissue-specificity are also useful for accurate identification in the absence of DNA sequence data (Molnár et al., 2002) and correlate better with SSU rDNA compared to spore morphology (Kent et al., 2001). While morphometrics of myxozoan spores, including total spore or spore body length, width, and thickness, are often suitable diagnostic features, they are sometimes inaccurate and ambiguous descriptive end points. This is particularly the case in myxozoans with asynchronous spore development such as species of *Henneguya*. Additionally, morphological variation has been noted in species of myxozoans that infect multiple hosts (Hallett et al., 2006). Thus, definitive identification of these histozoic parasites is sometime not possible, especially in the case of poorly described species. Presently, the application of molecular analyses, particularly utilization of small-subunit ribosomal RNA gene sequences in the case of myxozoans, is the most sensitive approach for definitive species identification.

The SSU rDNA sequence data for *H. gurlei* enable a clear distinction to be made from other described species of *Henneguya* that infect ictalurids. Utilization of genetic sequence information, such as SSU rDNA, clearly offers a powerful diagnostic tool capable of clarifying the identity and relationships between morphologically similar ictalurid *Henneguya* species. Such molecular approaches are likely necessary to describe the life cycle of these *Henneguya* species. Current evidence of this assertion is supported by the necessity for molecular data to conclusively identify *H. ictaluri* as the causative agent of proliferative gill disease in channel catfish (Pote et al., 2000). Unfortunately, only 20 SSU rDNA sequences from *Henneguya* spp. have been deposited in the NCBI GenBank, some of which are redundant. Sequence information from other, more rapidly evolving, loci may support a reassignment of species designa-
Histological section of a plasmodium containing mature spores with evident vacuoles in the sporoplasm (arrows) and coronal section of mature henneguya spores with well-defined sutural ridges (arrow heads; inlay).

ACKNOWLEDGMENTS
The authors would like to thank Blake Martin and the Pamlico Aquaculture Field Laboratory staff (Aurora, North Carolina) for collecting and holding brown bullheads for our laboratory stock population. Many thanks are due to Lorelei Ford (Mississippi State University) for her assistance with the initial sequencing of this isolate, and to the genetics laboratory of Dr. Tim King, U.S. Geological Survey Aquatic Ecology Laboratory, for time on their ABI for subsequent molecular analysis. We would also like to thank Drs. Alistair Dove, John Switzer, and Christine Densmore for critical review of the draft manuscript.
Figure 7. Histological sections of dermal cysts (P) in the caudal fin of an infected brown bullhead (a) that have disrupted (arrow heads) the normal pigment layer (arrows) (b). H&E and Geimsa staining, respectively.
Figure 8. Hyperplastic epithelial cells and alarm substance (As) cells in the epidermis, superficial to the well-vascularized layer of host cells surrounding plasmodium (P). Inflammatory lymphocytes can be seen in the epidermis and subdermal connective tissue (arrows). H&E staining.

Figure 9. Alignment of 18S rDNA emphasizing the diagnostic variable regions (DVR1, DVR2, and DVR3).
Figure 10. Phylogenies derived from ML (a) or BI (b) analyses of myxosporean 18S SSU rDNA (1694 bp including gaps). Bootstrap values are MP/ML and are reported on the same tree as the topology of the single most parsimonious tree (length = 3,985, CI = 0.5184, HI = 0.4816, RI = 0.6062; 899 parsimony informative characters), which was identical to that generated by ML analysis (−ln L = −17801.29, 1,000 replicates, GTR + I + G).

LITERATURE CITED


———, and A. Diamant. 2001. Ultrastructure and small-subunit ribosomal DNA sequence of Henneguya lesteri n. sp. (Myxosporea), a parasite of sand whiting Sillago analis (Sillaginidae) from the coast of Queensland, Australia. Diseases of Aquatic Organisms 46: 197–212.


SEQUENCE ANALYSIS OF RIBOSOMAL AND MITOCHONDRIAL GENES OF THE GIANT LIVER FLUKE Fascioloides magna (Trematoda: Fasciolidae): INTRASPECIFIC VARIATION AND DIFFERENTIATION FROM Fasciola hepatica

Ivica Králová-Hromadová, Marta Špakulová, Eva Horáčková*, Ludmila Turčeková, Adam Novobilský†, Relja Beck‡, Břetislav Koudela*, Albert Marinculič‡, Dušan Rajski‡, and Margo Pybus#

Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 04001 Košice, Slovakia. e-mail: hromadova@saske.sk

ABSTRACT: Complete sequences of ribosomal and mitochondrial genes of the giant liver fluke Fascioloides magna are presented. In particular, small subunit (18S) and internal transcribed spacers (ITS1 and ITS2) of the ribosomal gene (rDNA), as well as cytochrome c oxidase subunit I (cox1) and nicotinamide dehydrogenase subunit I (nad1) of the mitochondrial DNA (mtDNA), were analyzed. The 18S and ITS sequences were compared with previously published sequences of the liver fluke Fasciola hepatica. Fixed interspecific genetic differences were determined that allow molecular differentiation of F. magna and F. hepatica using either the PCR-RFLP method or PCR amplification of species-specific DNA regions. Additionally, intraspecific sequence polymorphism of the complete cox1 and nad1 mitochondrial genes in geographically distinct F. magna populations was determined. Based on the sequence divergences, short (<500 bp) variable regions suitable for broader biogeographical studies of giant liver fluke were designed.

The giant liver fluke Fascioloides magna is one of the largest digenetic flukes parasitizing the liver parenchyma of wild and domestic ruminants. It is generally accepted that F. magna is of northern U.S. origin and that it may have co-evolved with ancestral cervid host Odocoileus spp. (Pybus, 2001). Presently, the occurrence of F. magna in North America is restricted to 5 enzootic areas, which may be enlarged by the natural migration of obligate cervid hosts or by their translocation (Pybus, 2001).

In Europe, F. magna was first recorded in cervids in the natural park La Mandria near Turin in Italy, and it is still present in this area, with periodically fluctuating prevalence (Bassi, 1875; Balbo et al., 1987, 1989). Since then F. magna has established permanent foci in the Czech Republic (Ulrich, 1930; Erhardová-Kotrlá, 1971; Novobilský, Horáčková et al., 2007) and in the forest landscapes alongside the River Danube involving territories of Slovakia (Rajský et al., 1994, 2002), Hungary (Majoros and Sztojkov, 1994; Égri and Sztojkov, 1999), Austria (Winkelmayer and Prosl, 2001; Ursprung et al., 2006), and Croatia (Marinculič et al., 2002; Janicky et al., 2005; Slavica et al., 2006). At present, the parasite is spreading both up- and downstream along the Danube (Špakulová et al., 2003).

The closest relatives of F. magna are species of Fasciola, among which chiefly Fasciola hepatica represents the cosmo-politan parasite of great veterinarian and even medical impact (Mas-Coma, 2005). The discrimination of adults of both genera is easy because of significant differences in size, shape, and several other morphological characters (Erhardová-Kotrlá, 1971; Špakulová et al., 2003; Jones, 2005). The flukes share sympatric geographic ranges in some wetland habitats of North America and Europe and several definitive host species of cervids and bovids, utilizing snail intermediate hosts from the Lymnaeidae. Contrary to adult worms, intramolluscan larval stages and eggs of both flukes are morphologically almost indistinguishable (Pybus, 2001; Novobilský, Horáčková et al., 2007). Consequently, fecal examination, or a search of infected molluscs, or both using only morphological examination could not provide reliable differentiation of Fascioloides and Fasciola species.

A molecular approach, often used for quick and reliable differentiation among species of helminths, could be applied also in the case of the liver flukes. In particular, PCR-based techniques are an alternative approach to identify and separate eggs and larval stages of F. magna and F. hepatica. Moreover, an examination of intraspecific polymorphism of F. magna by sequence analysis of selected genes would provide meaningful information for ecological and phylogeographical studies. To date, there is limited information on F. magna sequence structure and variation. Adlard et al. (1993) published incomplete sequence of internal transcribed spacer 2 (ITS2) of a U.S. sample of F. magna. Recently, partial sequences of the small and large rDNA subunits and complete ITS1, 5.8S, and ITS2 sequences of another U.S. isolate of F. magna (www.ncbi.nlm.nih.gov; EF051080), as well as complete ITS2 sequence of an Austrian F. magna sample (DQ683545), have appeared in GenBank. However, these data have not been published.

Our primary goal was to obtain the complete sequences of selected ribosomal and mitochondrial genes of F. magna. The complete small subunit and internal transcribed spacers of ribosomal genes are compared with published data for corresponding genes of F. hepatica. Fixed interspecific genetic differences were determined, and a suitable molecular discrimination method for species delineation is proposed. We also sequenced the complete mitochondrial genes cox1 and nad1 of several geographically distinct F. magna samples. Based on the sequence divergence, shorter variable regions (<500 bp) suitable for molecular screening of high numbers of individuals from geographically distinct regions were selected.

MATERIALS AND METHODS

Materials
Adults of Fascioloides magna were isolated from liver tissue, rinsed in PBS buffer, and fixed in 96% ethanol. The giant liver flukes analyzed...
in the current work originated from 4 geographically distinct regions and following hosts species: (1) red deer (Cervus elaphus), Bodíky (Du­najská Streda), Slovak Republic; (2) red deer, Mirošov, Czech Republic; (3) black-tailed deer (Odocoileus hemionus columbianus), Salem, Oregon, U.S.A.; and (4) wapiti (Cervus elaphus canadensis), Banff, Alberta, Canada. Two isolates of the liver fluke *F. hepatica* came from cattle (Bos taurus f. domestica) from Snina, the Slovak Republic, and Limoges, France.

Trematodes of *F. magna* from Canada and the Slovak Republic and *F. hepatica* from the Slovak Republic were isolated directly from the livers of hunted animals and slaughtered cattle. The Czech and U.S. specimens of *F. magna* and the French sample of *F. hepatica* were obtained during the experimental infection as described by Novobilsky, Kasny et al. (2007).

**DNA isolation**

The genomic DNA of adult flukes (single worm from each sample) was isolated using incubation in extraction buffer (10 mM TRIS-HCl, pH 7.5; 10 mM EDTA; 50 mM NaCl; 2% sodium dodecyl sulphate and 20 mM dithiothreitol) and 900 μg/ml proteinase K at 56°C with gentle shaking for 12 hr. After lysis, phenol:chlorophorm:isoamylalcohol (25:24:1) extraction and ethanol precipitation (100% ethanol; 3 M sodium acetate, pH 4.8) were performed. Genomic DNA was finally diluted in deionized water and stored in −20°C.

**PCR and design of primers**

**PCR amplification:** For PCR amplification, a standard protocol was used. The total volume of amplification mixture was 50 μl and contained 10–20 ng of genomic DNA, 20 pmol of each of the 2 primers, 0.2 mM of each of the deoxynucleotide triphosphate (Fermentas UAB, Vilnius, Lithuania), 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, California) with corresponding reaction buffer and 1.5 mM MgCl2. The amplification was performed in the Techno-Gene thermal cycler programmed for 5 min at 94°C as the initial step, followed by 30 cycles 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. The final step was 5 min at 72°C. The annealing temperature of PCR amplification with species-specific primers was 55°C. The PCR products were loaded on the 1.5% agarose gel and purified using the Wizard PCR purification kit (Promega, Madison, Wisconsin).

**ITS primer design:** For amplification of the complete ITS1, the ITS-1 universal primer (5′-TTCCCGTAGGGTGAAACCTT-3′; Cunningham, 1997) annealing in flanking 18S rDNA, and 5.8S-1 primer (5′-CGCAATATGCGTCAAG-3′; Králová-Hromadová, Scholz et al., 2003) with annealing position in 5.8S rDNA were applied. Similarly for ITS2, the 5.8S-2 (5′-GTCGATGGAAGAGGCAG-3′; Králová-Hromadová, Scholz et al., 2003), and ITS-2 (5′-AGGAGGCGAATCACTAT-3′; Cunningham, 1997) primers with annealing positions in the 5.8S and 28S rDNA, respectively, were used (Fig. 1).

**18S primer design:** The complete 18S rDNA subunit was amplified using the conserved PCR/sequencing primers for small subunit (Littlewood and Olson, 2001) (Fig. 1). The 18S-E forward primer (5′-CCG AATTCTGCGACAACCTGTTTGATCGCCATT-3′) anneals at the very 5′ end of the 18S; the Worm-B reverse primer (5′-CTGTGATACGACTTTCATTCC-3′) has its annealing location at the very 3′ end of the 18S. To complete the small subunit sequences from both sides, 6 internal primers, in particular 18S-8 (5′-GCGCCTGGGATAATCCC-3′), Pace-A (5′-GCGTACTCCGGCTGTGAT-3′), Ael-5 (5′-TGGTTTACATTGACACACAGC-3′), 18S-11 (5′-GCTGTTACCGGCCTCTG-3′), 18S-1 (5′-AACGCGATCGCACCACCAC-5′), and 18S-5 (5′-CCCTTGGTACACCCCGCGCCG-3′) (Littlewood and Olson, 2001) were also applied.

**cox1 and nad1 primer design:** For amplification of the complete *cox1* and *nad1* mitochondrial genes of *F. magna*, the complete mt genomes of *F. hepatica* (GenBank AF216697 and NC002546; Le et al., 2000) and *Paragonimus westermani* (NC002354 and AF219379) were compared. Their comparison revealed highly conservative regions within rRNA genes and the large subunit of the ribosomal gene (*rrnL*.) (Fig. 1). In particular, primers *cox1int* and *cox1int* annealing in the rRNA-Ser

**Figure 1.** Schematic diagram of nuclear ribosomal gene with annealing positions of primers. Notes: gray boxes: desired genes; black boxes: amplified fragments; arrows: annealing positions and orientation of primers.

**Figure 2.** Schematic diagram of part of the mitochondrial DNA of *Fasciola hepatica* (Le et al., 2000). Notes: black boxes: desired genes cytochrome *c* oxidase subunit I (*cox1*) and nicotinamid dehydrogenase subunit I (*nad1*); gray boxes: amplified fragments; arrows: annealing positions and orientation of primers; empty boxes: surrounding genes; *nad2*, *nad3*: nicotinamid dehydrogenase subunit II and III, respectively; *rrnL*: large subunit of the ribosomal gene; three-letter codes: codes for rRNA for specific amino acids; Val: Valine; Ala: Alanine; Asp: Aspartic acid; Asn: Asparagine; Pro: Proline; Ile: Isoleucine; Lys: Lysine; Ser: Serine; Trp: Tryptophan; Thr: Threonine.
Table I. Originally designed primers used for PCR amplification and sequencing of mitochondrial cox1 and nad1 genes of Fascioloides magna.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence of primer</th>
<th>Annealing position</th>
<th>n1/n2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cox1</td>
<td>cox1See</td>
<td>5'--GCTTTGTCCGCTGCTAAGC--3'</td>
<td>tRNA-Ser mtDNA</td>
<td>17/16</td>
</tr>
<tr>
<td>cox1</td>
<td>cox1Int</td>
<td>5'--AACATATGATCGCAAAGCTA--3'</td>
<td>rrnL mtDNA</td>
<td>19/19</td>
</tr>
<tr>
<td>cox1</td>
<td>cox1Int2</td>
<td>5'--GCCTGCTGTTTCTGCTGTTG--3'</td>
<td>cox1 mtDNA</td>
<td>Internal primer</td>
</tr>
<tr>
<td>cox1</td>
<td>cox1Int3</td>
<td>5'--CCCCACGAGAATAACGAGCC--3'</td>
<td>cox1 mtDNA</td>
<td>Internal primer</td>
</tr>
<tr>
<td>cox1</td>
<td>cox1Int4</td>
<td>5'--CCCTATCCTAAATATTTAGAATGCG--3'</td>
<td>cox1 mtDNA</td>
<td>Internal primer</td>
</tr>
<tr>
<td>nad1</td>
<td>nad1Ala</td>
<td>5'--CTCTGCCAGACCTCGCT--3'</td>
<td>tRNA-Ala mtDNA</td>
<td>17/16</td>
</tr>
<tr>
<td>nad1</td>
<td>nad1Int</td>
<td>5'--ACCTGCTACGGCCACCCCTA--3'</td>
<td>tRNA-Ile mtDNA</td>
<td>19/19</td>
</tr>
<tr>
<td>nad1</td>
<td>nad1Int2</td>
<td>5'--GGTGCGTCTGCTTGGGCTCT--3'</td>
<td>nad1 mtDNA</td>
<td>Internal primer</td>
</tr>
<tr>
<td>nad1</td>
<td>nad1Int3</td>
<td>5'--AGAGCCAAAACGCAAGAAC--3'</td>
<td>nad1 mtDNA</td>
<td>Internal primer</td>
</tr>
</tbody>
</table>

* n1: number of nucleotides of the primer; n2: number of identical nucleotides between Fasciola hepatica and Paragonimus westermani (only for primers used for PCR amplification).

(Serine) and rrnL regions, respectively, were applied for amplification of complete cox1 gene of F. magna (Fig. 2; Table I).

For amplification of complete nad1 gene of F. magna, the primers nad1Int and nad1Int2 designed according to the tRNA-Ala (Alanine) and tRNA-Ile (Isoleucine) gene sequences, respectively, were used (Fig. 2; Table I). To complete sequences of cox1 and nad1 from both sides, 4 cox1 and 2 nad1 internal primers were applied (Fig. 2; Table I). The TAG stop codon and modified GTG start codon (instead of usual ATG) were determined according to the trematode mitochondrial code (Garey and Wolstenholme, 1989; Ohama et al., 1990).

PCR-RFLP

For RFLP analysis, ITS1 and ITS2 PCR products of F. magna from 4 allopatric populations and those of F. hepatica from 2 distinct localities (see Material) were applied. For restriction analysis, 20 μl of PCR products were directly digested with 10 U of MseI restriction enzyme (Promega) for 3 hr. The digestion products were loaded on 2% agarose gels stained with ethidium bromide.

Sequencing and sequence analysis

The amplified PCR products were loaded on the gel and purified using the Wizard PCR purification kit (Promega). They were directly sequenced using automatic genetic analyzer Applied Biosystems 3130xl (Applied Biosystems, Foster City, California) and sequencing kit BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems). The sequence alignment was performed using ClustalW (Thompson et al., 1994).

RESULTS

ITS1

PCR amplification with universal primers annealing in the 18S and 5.8S rDNA regions (Fig. 1) resulted in a 571-bp fragment, which included the complete ITS1 and the partial 18S and 5.8S rDNA sequences. For all 4, geographically distant F. magna samples (Slovak Republic, Czech Republic, Canada, and United States), the amplified regions were 100% identical, and no sequence variation was determined. The ITS1 sequences were deposited in GenBank under accession nos. EF534987, EF534988, EF534990, and EF534991. They were identical also with the ITS1 sequence of another U.S. sample of F. magna from Oregon (GenBank EF051080).

A corresponding 600-bp region including the complete ITS1 and partial 18S and 5.8S rDNA sequences of F. hepatica from Uruguay, Australia, Northern Ireland, and Korea (GenBank AB207139-41 and AB211236; Itagaki, Kikawa, Sakaguchi et al. 2005; Itagaki, Kikawa, Terasaki et al., 2005), a 433-bp ITS1 region of Bolivian and Spanish isolates (GenBank AJ243016; Mas-Coma et al., 2001), and a 422-bp ITS1 of 2 Chinese isolates (GenBank AJ628431-32) were included in the comparative analysis. The equivalent ITS1 regions of all of 8 geographically distinct F. hepatica isolates displayed no intraspecific sequence polymorphism and were 100% identical.

For a mutual comparison of ITS1 of F. magna and F. hepatica, the 430-bp region, matching with the 433-bp F. hepatica ITS1 sequence (Mas-Coma et al., 2001), was applied (Fig. 3). The level of interspecific sequence variation was 6.9%. The variation was due to 5 insertions/deletions and 25 nucleotide substitutions (Fig. 3). The analysis of restriction enzyme cut sites revealed differences in restriction sites for numerous restriction enzymes. For instance, the ITS1 region of F. magna possessed 1, and F. hepatica 2, cleavage site(s) for MseI (TTAA) within ITS1 region (Fig. 3), and an additional site is located in adjacent 5.8S rDNA of both species (data not shown). The restriction analysis of PCR products (PCR-RFLP) resulted in different restriction profiles for both species, namely, a 3-band profile in F. magna and a 4-band profile in F. hepatica. All analyzed geographic isolates of F. magna and F. hepatica (see Material) had species-specific RFLP patterns with no intraspecific variation.

Within the ITS1 region, short sequence motifs reflecting a rather high degree of variation between F. magna and F. hepatica were selected for design of species-specific primers (Fig. 3; Table II). The 2 F. magna-specific primers amplify a 127-bp fragment in the F. magna DNA and do not anneal in the F. hepatica DNA. Similarly, a 224-bp fragment is amplified with F. hepatica–specific primers with the F. hepatica DNA, while no amplification product was obtained with the F. magna DNA (Fig. 4).

ITS2

PCR amplification with universal primers annealing in the 5.8S and 28S rDNA regions (Fig. 1) resulted in 562-bp fragment, which included the complete ITS2 and the partial 28S and 5.8S rDNA sequences. For all 4, geographically distant F. magna samples, the amplified regions were 100% identical, and no sequence polymorphism was detected. The ITS2 sequences were deposited in GenBank under accession nos. EF534992-95. These sequences were identical with the ITS2 sequence of F. magna from Oregon, U.S.A. (GenBank EF051080), as well as with the Austrian F. magna sample (GenBank DQ683545). A partial sequence of ITS2 for another U.S. F. magna was ob-
**TABLE II. Primers originally designed for species identification and biogeography.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplified region</th>
<th>Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1</td>
<td>FmITS1.SPEC.F</td>
<td>FmITS1.SPEC.R</td>
<td>127 bp</td>
<td>F. magna</td>
</tr>
<tr>
<td></td>
<td>5’-TGTCATGCGATAAAATGTTT-3’</td>
<td>5’-CTGGACCGCCGCGAGGAA-3’</td>
<td>Identification</td>
<td></td>
</tr>
<tr>
<td>ITS1</td>
<td>FhITS1.SPEC.F</td>
<td>FhITS1.SPEC.R</td>
<td>224 bp</td>
<td>F. hepatica</td>
</tr>
<tr>
<td></td>
<td>5’-CATCGGATAAATTTGCGGACA-3’</td>
<td>5’-TGAAACAGTTCATAGGGCGGGA-3’</td>
<td>Identification</td>
<td></td>
</tr>
<tr>
<td>ITS2</td>
<td>FmITS2.SPEC.F</td>
<td>FmITS2.SPEC.R</td>
<td>152 bp</td>
<td>F. magna</td>
</tr>
<tr>
<td></td>
<td>5’-ACCGTTATGTTGTTGTTG-3’</td>
<td>5’-CCGTCTTTAAAACACAG-3’</td>
<td>Identification</td>
<td></td>
</tr>
<tr>
<td>ITS2</td>
<td>FhITS2.SPEC.F</td>
<td>FhITS2.SPEC.R</td>
<td>112 bp</td>
<td>F. hepatica</td>
</tr>
<tr>
<td></td>
<td>5’-CTATGATTTGCTCTGAAAAT-3’</td>
<td>5’-ATATCAACGACTAGCCC-3’</td>
<td>Identification</td>
<td></td>
</tr>
<tr>
<td>cox1</td>
<td>FmCOX1.VAR.F</td>
<td>FmCOX1.VAR.L</td>
<td>440 bp</td>
<td>Biogeography</td>
</tr>
<tr>
<td></td>
<td>5’-GGTCAAGGCGGATTATAC-3’</td>
<td>5’-ACACGTAGTAATTGCGG-3’</td>
<td>Biogeography</td>
<td></td>
</tr>
<tr>
<td>cox1</td>
<td>FmCOX1.VAR.R</td>
<td>FmCOX1.VAR.R</td>
<td>430 bp</td>
<td>Biogeography</td>
</tr>
<tr>
<td></td>
<td>5’-TATGCGGTTGTCATTTC-3’</td>
<td>5’-ATATCAACGACTAGCCC-3’</td>
<td>Biogeography</td>
<td></td>
</tr>
<tr>
<td>nad1</td>
<td>FmNAD1.VAR.F</td>
<td>FmNAD1.VAR.R</td>
<td>440 bp</td>
<td>Biogeography</td>
</tr>
<tr>
<td></td>
<td>5’-TATGCGGTTGTCATTTC-3’</td>
<td>5’-CTACACACATATAAAAAAC-3’</td>
<td>Biogeography</td>
<td></td>
</tr>
</tbody>
</table>
tained by Adlard et al. (1993). Its comparison with all 6 of the above mentioned sequences revealed 6 deletions in the later sequence, which was not included in the analysis (see Discussion).

The corresponding 506-bp region, which included complete ITS2, and partial 28S and 5.8S rDNA of *F. hepatica* from Uruguay, Australia, and Northern Ireland (GenBank AB010974 and AB207148; Itagaki and Tsutsumi, 1998; Itagaki, Kikawa, Sakaguchi et al., 2005), the 364-bp ITS2 sequence of *F. hepatica* from Spain and Bolivia (GenBank AJ272053; Mas-Coma et al., 2001), the 362-bp ITS2 sequence of *F. hepatica* from China and France (GenBank AJ557567-68; Huang et al., 2004), and the 523-bp partial 5.8S rDNA and ITS2 sequence of *F. hepatica* from Austria (GenBank DQ683546), were included into the analysis. The sequence alignment based on ITS2 sequences of 8 geographically distant *F. hepatica* samples revealed 4 nucleotide intraspecific substitutions (Fig. 5).

For ITS2 sequence comparison between *F. magna* and *F. hepatica*, the 363-bp region overlapping with the 364 bp *F. hepatica* ITS2 region, as determined by Mas-Coma et al. (2001), was applied (Fig. 5). *Fascioloides magna* ITS2 sequence was compared with each other of *F. hepatica* ITS2 sequences, and the level of interspecific sequence variation was 11.3–11.9%. The variation was due to 1 insertion/deletion and 38–42 nucleotide substitutions (Fig. 5). The analysis of restriction enzyme cut sites revealed differences in restriction sites of numerous restriction enzymes. For instance, the *F. magna* possesses 2, and *F. hepatica* 1, cleavage sites for *MseI* (TTAA) in the ITS2 region (Fig. 5), and both flukes also have a cleavage site in flanking 28S rDNA (Fig. 5) and 5.8S rDNA (data not shown). Thus, the 5-band PCR-RFLP profile of *F. magna* differed from 4-band pattern of *F. hepatica*. As for ITS1, samples from different geographic isolates of both species displayed no intraspecific variation in restriction profiles.

Within the ITS2 sequences of both flukes, regions that reflect a rather high degree of interspecific sequence variation were selected for primer design (Fig. 5; Table II). The 2 *F. magna*-specific primers amplify a 152-bp fragment not detected in the *F. hepatica* DNA. On the other hand, the *F. hepatica*-specific primers provide about 112-bp fragment in *F. hepatica* DNA, but do not anneal in the *F. magna* DNA (Fig. 4).

**18S rRNA**

Using universal PCR primers for 18S rDNA (Fig. 1), the 1,934-bp region of 18S rDNA of *F. magna* from the Czech Republic was obtained. The sequence was deposited in GenBank under accession no. EF534989 and was identical with 18S rDNA of *F. magna* from Oregon, U.S.A. (GenBank EF051080). A comparison with the 18S rDNA of *F. hepatica* (GenBank AJ004969; Fernandez et al., 1998) revealed rather low interspecific sequence variation (0.7%) caused by 1 deletion and 13 nucleotide substitutions in the *F. magna* 18S rDNA sequence (Table III). A slightly higher level of sequence polymorphism (2%) was revealed in the variable region V4 (data not shown).

**cox1**

The 2 designed primers *cox1* and *cox1* annealing in the tRNA-Ser and *rrnL* mt DNA regions (Fig. 2; Table I), respectively, amplified a 1,726-bp region, including partial tRNA-Ser, complete tRNA-Trp (Tryptophan), complete *cox1*, complete tRNA-Thr (Threonine), and partial *rrnL* sequences of *F. magna* mt DNA. The 1,545-bp *cox1* gene encoded for a protein of 515 amino acids (aa).
KRÁLOVÁ-HROMADOVÁ ET AL.—SEQUENCE ANALYSIS OF F. MAGNA DNA

5.8S

Fas.magna_ITS2_current
F. hepatica_ITS2_ltagaki1
F. hepatica_ITS2_ltagaki2
F. hepatica_ITS2_MasCom
F. hepatica_ITS2_Huang

ITTS2

GCCATGGTGTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTA~AACT
GCCATGGTGTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG~AOOAACT

GCCATGGTGTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG~AOOAACT
GCCATGGTGTAGCCTGTGGCCACGCCTGTCCGAGGGTCGG~AOOAACT

ATCACGACGCCCAAA0AGTCGTGGCTTGGGTTTTGCCAGCTGGCG~ATC
ATCACGACGCCCAAA0AGTCGTGGCTTGGGTTTTGCCAGCTGGCG~ATC

GTATCCGGATGCACCCTTGTCCTGGCAGAA~CGTG

GCCGAATCGTGGTTTAATAAI~GGGTrG(}T
GCCGAATCGTGGTTTAATAAI~GGGTrG(}T

MseI

FM_ITS2_SPEC_F

FH_ITS2_SPEC_F

S

FH_ITS2_SPEC_R

MseI

128S

CTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGAACTTAAGCATATCA
CTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGAACTTAAGCATATC/

ATACCCGCTGAACTTAAGCATATC/

CTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGAACTTAAGCATATC/
CTTCCTGACCTCGGTTCAGACGTGATTACCCGCTGAACTTAAGCATATC/

...}T

...}T
Table III. Nucleotide divergence between 18S rDNA gene sequences of *Fascioloides magna* and *Fasciola hepatica.*

<table>
<thead>
<tr>
<th>Position</th>
<th>167</th>
<th>291</th>
<th>330</th>
<th>616</th>
<th>684</th>
<th>691</th>
<th>772</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. magna</em></td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td><em>F. hepatica</em></td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Position</td>
<td>773</td>
<td>782</td>
<td>809</td>
<td>1,182</td>
<td>1,234</td>
<td>1,677</td>
<td>1,881</td>
</tr>
<tr>
<td><em>F. magna</em></td>
<td>C</td>
<td>—</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td><em>F. hepatica</em></td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>T</td>
</tr>
</tbody>
</table>

* Nucleotide sequence position is according to the sequence of *F. hepatica* (GenBank AJ004969; Fernandez et al., 1998).

The *cox1* sequences of *F. magna* from 3 different geographical localities (Slovak Republic, Czech Republic, and United States) were deposited in GenBank under accession nos. EF534996–98. Their mutual comparison revealed 28 point mutations (1.9% sequence divergence) (Table IV). Of these, 27 were silent, not affecting the translation into the amino acid sequence. Only 1 nucleotide substitution (Table IV, nucleotides in bold) resulted in Serine (alternative flavin mononuclear AGG codon) in the U.S. sample and Glycine (GGG codon) in the Slovak and Czech samples. The sequence polymorphism of *cox1* gene between the Slovak and Czech *F. magna* samples, as well as between the Slovak and U.S. ones was 1.0%. The sequence variation between the Czech and U.S. samples was slightly higher, 1.6%.

The nucleotide substitutions were unequally distributed along the *cox1* gene. The 2 regions within the complete *cox1* gene were selected as the ones with higher mutation rate (Table IV, italicized numbers). The primers proposed for amplification of the first variable region (Table II) amplify the 440-bp *cox1* fragment. It is about 180 bp downstream from the 5’ end of the gene and includes 10 of 28 polymorphic sites, which are responsible for a 2.3% sequence divergence. The second region with a higher mutation rate is at the 3’ end of the gene and involves 8 of 28 point mutations. Primers designed for its amplification (Table II) produce the 430-bp *cox1* fragment in which a 1.9% sequence divergence is due to 8 polymorphic sites.

**nad1**

The PCR amplification of the complete *nad1* gene of *F. magna* with *nad1*iso and *nad1*le primers (Table I) designed according to the tRNA-Ala and tRNA-Ile gene sequences (Fig. 2), respectively, resulted in 1,094-bp fragment including complete tRNA-Asp (Aspartic acid), complete *nad1*, complete tRNA-Asn (Asparagine), and partial tRNA-Pro (Proline) sequences of *F. magna* mtDNA. Of that, the 903-bp *nad1* gene encoded for a protein of 301 amino acids.

The *nad1* sequences of *F. magna* from 3 different geographical localities (Slovak Republic, Czech Republic, and United States) were deposited in GenBank under accession nos. EF534999, EF535000, and EF535001. Their mutual comparison revealed 13 polymorphic sites (a 1.5% sequence divergence) (Table IV). Of these, 11 were silent, not affecting the translation into the amino acid sequence. Two nucleotide substitutions were responsible for change in codon. The first (Table IV, nucleotides in bold) resulted in Threonine (ACG codon) in the Czech and Slovak samples and Methionine (ATG) in the U.S. sample. The second polymorphic site (Table IV, nucleotides in bold, closer to 3’ end) was responsible for coding Isoleucine (ATT codon) in the Slovak and U.S. samples, and Valine (GTG codon) in the Czech sample. The sequence variation of *nad1* gene between the Slovak and U.S. *F. magna* samples, as well as between the Czech and U.S. ones, was 1.0%. The nucleotide polymorphism between the Czech and Slovak samples was slightly lower, 0.9%.

Of 13 polymorphic sites, 8 were distributed near the 3’ end of the gene (Table IV, italicized numbers). This region was selected as the one with a high mutation rate. The primers proposed for amplification of this region (Table II) give the 440 bp *nad1* fragment, where 8 variable sites were responsible for a 1.9% variation.

Table IV. Nucleotide divergence among *cox1* and *nad1* sequences of *Fascioloides magna* from United States (US), Slovak Republic (SR), and Czech Republic CR.*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>1,155</th>
<th>1,167</th>
<th>1,308</th>
<th>1,386</th>
<th>1,413</th>
<th>1,431</th>
<th>1,473</th>
<th>1,492</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cox1</em></td>
<td></td>
<td>18</td>
<td>21</td>
<td>240</td>
<td>246</td>
<td>309</td>
<td>324</td>
<td>339</td>
<td>444</td>
</tr>
<tr>
<td>US</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>SR</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>CR</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>Position</td>
<td></td>
<td>492</td>
<td>567</td>
<td>651</td>
<td>711</td>
<td>735</td>
<td>771</td>
<td>831</td>
<td>958</td>
</tr>
<tr>
<td>US</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>SR</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>CR</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td><em>nad1</em></td>
<td></td>
<td>1,523</td>
<td>1,532</td>
<td>1,586</td>
<td>1,643</td>
<td>1,673</td>
<td>1,713</td>
<td>1,733</td>
<td>1,752</td>
</tr>
<tr>
<td>US</td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>SR</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>CR</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>G</td>
</tr>
</tbody>
</table>

* Nucleotides in bold: substitutions affecting the amino acid translations. Nucleotides included in the variable regions are below italicized numbers.
DISCUSSION

The giant liver fluke *F. magna* has attracted the attention of wildlife managers and veterinarians because of its unusual body size and potential to be detrimental to wild and domestic ruminants. In spite of this, it has never been investigated as intensively as the common liver fluke *F. hepatica*, which is not only a serious animal parasite but its prevalence in humans has been increasing in Central and South America, Europe, Africa, and Asia (Mas-Coma, 2005). The distribution of the 2 flukes overlaps in some areas in Europe (Erhardová-Kotrlá, 1971) and North America (Craig and Huey, 1984; Knapp et al., 1992). Whereas *F. magna* is primarily a parasite of wildlife ruminants and occurs in livestock in enzootic areas mainly in North America, *F. hepatica* is found predominantly in domestic sheep, goats, and cattle; infections in cervids are incidental, being reported more often in Europe (Pybus, 2001). Consequently, the possible simultaneous occurrence of both flukes in cervids or domestic animals has to be considered in enzootic areas.

The accurate identification of helminths at any developmental stage is central to many areas of parasitology, i.e., diagnosis, epidemiology, and control, as well as systematics, phylogeny, and ecology. Individual parasites traditionally are identified and distinguished on the basis of morphological features, the spectrum of hosts they infect, their geographical origin, and pathological effects on the host. However, these criteria often are inadequate for identification, particularly for eggs and larval stages (Gasser, 2001). In the case of *F. magna* and *F. hepatica*, the eggs and larval stages are morphologically similar (Hood et al., 1997), and epizootiological surveys based on copropathological examinations of wildlife ruminants or livestock do not provide reliable data. The overlapping spectrum of definitive hosts and geographical areas they inhabit make their differentiation difficult. Therefore, molecular approaches, often used for a quick and reliable differentiation of species of many animals, including parasitic flatworms (Olson and Tkach, 2005; Blair, 2006), should be applied also in the case of liver flukes.

In the current work, complete 18S rDNA, ITS1, and ITS2 sequences of *F. magna* were obtained and compared with the corresponding sequences of *F. hepatica*. The slight sequence divergence (0.7%) in the 18S rDNA did not permit a design of species-specific markers. On the other hand, ITS1 and ITS2 sequences provide many specific features for discrimination of *F. magna* and *F. hepatica*. Since these markers must rely on genetically fixed differences between species, a level of intraspecific variation of both *F. magna* and *F. hepatica* has to be established beforehand. For that reason, ITS sequences of geographically distant *F. magna* samples and *F. hepatica* populations were compared.

With respect to the ITS1 sequences, 4 currently analyzed isolates of *F. magna*, along with ITS1 sequence of a U.S. *F. magna* deposited in GenBank (EF051080), revealed no intraspecific sequence variation, similar to *F. hepatica* samples from 8 worldwide populations (Mas-Coma et al., 2001; Itagaki, Kikawa, Sakaguchi et al., 2005; Itagaki, Kikawa, Terasaki et al., 2005; AJ628431-32). Thus, fixed differences in ITS1 of both fluke species permit their reliable differentiation using either PCR-RFLP or PCR with species-specific primers.

A slightly more complicated situation was found in an analysis of the ITS2 structure of *F. magna*. No sequence polymorphism was detected in the ITS2 sequences of isolates from the Slovak Republic, Czech Republic, Canada, and United States, as well as in the ITS2 of the Austrian and the other U.S. samples of *F. magna* deposited in GenBank (DQ683545, EP051080). However, partial ITS2 sequence of another U.S. *F. magna* isolate published by Adlard et al. (1993) revealed 6 deletions in the latter sequence. The most likely explanation for these discrepancies is that during manual sequencing, common in the early 1990s, fusion of bands developed on X-ray films may have occurred, consequently causing misinterpretation of the sequence. Therefore, we have excluded the data of Adlard et al. (1993) from further comparative analyses. From the same reason, the ITS2 sequence of *F. hepatica* (LO7844) published by Michot et al. (1993) also has not been included in our analysis. In comparison with all other ITS2 sequences of *F. hepatica*, it contains several deletions, substitutions, and a mismatch at the very 3' end of ITS2.

With respect to the intraspecific ITS2 variation of *F. hepatica* assessed from the GenBank data, 4 polymorphic sites were detected after sequence alignment of 8 distant isolates of *F. hepatica* (Itagaki and Tsutsumi, 1998; Mas-Coma et al., 2001; Huang et al., 2004; Itagaki, Kikawa, Sakaguchi et al., 2005; GenBank DQ683546). For a credible design of interspecific *F. magna* and *F. hepatica* markers, the regions containing point mutations were not considered as appropriate target spots either for primer design or for selection of restriction enzyme for RFLP.

The above data enabled discrimination of *F. magna* and *F. hepatica* by PCR amplification of the complete ITS1 and/or ITS2 and its consequent RFLP analysis, and PCR amplification of the selected ITS1/ITS2 region with highly species-specific primers. Although the first approach required 2 successive methodological steps, the use of specific primers was more advantageous since only a single step was sufficient. Besides technical benefits, this method has a potential to be more specific in the case of mixed infections of hosts with a variety of parasite species.

Primers applied in the present study for retrieval of complete ITS1 and ITS2 regions of *F. magna* have their annealing positions in highly conservative regions of flanking genes of various groups of Platycladinos and even Acanthocephala. In particular, ITS-1 and ITS-2 primers, designed according to the conservative 3' end of 18S and 5' end of 28S, were originally used for amplification of ITS sequences in monogeneans (Cunningham, 1997), and subsequently applied in cestodes (Králova-Hromadová, Scholz et al., 2003) and acanthocephalans (Králova-Hromadová, Tietz et al., 2003). The 2 other primers, 5.8S-1 and 5.8S-2, were designed on 5.8S sequences of cestodes (Králova-Hromadová, Scholz et al., 2003), and also were successfully employed for acanthocephalans (Králova-Hromadová, Tietz et al., 2003). Therefore, the PCR-RFLP can be applied for reliable differentiation between *F. magna* and *F. hepatica*. However, this method does not seem to be suitable in the case of routine examination of deer feces or surveys of intermediate hosts when other parasites of cervids and snails can be present in the same locality as well.

The second methodological approach proposed, i.e., differentiation of *F. magna* and *F. hepatica* after PCR with species-specific primers, is more distinctive. The primers were designed in ITS1/ITS2 regions with higher frequency of interspecific
polymorphic sites without any variation within each of the species. According to the available data from GenBank, they should not anneal in another liver fluke of ruminants, Dicrocoelium dendriticum (GenBank DQ3779986) (data not shown).

The present work provides the first data on mitochondrial DNA, suitable for studies on the geographic and/or historic distribution of *F. magna* populations. For that purpose, mitochondrial DNA genes with a high evolutionary rate have most frequently been applied (Hu et al., 2004). Since no information for any mitochondrial genes of *F. magna* was available, we sequenced the complete protein-encoding genes, i.e., cytochrome c oxidase subunit I (*cox1*) and nicotinamide dehydrogenase subunit I (*nad1*), and determined their intraspecific nucleotide polymorphism. The primers used for PCR amplification were designed according to GenBank data on the complete mtDNA sequences of the digenetic flukes *F. hepatica* and *P. westermani*. The complete mtDNA of 5 other digenenns have been sequenced; however, they are schistosome species, dioecious flukes that possess a different order of mitochondrial genes (Le et al., 2000; Littlewood et al., 2006).

Within the complete *cox1* and *nad1* sequences of *F. magna*, distribution of polymorphic sites enabled selection of 3 variable regions. When comparing a ratio of the number of variable sites to the length of the polymorphic fragment with that of the complete genes, the selected *cox1* and *nad1* polymorphic fragments displayed at least the same, or an even higher, level of sequence divergence (1.9–2.3%) than the complete genes (1.5–1.9%). Analyzing 3 allopatric populations of *F. hepatica*, Morozova et al. (2004) ascertained similar intraspecific sequence divergence (up to 2.3%) within selected variable regions of *cox1* and *nad1* genes. In the subsequent work of the same Russian research group (Semyenova et al., 2006), 20 populations of *F. hepatica* were involved in the analysis, and the sequence divergence increased up to 4.1%. It can also be expected that in case of *F. magna* a higher level of variation within the fragments will be detected after more populations and individuals are examined.

The only previous works on intraspecific genetic diversity of *F. magna* were performed by Lydeard et al. (1989) and Mulvey et al. (1991). Allozyme studies on variation of population structure of the parasite and its deer host revealed that patterns of genetic distance in flukes were not concordant with those of deer. Moreover the tendency for individual deer to harbor multiple fluke individuals from a limited number of parasite clones was demonstrated. This is consistent with the most recent DNA analysis of *F. hepatica* in naturally infected cattle (Walker et al., 2007), where high genetic diversity of the parasite gene pool and existence of multiple mitochondrial haplotypes within a single host were revealed.

The current *cox1* and *nad1* variable regions are suitable for further studies on genetic polymorphism of *F. magna* within a single infrapopulation (host) as well as within geographically distant populations. Frequencies of mitochondrial haplotypes, thus determined, will help reveal historic biogeography of giant liver fluke in its native North America, as well as to understand the course of colonization of Europe by this alien parasite.

**ACKNOWLEDGMENTS**

The authors would like to thank Daniel Rondelaud, University of Limoges, France, and Norman Baldwin, Baldwin Aquatics, Oregon, U.S.A., for providing the material, Anna Faltyňková and Aleš Horák, Biology Centre AS CR, Institute of Parasitology, České Budějovice, Czech Republic, for help in laboratory, and Tomáš Scholz from the same institution is acknowledged for critical reading and valuable comments to the manuscript. This work was supported by the Slovak Research and Development Agency under contract APVV-51-062205 and by the Grant Agency of the Academy of Sciences of the Czech Republic (Projects A6022409 and 524/03/H133). E.H. was funded by the Research Project of the Institute of Parasitology, AS CR (Z60220518).

**LITERATURE CITED**


———, AND K. TSUTSUMI. 1998. Triploid form of Fasciola in Japan:
Genetic relationships between Fasciola hepatica and Fasciola gigantica determined by ITS-2 sequence of nuclear rDNA. International Journal for Parasitology 28: 777–781.


SEROPREVALENCE AND ISOLATION OF TOXOPLASMA GONDII FROM FREE-RANGE CHICKENS IN GHANA, INDONESIA, ITALY, POLAND, AND VIETNAM

U.S. Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, Maryland 20705-2350. e-mail: jitender.dubey@ars.usda.gov

ABSTRACT: The prevalence of Toxoplasma gondii in free-ranging chickens (Gallus domesticus) is a good indicator of the prevalence of the parasite’s oocysts in soil because chicken feed from the ground. The prevalence of T. gondii in free-range chickens from Ghana, Indonesia, Italy, Poland, and Vietnam was determined using the modified agglutination test (MAT). Antibodies to T. gondii were found in 41 (64%) of 64 chickens from Ghana, 24 (24.4%) of 98 chickens from Indonesia, 10 (12.5%) of 80 chickens from Italy, 6 (30%) of 20 chickens from Poland, and 81 (24.2%) of 330 chickens from Vietnam. Hearts and brains of chickens were bioassayed for T. gondii. Viable T. gondii was isolated from 2 chickens from Ghana, 1 chicken from Indonesia, 3 chickens from Italy, 2 chickens from Poland, and 1 chicken from Vietnam. Toxoplasma gondii isolates from 9 chickens were genotyped using 10 PCR-RFLP markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico. A total of 7 genotypes was identified; the 3 isolates from chickens from Italy were clonal type II, and the others were nonclonal. This is the first report of genetic characterization of T. gondii isolates from animals from these countries.

Toxoplasma gondii infections are widely prevalent in humans and other animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. Felids are the most important host in the life cycle of T. gondii because they excrete environmentally resistant oocysts. Cats become infected with T. gondii by eating infected tissues from intermediate hosts. Birds and rodents are considered an important source of T. gondii infection for cats.

We have initiated a worldwide study of T. gondii population structure (Dubey, Gennari et al., 2006, and references contained; Lehmann et al., 2006). For this purpose, we chose the free-range chicken as the best indicator for soil contamination with T. gondii oocysts because they feed from the ground. In the present study, we report seroprevalence of T. gondii in chickens from Ghana, Indonesia, Italy, Poland, and Vietnam, and results of our effort to isolate viable T. gondii from chickens in Kumasi during August 2006 and January 2007 and sent to Beltsville in 4 batches. Kumasi is the second largest city of Ghana, with a population of about 3 million. It is located between 6°30’ and 7°00’N, and 1°30’ and 2°00’W, at an altitude of 287 m above sea level. It is a humid area (relative humidity ranges between 53 and 93%). Chickens from Indonesia were obtained from 19 properties in 12 towns, killed 7–13 July 2003 and sent as a single batch. Chickens from Italy were from 16 rural livestock farms located in the southern part of the country, and the sampling was from November 2003 to 2004; 5 chickens were obtained from each of the 16 farms. Chickens from Poland were from 2 small farms near Kartuzy (54°20’N, 18°11’E) in the Pomeranian Province (the northern part of Poland). Hearts and sera from 20 chickens from Poland were received in February 2005. Chickens from Vietnam were from rural Dong Nai, Ben Tre, Tien Giang, Long An, Trà Vinh, and Vinh Long and were purchased during April–September 2003 from 38 properties that were at least 1 km apart.

Serological examination
Sera of chickens were tested for T. gondii antibodies using the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Bioassay of chicken tissues for T. gondii
Tissues of chickens were bioassayed in mice or cats for T. gondii either individually for each chicken or in groups. Tissues were homogenized, digested in an acidic pepsin solution, neutralized, and centrifuged (Dubey, 1998), and the homogenate was inoculated subcutaneously into 4–5 outbred female Swiss Webster (SW) mice (Taconic Farms, Germantown, New York) as described by Dubey et al. (2002). The recipient mice were examined for T. gondii infection. Tissue imprints of mice that died were examined for T. gondii tachyzoites or tissue cysts. Survivors were bled on days 40–42 postinoculation (PI) and a 1:25 dilution of serum from each mouse was tested for T. gondii antibodies with the MAT. Mice were killed 45–48 days PI and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with T. gondii when tachyzoites or tissue cysts were found in tissues. For further propagation of T. gondii, tissues from some SW infected mice were inoculated in to interferon gamma gene knock out (KO) mice; the KO mice are highly susceptible to protozoa because they lack the cytokines needed for protection against T. gondii (Dubey et al., 2005). For bioassay in cats, tissues from chickens were fed to T. gondii–free cats (Dubey et al., 2002). Feces of cats were examined for shedding of T. gondii oocysts 3–14 days postingestion of chicken tissues. Fecal floats were incubated for 1 wk at room temperature to allow sporulation of oocysts and were bioassayed in mice (Dubey and Beattie, 1988). The inoculated mice were examined for T. gondii infection.

Received 20 June 2007; revised 9 July 2007; accepted 10 July 2007.
* Department of Parasitology and Pathobiology, Faculty of Veterinary Medicine, University of Agriculture and Forestry, Ho Chi Min City, Vietnam.
† Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, West Africa.
‡ Research Institute for Veterinary Science, Martadinata 30, Bogor 30161, Indonesia.
§ Dipartimento di Sanita e Benessere degli Animali, Universita di Bari, Strada Prov. Per Casamassima, km 3, 70010 Valenzano, Italy.
¶ Witold Stefanski Institute of Parasitology, Polish Academy of Sciences, 00-818 Warszawa, Twarda 51/55, Poland.
# Department of Microbiology, The University of Tennessee, 1414 West Cumberland Ave., Room 409, Knoxville, Tennessee 37996-0845.
Table I. Seroprevalence of Toxoplasma gondii antibodies in chickens from different countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>No. of chickens</th>
<th>No. positive (%)</th>
<th>No. of chickens with MAT* titers of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:5</td>
</tr>
<tr>
<td>Ghana</td>
<td>64</td>
<td>41 (64)</td>
<td>9</td>
</tr>
<tr>
<td>Italy</td>
<td>80</td>
<td>11 (13.7)</td>
<td>4</td>
</tr>
<tr>
<td>Indonesia</td>
<td>90</td>
<td>24 (26.6)</td>
<td>11</td>
</tr>
<tr>
<td>Poland</td>
<td>20</td>
<td>6 (30)</td>
<td>2</td>
</tr>
<tr>
<td>Vietnam</td>
<td>330</td>
<td>80 (24.2)</td>
<td>34</td>
</tr>
</tbody>
</table>

* MAT, modified agglutination test.

Genetic characterization of T. gondii isolates

Toxoplasma gondii DNA was extracted from the tissues of infected mice and strain typing was performed using PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB, GRA6 c22-8, c29-2, L358, PK1, and Apico (Dubey, Pattucci et al., 2006; Su et al., 2006; Dubey, Sundar et al., 2007).

RESULTS

Toxoplasma gondii prevalence

Ghana: Tissues of 32 of the 41 seropositive chickens were bioassayed in mice. Toxoplasma gondii was isolated from tissues (brain and heart mixed) from 2 chickens (nos. 54 and 55). Chicken 54 had a MAT titer of 1:160, and chicken 55 had a MAT titer of 1:40. Only a few tissue cysts were found in the brains of the 4 mice killed 43 day after inoculation with tissues of chicken no. 54. To propagate and cryopreserve this isolate, brain of each SW mouse was subinoculated into an interferon gamma gene KO mouse; all 4 KO mice inoculated with isolate TgCkGl died of acute toxoplasmosis 15 day PI, and tachyzoites were found in lungs of the mice. The T. gondii strain from chicken 54 was designated TgCkGt1 and TgCkGt2. In the second batch of 20 chickens, 6 were seropositive, and tissues of all 20 chickens were bioassayed for T. gondii. For this bioassay, tissues of all 6 seropositive chickens were fed to 6 cats individually, and tissues of the 14 seronegative chickens were mixed and fed to 1 cat. One cat fed tissues of a chicken with MAT titer of 1:5 shed T. gondii oocysts; that chicken was from Farm G located in Santeramo. Oocysts of this T. gondii isolate were infective to mice; mice fed oocysts remained asymptomatic, and tissue cysts were found in the brains of the mice killed 31 day PI. This isolate was designated TgCkIt3. Thus, all 3 isolates from chickens from Italy were avirulent for mice.

Poland: Six of 20 chickens had T. gondii antibodies, and tissues of all 20 birds were bioassayed. Hearts of 15 seronegative chickens were mixed and fed to 1 cat; the cat did not shed oocysts. Hearts of 5 seropositive chickens were bioassayed individually in mice. Toxoplasma gondii was isolated from 2 chickens (birds 9 and 18). One of the 5 mice inoculated with the heart of bird 9 (MAT titer 1:5) and 1 of the 5 mice inocu-

Table II. Genotyping of Toxoplasma gondii isolates from chickens.

<table>
<thead>
<tr>
<th>Country</th>
<th>T. gondii isolate designation</th>
<th>SAG1</th>
<th>SAG2</th>
<th>SAG3</th>
<th>BTUB</th>
<th>GRA6 c22-8</th>
<th>c29-2</th>
<th>L358</th>
<th>PK1</th>
<th>Apico</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghana</td>
<td>TgCkGl</td>
<td>u-1</td>
<td>II</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>TgCkGt2</td>
<td>II or III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indonesia</td>
<td>TgCkKt1</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Italy</td>
<td>TgCkKt2</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>TgCkKt3</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td>Poland</td>
<td>TgCkPo1</td>
<td>u-1</td>
<td>II</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>I</td>
<td>III</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>TgCkPo2</td>
<td>u-1</td>
<td>II</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Vietnam</td>
<td>TgCkKv1</td>
<td>u-1</td>
<td>II</td>
<td>nd*</td>
<td>III</td>
<td>nd</td>
<td>nd</td>
<td>II</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* nd, No data because the quantity of DNA was insufficient.
lated with heart of chicken 18 became infected with *T. gondii*, and many tissue cysts were found in mouse brains when killed day 49 PI; these isolates were designated TgCkPo1 and TgCkPo2.

**Vietnam:** Tissues of 289 of 330 chickens were bioassayed for *T. gondii*. Tissues of 80 seropositive chickens were bioassayed in mice individually, and *T. gondii* was not isolated from any of these chickens. Tissues of 205 chickens with titers of 1:5 or less were mixed into 7 pools and fed to 7 cats. One cat fed tissues of 15 chickens shed oocysts; mice fed oocysts were killed 5 days later, and *T. gondii* tachyzoites were found in their mesenteric lymph nodes.

**Pathogenicity of T. gondii isolates to mice**

All isolates of *T. gondii* from these chickens were avirulent for mice because none of the infected mice died of toxoplasmosis.

**Genotyping of T. gondii**

Genotyping revealed that the 3 isolates from Italy were clonal type II with difference at locus Apico; both TgCkIt1 and TgCkIt2 have type I allele, whereas TgCkIt3 has a type II allele (Table II). The isolates from Poland, Ghana, Indonesia, and Vietnam were nonclonal. Both genotypes from the 2 Ghana isolates (TgCkGh1, TgCkGh2) are unique and have not been previously identified. The genotype of the isolate (TgCkId1) from Indonesia is also unique. The 2 isolates (TgCkPo1, TgCkPo2) share a genotype that is identical to a sheep isolate, CAST-TELLS, from Uruguay (Su et al., 2006). Genotyping results for the only chicken isolate (TgCkVn1) from Vietnam were incomplete, but the available data suggest it may be identical to dog isolates studied previously (Dubey, Huang et al., 2007).

**DISCUSSION**

The low isolation rate of *T. gondii* from chickens from Ghana, Indonesia, and Vietnam is probably due to autolysis of tissues during delay in transport from these countries to Beltsville. Unfortunately, it was not possible to extend this study to more samples because of the devastation of the poultry industry in southeastern Asia from the epidemic of avian influenza. Researchers in the United States on international transport of chicken tissues.

In the present study, sera of chickens were tested starting with a low (1:5) dilution of serum because *T. gondii* has been isolated occasionally from chickens with low titers (Dubey et al., 2005, 2006). Generally, titers of <1:25 are regarded as nonspecific, and sera are generally screened starting at 1:20 or 1:25 serum dilution in MAT. However, in any population, there will be a few infected individuals with a low titer, and these are likely missed in a routine serological screen. Therefore, Table I has data inclusive of all titers. It is of interest that *T. gondii* was isolated from 2 chickens from Poland with MAT titers of 1:5 and 1:10; these sera were of good quality, and there was no prozone.

The genotypes of the 2 isolates (TgCkGh1 and TgCkGh2) from Ghana and the genotype of isolate (TgCkId1) from Indonesia are unique and were not identified from isolates collected from a variety of hosts in South America and Asia, including Brazil, Colombia, Chile, Costa Rica, Nicaragua, Guatemala, Vietnam, China, and Sri Lanka. The limited typing result from the Vietnam chicken isolate TgCkVn1 suggests it may be identical to a genotype previously identified in dogs in that area (Dubey, Huang et al., 2007). The 3 isolates from Italy are identical to the clonal type II lineage that is predominant in North America and Europe. Interestingly, the 2 isolates (TgCkPo1 and TgCkPo2) from Poland are identical to a sheep isolate CAST-TELLS from Uruguay in South America (Su et al., 2006).

**Toxoplasma gondii** isolates have been classified into 3 genetic types (I, II, III) based on restriction fragment length polymorphism (RFLP). The parasite used to be considered clonal, with very low genetic variability. However, most of the previous information was derived from isolates from Europe and North America. Based on newer markers for genetic characterization and using recently isolated strains from Brazil and French Guiana, a higher genetic variability has been revealed than previously reported (Ajzenberg et al., 2004; Lehmann et al., 2006; Dubey, Applewhaite et al., 2007). Most of the isolates from animals and humans from Europe were type II (Owen and Trees, 1999; Ajzenberg, Bañuls et al., 2002; Ajzenberg, Cogné et al., 2002; Jungersen et al., 2002; Ajzenberg et al., 2004; de Souza et al., 2005; Dubey et al., 2005; Dubey, Vianna et al., 2006; Dumetre et al., 2006). Similarly, all 3 isolates from Italy in the present study were type II. It is also of interest that the 2 isolates from Polish birds were nonclonal and identical to a sheep isolate from Uruguay in South America. New, unique genotypes identified from Ghana and Indonesia warrant further study in Africa and Asia to more completely identify *T. gondii* genetic diversity in these regions.

**LITERATURE CITED**


Toxoplasma gondii infections in free-range chickens from Austria. Veterinary Parasitology 133: 299–306.


ABSTRACT: *Toxoplasma gondii* is a protozoan pathogen of birds and mammals, including humans. The infective stage, the bradyzoite, lives within cysts, which occur predominantly in cells of the central nervous system and skeletal and cardiac muscles, characterizing the chronic phase of toxoplasmosis. In the present study, we employed for the first time primary mouse culture of skeletal muscle cells (SkMC) infected with bradyzoites, as a cellular model for cystogenesis. The interconversion of bradyzoite and tachyzoite was analyzed by immunofluorescence using 2 stage-specific antibodies, i.e., anti-bradyzoite (anti-BAG1) and anti-tachyzoite (anti-SAG1). After 24 hr of interaction only bradyzoites were multiplying, as revealed by anti-BAG1 incubation; interconversion to tachyzoites was not observed. After 48 hr of infection, 2 types of vacuoles were seen, i.e., BAG1⁺ and SAG1⁺, indicating the presence of bradyzoites as well as their interconversion to tachyzoites. After 96 hr of infection, BAG1⁺ vacuoles presented a higher number of parasites when compared to 48 hr, indicating multiplication of bradyzoites without interconversion. Using ultrastructural analysis, bradyzoites were found to adhere to the cell membranes via both the apical and posterior regions or were associated with SkMC membrane expansions. During bradyzoite invasion of SkMC, migration of the rough endoplasmic reticulum (RER) profiles to the parasite invasion site was observed. Later, RER profiles were localized between the mitochondria and parasitophorous vacuole membrane (PVM) that contained the parasite. After 31 days of parasite-host cell infection, RER profiles and mitochondria were not observed in association with the cyst wall. Alterations of the PVM, including increased thickness and electron density gain on its inner membrane face, were observed 48 hr after infection. Cystogenesis was complete 96 hr after infection, resulting in the formation of the cyst wall, which displayed numerous membrane invaginations. In addition, an electron-dense granular region enriched with vesicles and tubules was present, as well as numerous intracytic bradyzoites. These results show that the in vitro *T. gondii* model and SkMC are potential tools for both the study of cystogenesis using molecular approaches and the drug screening action on tissue cysts and bradyzoites.

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that infects warm-blooded vertebrates, including humans. The parasite is distributed widely in the human population and is estimated to affect more than one billion people worldwide (Switaj et al., 2005). *Toxoplasma gondii* has 2 different asexual forms in its life cycle, i.e., rapidly multiplying tachyzoites and slowly multiplying bradyzoites. Tachyzoites invade host cells and are responsible for the acute phase of toxoplasmosis. Bradyzoites, characterized by slow metabolic processes, remain in the tissues as cysts. Bradyzoites develop in cysts within host cells in a variety of tissues, but they are more common in neural and muscular tissues. Infection is typically acquired by ingestion of undercooked meat containing tissue cysts for intermediate hosts and feline definitive hosts, or oocysts in food or water contaminated with cat feces (Dubey and Frenkel, 1976; Dubey and Beattie, 1988; reviewed in Hill et al., 2005). Tissue cysts have been isolated from skeletal muscle during autopsies of humans (Remington and Cavanaugh, 1965). Muscular toxoplasmosis in patients with HIV has been evidenced by immunocytochemistry techniques and electron microscopy (Gherardi et al., 1992). However, the preferred or predominant cell in which cysts are formed is still unclear, as well as whether host cells influence cyst formation (Lindstády et al., 1991; McHugh et al., 1993). Cell culture and the acquisition and purification of stage-specific antibodies against bradyzoites, tachyzoites, and cysts of *T. gondii* permitted the study of the interconversion of bradyzoites into tachyzoites and the progress of cystogenesis in vitro (Dubey et al., 1998; Weiss and Kim, 2000). The maintenance of the parasite in the host tissues is a key event for the establishment of chronic toxoplasmosis and is also responsible for the parasite reactivation due to an immunosuppression state, with cysts rupturing and producing toxoplasmic encephalitis (Luft and Remington, 1992; Lyons et al., 2002; Montoya and Liesenfeld, 2004).

Our proposal in this work is to use skeletal muscle cell as a model for studies of *T. gondii* cystogenesis in vitro (Monteiro-Leal et al., 1998; Andrade et al., 1999, 2001; Guimarães et al., 2002; Barbosa et al., 2005), in which the parasite remains during the chronic phase of the disease and is responsible for horizontal transmission by ingestion of raw or undercooked meat (Remington and Cavanaugh, 1965; Jacobs, 1967; Mehlihorn and Frenkel, 1980; Dubey, 1988; Frenkel, 1990; Weiss and Kim, 2000).

**MATERIALS AND METHODS**

**Parasites**

*Toxoplasma gondii* cysts from ME-49 strain (Type II) isolated from sheep were maintained in C57BL/6 female mice (12–18 g) (obtained from the Animal Facility (CECAL) of the Fundação Oswaldo Cruz-Fiocruz) with intraperitoneal inocula of 50–100 cysts/animal. The mice were killed 4–8 wk postinfection (PI), and the brain cysts were isolated as described below.

**Cyst isolation and purification**

The methodologies used were based on protocols developed by Freyre (1995) and Popiel et al. (1996) and modified by Guimarães et al. (2003). Mice were killed in CO₂ chambers, and their brains were surgically removed under aseptic conditions. After immersion in phosphate-buffered saline (PBS) at 4 C, the brains were washed to remove blood cells, then fragmented using scissors, and macerated by successive passages through 18- to 23-gauge needles. The tissue suspension was filtered with a cell dissociation sieve-tissue grinder kit (Sigma Chemical Co., St. Louis, Missouri) to remove small fragments and cellular debris. Afterward, the suspension was centrifuged at 400 g for 10 min, and the pellet was resuspended in PBS supplemented with 25% Dextran (Sigma Chemical Co.), with 1 brain per 2.5 ml of final solution, and submitted to centrifugation at 2,200 g for 10 min. The sediment containing the
cysts was recovered and resuspended in PBS, centrifuged at 400 g for 10 min to remove the Dextran solution, and then diluted in the same buffer. After that, 20 μl of the total suspension was placed between slide/coverglasses (24 × 32 mm), and the number of cysts was counted in the total area of the coverslip using a light microscope equipped with an X10 objective.

**Bradyzoite isolation**

To obtain viable bradyzoites from isolated tissue cysts (Popiel et al., 1996), the cyst wall was digested by adding pepsin digestive fluid (0.01% pepsin, 1% NaCl, and 0.28% HCl in distilled water) diluted 1:5 in PBS, and the suspension was incubated for 1–5 min at 37°C under agitation. Digestion was stopped by placing the tubes on ice and adding 1% sodium carbonate in water. After the neutralization, the material was resuspended in Eagle’s medium and centrifuged at 250 g for 10 min. The number of isolated parasites was determined using a Neubauer chamber, and the parasites were immediately used in the experiments involving T. gondii-host cell interaction. The degree of purification of the bradyzoites obtained from the T. gondii-infected-mice brain cysts was monitored using immunofluorescence approaches (see Fig. 1A, B).

**Primary culture of skeletal muscle cells**

Skeletal muscle cells were obtained from thigh muscles of 18-day-old mouse embryos. The tissues were minced and incubated 5–7 times for 7 min in a solution containing 0.05% trypsin and 0.01% versene in PBS (pH 7.2). The enzyme digestion was interrupted by the addition of 10% fetal bovine serum in Dulbecco’s modified Eagle medium (DMEM) and maintained at 4°C. The suspension containing isolated cells was centrifuged for 7 min at 650 g and resuspended in DMEM supplemented with 10% horse serum, 5% fetal calf serum, 2% chick embryo extract, 1 mM l-glutamine, 1,000 U/ml penicillin, and 50 μg/ml streptomycin. This new suspension was then incubated for 30 min at 37°C in a 5% CO2 atmosphere. After incubation, the culture flask was gently shaken to release nonattached cells, and the supernatant enriched with myoblasts was seeded in 0.02% gelatin-treated 24-well culture plates for the fluorescence assays. The cultures were maintained at 37°C for 3–5 days to obtain the muscle fibers; fresh culture medium was added every 2 days (see Barbosa et al., 2000).

**Immunofluorescence analysis of the interconversion of T. gondii in SkMC**

Immunofluorescence assays were performed using specific monoclonal antibodies for bradyzoites 7E5 (anti-BAG1) and tachyzoites (anti-SAG1). Briefly, bradyzoites were allowed to interact with 4-day-old SkMC in the ratio of 10:1. After 12 to 16 hr of interaction, the cultures were fixed for 30 min at room temperature in 4% paraformaldehyde diluted in PBS and then washed 3 times (10 min each) with PBS. The cultures were incubated for 1 hr at room temperature in a blockade solution containing 4% bovine serum albumin (BSA), 2% normal sheep serum, and 0.5% Triton X100 (Sigma Chemical Co.) in PBS, followed by incubation for 4 hr at 37°C with anti-BAG1 (1:50) or anti-SAG1 (1:100) diluted in PBS/BSA/Triton. After incubation with the first antibody, the cultures were washed 3 times for 10 min in PBS/BSA/Triton and incubated for 1 hr at 37°C with goat anti-mouse antibody labeled with tetramethylrhodamine (TRITC; Sigma Chemical Co.), a DNA stain that enables the visualization of host and parasite nuclei. Then, they were washed again in PBS. The coverslips were then mounted on slides with 2.5% DABCO (1,4-Diazabicyclo[2.2.2]octane-Triethylenediamine antifading; Sigma Chemical Co.) in PBS containing 50% glycerol (pH 7.2). Controls were performed by omission of the primary antibody. The samples were examined under a Nikon Eclipse E800 microscope; images were obtained with a CoolSNAP-Pro color camera and processed with Image-Pro Plus 4.5,1.29 software in the Production and Image Management Laboratory/IOC-FIOCRUZ, RJ (Rio de Janeiro, Brazil).

**Transmission electron microscopy**

SkMC were infected with T. gondii bradyzoites (1:10 parasite-host cell ratio). After different periods of infection (1, 4, 48, and 96 hr and 31 days), the culture cells were washed 3 times for 10 min with PBS and fixed for 1 hr at 4°C in 2.5% glutaraldehyde (GA) diluted in 0.1 M sodium cacodylate buffer containing 3.5% sucrose and 2.5 mM CaCl2 (pH 7.2). After fixation, the cells were washed in the same buffer and post-fixed for 30 min at room temperature in 1% osmium (OsO4) diluted in 0.1 M cacodylate buffer. After post-fixation, the cells were washed in the same buffer and scraped at 4°C from the plastic dish and centrifuged for 5 min at 10,000 g. Thereafter, the cells were dehydrated in graded acetone and embedded in Epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and then examined under a Zeiss EM10C transmission electron microscope.

**Scanning electron microscopy**

SkMC were infected with T. gondii bradyzoites (10:1 parasite-host cell ratio) for periods from 15 min to 2 hr and then fixed for 30 min at...
RESULTS

Immunofluorescence assays

The immunofluorescence studies allowed evaluation of bradyzoite-tachyzoite interconversion and cystogenesis of *T. gondii* in SkMC. The infection was initiated using 100% bradyzoites isolated from ME-49 strain cysts (Fig. 1A, B). Immunofluorescence analysis was employed by incubation of the infected cultures with an antibody-specific stage, i.e., anti-BAG1 and anti-SAG1, for periods from 12 to 168 hr. The analysis of the infected cultures after 24 hr of interaction with bradyzoites revealed that (1) all intracellular parasites expressed bradyzoite antigen (Fig. 2A–C); (2) the interconversion of bradyzoites to tachyzoites did not occur, as verified by the absence of SAG1 labeling (Fig. 2D–F); and (3) parasite multiplication occurred as bradyzoites and most parasitophorous vacuoles (PVs) were seen with 2–4 bradyzoites (Fig. 2A–C).

At 48 hr PI, there were some vacuoles with parasites expressing BAG1 (Fig. 3A–C) and others labeled with anti-SAG1 (Fig. 3D–F), demonstrating the conversion of some bradyzoites into tachyzoites. PVs with parasites positive for anti-SAG1 contained a larger number of parasites (Fig. 3E, F) when compared with those expressing BAG1 antigen (Fig. 3B, C). In a single culture (Fig. 3B, C), a small vacuole with parasites marked for anti-BAG1 was observed next to a larger vacuole with parasites not marked for anti-BAG1, probably containing tachyzoites.

After 72 hr of infection, 2 forms of *T. gondii*-infected myotubes were observed. Figure 4A–C shows, in the same host cell, a PV with BAG1-positive parasites close to another PV containing parasites without staining. Infected cells possessed fewer bradyzoites (Fig. 4B, C) when compared with those containing parasites marked with anti-SAG1 (Fig. 4E, F).

At 96 hr PI (Fig. 5A–F), some vacuoles with parasites positive for anti-BAG1 were larger in comparison with those analyzed after shorter interaction times (Figs. 2B, 3B, 4B), possibly suggesting that in these vacuoles the multiplication of bradyzoites is occurring without the previous interconversion to tachyzoites. PVs with SAG1-positive parasites also were observed (data not shown) at 96 hr PI.

The cultures infected for 120 hr also possessed cells that were anti-BAG1 positive for parasites in PVs as well as negative (Fig. 6A–C). When these cultures were incubated with anti-SAG1, both stages of the parasite were present inside the cells (Fig. 6D–F).

After 144 hr of infection, large numbers of parasites expressing...
GUIMARÃES ET AL. - TOXOPLASMA GONDII CYSTOGENESIS IN SKELETAL MUSCLE CELLS

Skeletal muscle cells + bradyzoites 72 hours

**Figure 4.** (A–F) SkMC infected for 72 hr with bradyzoites. (A, D) Phase contrast microscopy of infected myotubes. (B, E) Immunofluorescence with anti-BAG1 and anti-SAG1, respectively. (B) Bradyzoites inside PVs labeled (arrow) and others unlabeled with anti-BAG1 antibodies, were present in the same host cell. (E) Myotube with a PV containing many parasites labeled (dotted circle) with anti-SAG1 antibodies. (C, F) Infected SkMC: Double labeled with DAPI plus anti-BAG1 and DAPI plus anti-SAG1, respectively. Bar = 5 µm.

Ultrastructural analysis

The interaction of bradyzoites with SkMC was examined from 15 min to 31 days by scanning (SEM) and transmission electron microscopy (TEM). After a brief interaction (15 min to 2 hr), it was possible to observe the close association of parasites with the host cell membrane (Fig. 8A–D). Bradyzoites adhered via the apical region, where the conoid is located (Fig. 8A), via the posterior region, or by the filopodial surface of SkMC (Fig. 8B). When the parasite was apically oriented, the extrusion of the conoid was evident (data not shown). Expansion of the host cell membrane during the adhesion step could be seen by SEM (Fig. 8C, D). From 1 hr up to 4 hr, TEM showed that bradyzoites were attached to the host cell membrane (Fig. 9A, B). During this process, a concentration of rough endoplasmic reticulum (RER) was observed near the parasite invasion sites surrounding the PV that was under formation (Fig. 9B). During this period, no mitochondria were observed in association with the parasitophorous vacuole membrane (PVM) containing bradyzoites, although RER profiles were localized between the mitochondria membrane and PV (Fig. 9C–E). The internalized parasites were always surrounded by a membrane in a typical PV (Fig. 9C–E). The identification of bradyzoites was easy, due to the presence of large amounts of amylopectin granules in the cytoplasm (Fig. 9C–E). The association of host cell organelles with the PVM is observed in Figure 9C–E. RER profiles were frequently attached to the PVM, being more evident in the longitudinal sections of the parasite (Fig. 9E).

After 48 hr of interaction, dividing parasites (by endodyogeny) were found within the PV of SkMC (Fig. 10A, B). The parasites were surrounded by a thick membrane of increased electron density both on the inner face and in the matrix (Fig. 10A). In addition, small membrane invaginations of the PV were also observed (Fig. 10B). After this time, some parasites did not present the typical morphological characteristics of bradyzoites (data not shown).

After 96 hr of infection, intracellular parasites with bradyzoite morphology were surrounded by a membrane with the typical morphology of a cyst wall (Fig. 11A, B). As observed at 48 hr PI, the PVM displayed many invaginations and was also very thick due to the increased electron density on its inner face (Fig. 11A, B). Parasites with bradyzoite morphology were found immersed in the electron-dense matrix surrounded by a structure similar to a cyst wall (Fig. 11A, B). These data indicate that the complete *T. gondii* cystogenesis (bradyzoites of the ME-49 strain inside SkMC) occurred in 96 hr.

Our system was studied for 31 days. The ultrastructural analysis demonstrated that the cysts in vitro had the same characteristics as in vivo, with bradyzoites presenting typical organelles, a large amount of amylopectin granules, micronemes,
electron dense rhoptries, and nuclei toward the posterior end (Fig. 12A, B). The parasites were always surrounded by an electron-dense matrix and an invaginated membrane constituting the cyst wall (Fig. 12A, B). In addition, vesicles and tubules were also present in a granular region (Fig. 12B). An analysis of various thin sections showed no RER profiles or mitochondria associated with the cyst walls (Fig. 12A, B).

**DISCUSSION**

In the present work, we report, for the first time, the use of SkMC as a model for studying *T. gondii* cystogenesis. Most of the available knowledge concerning the interaction of this parasite with host cells in vitro has employed tachyzoites, but little information exists regarding the use of bradyzoites and sporozoites (reviewed in Dubey et al., 1998). Here, the infection of SkMC with bradyzoites is justified by the fact that this is the stage that emerges from tissue cysts after the latter are ingested in raw or undercooked meat (reviewed in Weiss and Kim, 2000).

The evaluation of the bradyzoite-tachyzoite interconversion and cyst formation by immunofluorescence was carried out from 12 to 168 hr, with daily analysis. For the first 24 hr of in vitro culture, all intracellular parasites expressed BAG1 protein, which is specific for the bradyzoite forms. Until 12 hr PI, the majority of BAG1+ vacuoles presented only 1 parasite. However, after 24 hr, the multiplication of this form occurred within vacuoles containing up to 4 parasites. These results suggest that at least some bradyzoites multiply without their transitory differentiation to tachyzoites, and thus produce cysts in culture. Soète et al. (1993), using cultures of human fibroblasts infected with bradyzoites, reported that at the beginning of the infection, 100% of the parasites were positive for 4 different antigens of bradyzoites and negative for SAG1 protein. These authors reported that after 15 hr of infection, intermediate forms (parasites expressing bradyzoites and tachyzoite antigens) could be found. The existence of an intermediate stage is not surprising, because it has been demonstrated that surface proteins are gradually modified during the conversion (Soète et al., 1993). In contrast to the results obtained by these authors, at 48 hr PI we found vacuoles with parasites expressing SAG1.

At 48 hr PI, evidence for vacuoles possessing parasites that are positive for anti-BAG1, together with a lower parasite number as compared to those cells with vacuoles harboring parasites expressing SAG1 antigen, suggests that these bradyzoites had not converted into tachyzoites and that they are in a slow process of multiplication. The parallel analysis by fluorescence and
phase contrast microscopies allowed us to observe small vacuoles in a single cell containing parasites expressing BAG1 antigen; adjacent were larger vacuoles with parasites positive for SAG1 antigen. Our data are in agreement with other studies that have reported a cell cycle time for tachyzoites of about 7–10 hr (Hu et al., 2002; Woodmansee, 2003) and confirm the results of Soëte et al. (1993), who showed that bradyzoites have a low multiplication capacity and generate daughter cells expressing antigens that are specific for these forms.

The observation after 72 hr of infection showed vacuoles with positive and negative parasites for BAG1 in the same myotube, probably containing tachyzoites, in agreement with assays using other cellular models (Tomavo et al., 1991; Bohne et al., 1993; Soëte et al. 1993, 1994; Weiss et al., 1995). Moreover, in different cellular types infected with bradyzoites, parasites have also been identified that express specific antigen for both forms inside the same vacuole (Bohne et al., 1993). Thus, if one considers that all the parasites inside a PV are derived from just a single parasite, the trigger of bradyzoite-tachyzoite interconversion does not seem to be a synchronized event as has been suggested (Darde et al., 1989; Bohne et al., 1993; Soëte et al., 1993). Thus, it appears that parasites originating from an apparently homogeneous population (bradyzoites isolated from brain or tachyzoites obtained from peritoneal exudate) in the same culture conditions can develop via different routes, resulting in a heterogeneity of stages in the same cell and in the same PV, as espoused by Soëte et al. (1993). In SkMC, we did not observed vacuoles simultaneously containing bradyzoites and tachyzoites.

At 96 to 144 hr PI, multiplication of bradyzoites was observed, i.e., large vacuoles were full of anti-BAG1+ parasites as previously described by Weiss et al. (1994, 1995), who also used bradyzoites from ME-49 strain, and by Soëte et al. (1993) using other parasite strains. The suggestion that these vacuoles would give rise to cysts was supported by ultrastructural images in the present study, which showed PVM modifications with morphological characteristics compatible with a cyst wall. Both bradyzoites and tachyzoites were still observed after 96 and 120 hr, because vacuoles with BAG1+ and BAG1− parasites were found in the same myotube. An explanation for such a finding, however, could be the reinfection of these cells by tachyzoites because of the disruption of adjacent infected cells. Alternatively, these vacuoles with a larger number of positive parasites for SAG1 antigen may be derived from the initial infection, with bradyzoites converted into tachyzoites.

In all cell models infected with bradyzoite forms, including SkMC, similar phenomena were reported, including (1) the presence of large numbers of tachyzoites, long after the initial exposure to infection, possibly due to the absence of an immune response in this in vitro system; (2) formation of tissue cysts, independent of immunological factors, as was also described by Darde et al. (1989); and (3) a reduction in the number of vacuoles expressing antigens for bradyzoites.

The studies with MDBK (Madin-Darby bovine kidney) cells demonstrated an active participation of the host cell with emission of filopodium on the lateral region of bradyzoites and their posterior reorientation, directing the apical region to the invasion site (Sasono and Smith, 1998). This description, associated with our present results (parasite adhesion occurring by apical region with conoid extrusion and by the posterior region of the parasite with or without expansion of the host cell membrane), are opposed to “the classic” description of active penetration of tachyzoite invasion into the host cell by a process dependent on the actin and myosin of the parasite (reviewed in Carruthers, 2002). Additionally, the conoid extrusion observed in this present study suggests that, as observed with tachyzoites, the success of parasite internalization involves the secretion of parasite protein by organelles such as rhoptries and micronemes. Our ultrastructural analysis points out that both invasion mechanisms, i.e., active penetration and endocytosis, could occur and be strategic for bradyzoite internalization.

In our model, cyst formation was initially observed at 48 hr PI. During this time, modifications of the PVM and the presence of multiplication parasites were seen. These data suggest that although some parasites do not present morphological characteristics of bradyzoites, they may maintain the expression of specific proteins driving the signaling for cyst wall formation, as demonstrated here and previously (Bohne et al., 1993; Weiss et al., 1994, 1995; Lane et al., 1996; Sahm et al., 1997; Lüder et al., 1999).

The data obtained in the present study suggest that the complete formation of cysts in SkMC is achieved in 96 hr. After this time, we observed a cyst wall membrane with invaginations and a large quantity of electron-dense material below the membrane and in the cyst matrix. These are characteristics that are in accordance with the ultrastructural analysis of cysts performed by Lindsay et al. (1991). However the electron-dense material in the cyst matrix, filling all the free space between the parasites as described here, was not observed when human fibroblasts were used (McHugh et al., 1993). Moreover, depending on the methodology used for the evaluation of cysto-
FIGURE 9. (A–E) Ultrastructural analysis of SkMC- *T. gondii* bradyzoites interaction from 1 to 4 hr. (A, B) Electron micrography showing the adhesion of bradyzoites at SkMC membrane (A). Rough endoplasmic reticulum (RER) profiles were observed surrounding the PV that is under formation (B). (C, E) RER profiles were also observed surrounding the PV containing bradyzoites. Details in (E).
FIGURE 10. (A, B) Ultrastructural aspects of SkMC after 48 hr of infection with bradyzoites. (A) Host cell containing parasites (P) in process of cellular division (endodyogeny) can be seen. Parasitophorous vacuole membrane (PVM) presenting structural modifications: A dense matrix (*) can also be seen inside this vacuole, indicative that cystogenesis is occurring. (B) Detail of the modification in PVM showing several invaginations (arrow) and higher electrondensity below this membrane suggestive of cyst wall (CW) formation inside the host cell. Nucleus (N).
Figure 11. (A, B) Ultrastructural aspects of the T. gondii cystogenesis in SkMC after 96 hr of infection showing the modification of the PVM into CW. (A) Parasites presenting compatible morphology with bradyzoites inside a cyst. (B) TEM image showing: (i) electron-dense material (inside PV) that corresponds to the cyst matrix (*); (ii) the cyst wall; and (iii) the bradyzoites displaying electron-dense rhoptries (R) and a large amount of micronemes (Mic). Amylopectin granules (AG), conoid (C).
FIGURE 12. (A, B) TEM analysis of SkMC after 31 days of infection with *T. gondii* bradyzoites. Besides having many typical bradyzoites, the cysts displayed membrane invaginations (arrow) and tubules (T) in the granular region (GR). Rare profiles of RER or mitochondria (Mt) associated with the CW were observed. Cyst matrix (*), nucleus (N), amylopectin granules (AG), micronemes (Mic), rhoptries (R).
genesis kinetics, different results were reported, i.e., 6–7 days (Shimada et al., 1974; Hoff et al., 1977), 20–21 days (Hogan et al., 1960; Kambara et al., 1971), and 40–80 days (Jones et al., 1986). These differences can be related to the virulence of the strains and the infective stage of the parasite employed in the infection, as well as the host cell type. The velocity of the bradyzoite→tachyzoite interconversion and the production of a great number of cysts in vitro have been related to the T. gondii strain (McHugh et al., 1993; Soête et al., 1994). Strains of low virulence (such as the ME-49) form cysts more efficiently in mice when compared to strains of high virulence, such as RH (Soête et al., 1994). Our hypothesis is that the use of a cell model involved in the chronic phase of toxoplasmosis, such as skeletal muscle associated with the low-virulence strain ME-49, can present determining factors for reaching cystogenesis in 96 hr, as described here.

In the present model, we found an association of the RER with nascent PVM during the bradyzoites invasion, as well as during the formation of the cyst wall. These data differ from the in vivo studies of Ferguson and Hutchison (1987) in which the ultrastructural analysis did not show RER or mitochondria surrounding cysts in formation. Additionally, no evidence regarding the association of host cell organelles with the cysts already formed were observed. Our results are partially in accordance with the descriptions of Wanko et al. (1962), Sasono and Smith (1998), and Speer et al. (1999), who observed an association of the RER with already formed cysts. Moreover, an association of organelles, cytoskeleton elements, and intermediate filaments of astrocytes with T. gondii has been demonstrated, as well as the exclusion of mitochondria and endoplasmic reticulum from the surface of the cyst (Halonen et al., 1998). In mice brains, the cyst tissues were surrounded by an organized layer of microtubules and neurofilaments lined up in parallel to the cyst wall (Sims et al., 1988); however, they were absent in skeletal muscle cells of infected mice (Mehlhorn and Frenkel, 1980). The presence of microtubules surrounding PVs containing tachyzoites has been described in SkMC (Andrade et al., 2001) and in cellular lineage (De Melo et al., 2001). The conflicting data related to the association of host cytoskeleton elements and organelles with T. gondii is possibly due to differences in the interaction of tachyzoites and bradyzoites during the forming of the PVM (Sinai et al., 1997; Sinai and Joiner, 2001; reviewed in Carruthers, 2002; Coppins and Joiner, 2003; Magno et al., 2005).

In conclusion, our data analyzing the interaction of SkMC with bradyzoite of T. gondii from 15 min up to 31 days showed that this cellular model (1) is viable and suitable for studies regarding the mechanisms of Toxoplasma development in host cells involved in chronic toxoplasmosis; (2) has a potential use in cystogenesis studies; and (3) is applicable for the investigation of drug activities against cysts and bradyzoites in vitro.

ACKNOWLEDGMENTS

We are grateful to Sandra Maria de Oliveira Souza, Marielle Moreira, and Genesio Lopes for their technical assistance and to David Straker for the English revision. This work was supported with grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Oswaldo Cruz (Programa Estratégico de Apoio à Pesquisa em Saúde – PAPES IV) and Instituto Oswaldo Cruz/FIOCRUZ. The monoclonal antibodies anti-SAG1 and anti-BAG1 were kindly supplied by Dr. José Roberto Mineo (Laboratório de Imunoparasitologia, Universidade Federal de Uberlândia, MG, Brazil) and by Dr. Wolfgang Böhne (Institut für Medizinische Mikrobiologie, Universität Göttingen, Germany), respectively.

LITERATURE CITED


——, L. CARVALHO, AND H. S. BARBOSA. 2003. An alternative tech-
nique to reveal polysaccharides in Toxoplasma gondii tissue cysts. Memórias do Instituto Oswaldo Cruz 98: 915–917.


PARASITOLOGY 93: 23–32.


TRANSCRIPTIONAL INHIBITION OF INTERLEUKIN-12 PROMOTER ACTIVITY IN LEISHMANIA SPP.-INFECTED MACROPHAGES

Asha Jayakumar*, Robyn Widenmaier†, Xiaojing Ma†, and Mary Ann McDowell‡

215 Galvin Life Sciences, Center for Global Health and Infectious Diseases, Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556. e-mail: mcdowell.11@nd.edu

ABSTRACT: To establish and persist within a host, Leishmania spp. parasites delay the onset of cell-mediated immunity by suppressing interleukin-12 (IL-12) production from host macrophages. Although it is established that Leishmania spp.-infected macrophages have impaired IL-12 production, the mechanisms that account for this suppression remain to be completely elucidated. Using a luciferase reporter assay assessing IL-12 transcription, we report here that Leishmania major, Leishmania donovani, and Leishmania chagasi inhibit IL-12 transcription in response to interferon-gamma, lipopolysaccharide, and CD40 ligand and that Leishmania spp. lipophosphoglycan, phosphoglycans, and major surface protein are not necessary for inhibition. In addition, all the Leishmania spp. strains and life-cycle stages tested inhibited IL-12 promoter activity. Our data further reveal that autocrine-acting host factors play no role in the inhibitory response and that phagocytosis signaling is necessary for inhibition of IL-12.

The hallmark of an intracellular pathogen is its ability to survive within the intracellular niche. These organisms must be resistant to, or able to evade, the host cell's microbiocidal mechanisms. This dilemma is particularly relevant to Leishmania spp. because these organisms primarily reside within vertebrate macrophages (MP). These host cells become activated for microbial destruction and cytokine secretion by exposure to immune modulators, such as interferon-gamma (IFN-γ) and CD40 ligand (CD40L) found on activated T-cells. One strategy Leishmania spp. parasites use to avoid host cell activation is to interfere with the signaling pathways that induce MP to become microbiocidal (Gregory and Olivier, 2005); another is inhibiting or delaying the production of activating cytokines (Belkaid et al., 2000).

The most consistent dysfunction reported from Leishmania spp.-infected MP is the aberrant production of inflammatory cytokines, specifically an inhibition of interleukin-12 (IL-12) production (McDowell and Sacks, 1999). Being essential for T-helper 1 (Th1) cell differentiation, the inability of Leishmania spp.-infected MP to produce IL-12 allows Leishmania spp. to evade acquired resistance by postponing IL-12 production and the induction of IFN-γ, thereby allowing the establishment of the infection; both clinical and experimental studies indicate that the onset of Th1-mediated immunity and Leishmania spp. killing is indeed delayed (Melby, 1991). Inhibition of MP IL-12 production and resulting Th1 responses is not unique to Leishmania; viruses (Chehimi et al., 1994; Chouignet et al., 1996; Karp et al., 1996) and bacteria (Marth and Kelsall, 1997; Sutterwala et al., 1997; Matsunaga et al., 2003) also exploit this mechanism to avoid clearance.

The general nature of impaired IL-12 production in Leishmania spp.-infected MP has extended to every IL-12 agonist that has been tested. Leishmania spp.-infected MP are unable to produce IL-12 even in response to strong inflammatory stimuli, including microbial stimuli, e.g., lipopolysaccharide (LPS), Staphylococcus aureus (SAC), Toxoplasma gondii antigen, and mycobacteria (Carrera et al., 1996; Sartori et al., 1997; Belkaid et al., 1998; Weinheber et al., 1998; Piedrafita et al., 1999), and T-cell-dependent agonists, e.g., IFN-γ and CD40L (Carrera et al., 1996; Belkaid et al., 1998; Weinheber et al., 1998; Piedrafita et al., 1999). Furthermore, the inhibition observed in Leishmania spp.-infected MP is selective, that is, other proinflammatory cytokines and chemokines are not affected (Carrera et al., 1996).

Several studies have shown that Leishmania spp. infection interferes with IL-12 production in MP; however, conflicting data have been reported concerning the role of different Leishmania spp. life-cycle stages in the inhibitory process. Reiner et al. (1994) were the first to report that Leishmania major amastigotes stimulate, rather than inhibit, IL-12 production in murine bone marrow-derived MP (BMDM), an observation that was confirmed in the murine MP cell line J774 (Piedrafita et al., 1999). Conversely, Leishmania mexicana amastigotes inhibit IL-12 secretion in murine BMDM (Weinheber et al., 1998). Although L. major and Leishmania panamensis stationary-phase promastigotes induce small amounts of IL-12 secretion in human peripheral blood mononuclear cells (PBMC), these parasites, as well as Leishmania braziliensis, L. mexicana, and Leishmania guyanensis promastigotes, inhibit IL-12 release in response to SAC (Sartori et al., 1997). Furthermore, L. major and Leishmania donovani metacyclic promastigotes inhibit IL-12 production in murine MP in response to IFN-γ and LPS (Belkaid et al., 1998) and mycobacterial products (Carrera et al., 1996).

The fact that Leishmania spp.-conditioned medium is able to inhibit SAC-induced IL-12 production in human PBMC (Sartori et al., 1997) suggests that soluble parasite components mediate IL-12 inhibition. Specifically, both purified and synthetic phosphoglycans (PG) have been reported to inhibit IL-12 production in murine MP (Piedrafita et al., 1999). The leishmanial surface expresses other molecules that interact with the host cell, such as lipophosphoglycan (LPG), glycosylinositol phospholipids (Orlandi and Turco, 1987; McConville et al., 1995), and a surface protease (GP63, MSP) (Guha-Niyogi et al., 2001), that may mediate IL-12 inhibition.

IL-12p70 is a covalently linked heterodimer composed of 2 chains, p40 and p35, encoded by separate genes (Ma, Atemezaga et al., 1996). Whereas p40 transcripts are highly regulated and found only in cells producing biologically active IL-12, the p35-encoding gene is constitutively expressed in many cell types (Ma et al., 1995). Until recently, it was believed that...
expression of IL-12p40 alone was predictive of IL-12p70 production; however, it is now evident that IL-12p35 gene transcription can be tightly regulated (Hayes et al., 1995; Snijders et al., 1996). For most Leishmania species, the mechanism of IL-12 inhibition is mediated at the level of IL-12p40 mRNA accumulation (Carrera et al., 1996; Sartori et al., 1996, 1997; Piedrafita et al., 1999). However, steady-state IL-12p40 mRNA levels are not affected by L. mexicana infection (Weinheber et al., 1998), indicating that a posttranscriptional regulation mechanism may be occurring.

Although many studies indicate that Leishmania spp. infection inhibits MP production of IL-12, several discrepancies have been reported. The reason for the reported differences in the literature is unclear; explanations may lie in the differences between the cell types investigated, differences in the Leishmania species and life cycle stages used, the use of nonphysiological amounts of purified parasite molecules, or the utilization of different assays assessing either mRNA levels, protein production, or IL-12 secretion. In the present work, we examine several different parameters using a single assay to elucidate the parasite and host factors that mediate impaired IL-12p40 promoter activity in Leishmania spp.-infected MP. This assay consists of the RAW264.7 murine MP cell line, stably transfected with the human IL-12p40 promoter tagged to a firefly luciferase construct; this system was previously shown to recapitulate endogenous IL-12p40 production in terms of cell type specificity and stimulus responsiveness (Ma, Chow et al., 1996) and allows us to assess suppression of IL-12p40 promoter activity and thus implicate IL-12p40 transcription. Here, we demonstrate that inhibition of IL-12 in MP by Leishmania spp. infection extends to all Leishmania species and strains that were tested, does not require LPG, PG, or MSP, involves phagocytosis signaling, and likely requires cell contact.

MATERIALS AND METHODS

Cell lines and reagents

The murine macrophage cell line RAW264.7 stably transfected with human IL-12p40 promoter tagged to luciferase construct (RAWp40LUC) was previously generated (Ma, Chow et al., 1996). Cells were maintained in complete RPMI (10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% t-glutamine) at 37 °C and 5% CO2. Recombinant mouse (rm) IFN-γ purchased from Peprotech (Rocky Hill, New Jersey) was used at 1,000 U/ml. Escherichia coli LPS (Sigma Aldrich, St. Louis, Missouri) was used at 1 µg/ml. Recombinant mouse CD40L trimer was donated by Immunex (Seattle, Washington). Cytochalasin D (MP Biomedicals, Solon, Ohio) and phospholipase C (PLC) inhibitor U73122 (Calbiochem, San Diego, California) were used at a concentration of 5 µM.

Parasites

Infections were performed with L. major NIH Friedlin V1 strain (MHOM/IL/80/FN), isolated from a patient with localized cutaneous leishmaniasis in Israel; L. major substrain IR173 (MHOM/IR/173), isolated from patients with localized cutaneous leishmaniasis in Iran; L. major LV39 (MH0/SU/59/P), isolated from a gerbil reservoir in southern Russia; L. major NIH S strain (MHOM/SN/74/Seidman), isolated from a patient from Senegal, West Africa, with multiple subcutaneous nodules; L. donovani strain 9515 (MHOM/IN/95/9515), isolated from splenic aspirates of patients with visceral leishmaniasis in India; L. donovani strain Mongi (MHOM/IN/83/Mongi-142), isolated from bone marrow biopsies of patients with visceral leishmaniasis in India; L. donovani strain 1S isolated from a patient with visceral leishmaniasis in Sudan; L. donovani mutants LPG+/− (R2D2) and PG+/− (C3PO) generated by chemical mutagenesis of L. donovani (1S) (King and Turco, 1998); wild-type Leishmania chagasi was originally isolated from a patient in Brazil; and the attenuated L. chagasi strain (LS) was generated previously by exconjugation (Wilson et al., 1989). Infective-stage metacyclic promastigotes were isolated by ficoll gradient (Spaith and Beverley, 2001) and opsonized in 3 ml of Hank’s balanced salt solution media (0.15 mM CaCl2 and 1.0 mM MgCl2) containing 5% normal mouse serum at 37 °C for 30 min. Procyctic promastigotes were obtained from 2-day-old parasite cultures. Lesion-derived amastigotes were isolated from footpads of BALB/c mice. All parasite strains were cultured at 26 C without CO2, in medium 199 containing 10% heat-inactivated FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml boitin. Parasites tested negative for mycoplasma (polymerase chain reaction (PCR) detection method) and tested below the detection limits for endotoxin (LAL assay; Endosafe, Charles River Laboratories, Charleston, South Carolina).

Parasite preparations

Parasite lysates were prepared by subjecting isolated metacyclics to alternating cycles of freezing (dry ice/90% ethanol bath) and thawing (37 °C) for 5 min each. Parasites were heat killed at 56 C for 1 hr. All parasite preparations were examined by light microscopy to ensure completeness of parasite death. Supernatant cultures were filter sterilized before use. Amicon ultracentrifugal filter devices (Millipore, Billerica, Massachusetts) were used to concentrate parasite supernatants.

Luciferase assay

RAWp40LUC cells were plated at 2.5 × 106 cells per well in 6-well plates, lower and upper chambers of the transwell system. Cells were primed with recombinant mouse IFN-γ (Peprotech) at 1,000 U/ml overnight followed by LPS (1 µg/ml) or recombinant mouse CD40L (Immunex, Seattle, Washington) at 0.5 µg/ml stimulation for 8 hr. Infections were carried out at multiplicity of infection of 5–10 parasites to 1 cell at the time of rm IFN-γ addition. One well was reserved for quantifying infection rates. After adherence to slides by cytopsin centrifugation, cells were methanol fixed, stained with Diff-Quick, and visualized with light microscopy. Infection rates for all infections did not vary by more than 5% between the different infecting strains. Cells were lysed and luciferase activity determined according to instructions of the luciferase assay kit (Promega, Madison, Wisconsin) using LMax ll 384 (Molecular Devices, Sunnyvale, California). Triplicate samples were run and Student’s t-test was used to determine statistical significance. Data were normalized to the mean luciferase units of the uninfected/untreated controls within a single assay and expressed as relative luciferase units. All cells were examined by Wright–Giemsa staining and light microscopy to ensure equal uptake of parasites, or, in the case of transwell system, to ensure that parasites did not cross the membrane from the upper chamber into the lower chamber.

Real-time quantitative reverse-transcription (RT)-PCR

Relative levels of IL-12p40 mRNA were determined by real-time PCR. mRNA levels were measured after 4 hr of infection. Total mRNA was prepared with the RNeasy Mini Kit (Qiagen). One microgram of total mRNA was reverse transcribed using random primers with SuperScript III Synthesis System for RT-PCR (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. For analysis of IL-12p40 and hypoxanthine–guanine phosphorylase transferase (HPRT) mRNA expression, real-time PCR was performed using SYBR green chemistry with the following primers: murine IL-12p40 forward: 5′-AAC CAT TTC CTG TCG TTC CA-3′; murine IL-12p40 reverse: 5′-CCG GAG TCC AGTCCA CTG C-3′; murine IL-10 forward: 5′-GCAC AAA GCA GAC GAC ACT CTT A-3′; murine IL-10 reverse: 5′-CTG GCC CCT GCT GAT C-3′; tumor necrosis factor (TNF)-α forward: 5′-GAA ACA CAA GAT GCT GGC A-3′; TNF-α reverse: 5′-TCG AGG CTC CAG TGA ATT C-3′; murine HPRT forward: 5′-CAA AGC CTA AGA TGA CCG A-3′; murine HPRT reverse: 5′-AGG CAC ATG GCC ACA GGA C-3′. Real-time PCR reactions were performed according to the manufacturer’s recommendations with an ABI Prism 7500 sequence detection system (Perkin Elmer). The relative number of mRNA copies for p40 was determined by the fol-
then stimulated with purified CD40L trimer or LPS with and without IFN-γ stimulation. Similar to primary macrophages, *L. major* (Figs. 1, 2B) and *L. donovani* (Fig. 2A) infection had a significant inhibitory effect on LPS ± IFN-γ-induced IL-12p40 promoter activity in RAWp40LUC cells. Furthermore, both species were able to inhibit IL-12 promoter activity in response to LPS ± IFN-γ stimulation (Fig. 1; data not shown), indicating that *Leishmania* spp. inhibition is able to block multiple signaling pathways, including nonmicrobial agonists. As previously reported (Carrera et al., 1996), IL-12p40 mRNA accumulation is inhibited by *Leishmania* spp. infection (Fig. 7); TNF-α mRNA was not affected and IL-10 mRNA expression was augmented (data not shown).

**Leishmania** spp.-induced inhibition of IL-12p40 promoter activity is independent of species, strain, or life-cycle stage

It is apparent that different *Leishmania* substrains express structurally distinct surface molecules (Dobson, Mengeling et al., 2003) and that the clinical manifestations caused by these substrains also differ (Neva et al., 1979). To ensure that the *Leishmania* spp.-induced inhibition of IL-12 promoter activity was not just an anomaly due to the *L. major* and *L. donovani* strains utilized, we tested whether the inhibition was produced by infecting with other species-defined *L. major* and *L. donovani* isolates. In all cases, *Leishmania* spp. infection abrogated luciferase induction in response to IFN-γ + LPS (Fig. 2) and IFN-γ + CD40L (data not shown). It previously has been shown that *L. major* amastigotes induce IL-12 production in murine MP (Reiner et al., 1994; Piedrafita et al., 1999) and *L. major* log-phase (procyclic) promastigotes induce IL-12p40 in human PBMC (Sartori et al., 1996). In our studies, *L. major* metacyclic and log-phase promastigotes, as well as amastigote infection, inhibited luciferase production (Fig. 3), indicating that all *L. major* life cycle stages can inhibit IL-12p40 production in murine MP. Furthermore, amastigotes were unable to induce transcription via the human IL-12p40 promoter in this system.

**Leishmania** spp. products inhibit IL-12p40 promoter activity

To determine if inhibition of IL-12p40 promoter activity required a live *L. major* infection, we infected RAWp40LUC cells with *L. major* metacyclic promastigotes and with parasites...
stages for 16 hr followed by 8 hr of LPS stimulation. We further determined if live infection is not required for IL-12p40 inhibition, we infected RAWp40LUC cells with wild-type L. chagasi and an attenuated strain of L. chagasi that lacks MSP surface expression (Wilson et al., 1989) (Fig. 5B). Both attenuated and wild-type L. chagasi inhibited LPS-induced luciferase production, indicating that the most prominent Leishmania spp. molecules, LPG and MSP, are not necessary for inhibition of IL-12p40 promoter activity.

**Autocrine regulation is not responsible for IL-12p40 inhibition**

To investigate the role of autocrine or paracrine regulation of IL-12 production, we utilized a transwell system, whereby cells in the upper chambers could produce soluble mediators to affect luciferase production in cells in the lower chambers (Fig. 6). The upper chambers contained either uninfected or L. major-infected RAWp40LUC cells and the lower chambers contained RAWp40LUC cells either unstimulated or stimulated with IFN-γ + LPS. As a positive control for inhibition, we infected cells in the lower chamber with L. major parasites. Lower-chamber cells from wells containing uninfected cells in the upper chambers exhibited the typical pattern of luciferase production; that is, high amounts of luciferase from uninfected cells stimulated with IFN-γ and LPS and an inhibition of luciferase production from L. major-infected cells. The same pattern is found in lower-chamber cells from wells containing infected cells in the upper chambers, indicating that L. major infection is not inducing MP autocrine/paracrine regulation of IL-12 promoter activity.

**Inhibitors of phagocytosis block Leishmania spp.-induced IL-12p40 inhibition**

We investigated the role phagocytosis plays in Leishmania spp.-induced inhibition of IL-12 in response to IFN-γ and LPS stimulation by using 2 different phagocytosis inhibitors, cytochalasin D and the PLC inhibitor, U73122. Because both of

- **Figure 3.** Inhibition of IL-12p40Luc by different Leishmania spp. life-cycle stages. RAW264.7 cells stably transfected with human IL-12p40-LUC construct were infected with L. major strain V1 life-cycle stages for 16 hr followed by 8 hr of LPS (1 μg/ml) stimulation. Life-cycle stages (met = metacyclic; log = log-phase promastigotes; am = amastigotes). Samples were run in triplicate and mean relative luciferase units ± standard deviation is reported. * Infected values were significantly different from uninfected, stimulated controls (P < 0.05). † Untreated, stimulated values were different from untreated, media controls (P < 0.05). One representative of 2 independent experiments is shown.

- **Figure 4.** Inhibition of IL-12p40Luc by Leishmania spp. products. RAW264/p7 cells stably transfected with human IL-12p40-LUC construct were infected with L. major strain V1 or treated with parasite products for 16 hr followed by 8 hr of LPS (1 μg/ml) stimulation. (A) Parasite products (Live = live metacyclic infection; Lysate = freeze-thaw lysates; HK = heat-killed metacyclics). (B) Secreted products (V1 = live metacyclic infection; Neat, Dil, Conc = cultured parasite supernatants either diluted or concentrated). Samples were run in triplicate and mean relative luciferase units ± standard deviation is reported. * Infected values were significantly different from uninfected, stimulated controls (P < 0.05). † Untreated, stimulated values were different from untreated, media controls (P < 0.05). In each case 1 of 3 independent experiments is shown.

metacyclic promastigotes also diminished luciferase production, indicating that the Galα(1,4)Manα(1)-PO₄ repeat motif present in all PG is not required for IL-12 inhibition.

Being the most abundant surface glycoproteins on the Leishmania spp. surface, the MSPs are likely candidates for modulating host cell functions. To test the role of MSP in IL-12 inhibition, we infected RAWp40LUC cells with wild-type L. chagasi and an attenuated strain of L. chagasi that lacks MSP surface expression (Wilson et al., 1989) (Fig. 5B). Both attenuated and wild-type L. chagasi inhibited LPS-induced luciferase production, indicating that the most prominent Leishmania spp. molecules, LPG and MSP, are not necessary for inhibition of IL-12p40 promoter activity.

**Autocrine regulation is not responsible for IL-12p40 inhibition**

To investigate the role of autocrine or paracrine regulation of IL-12 production, we utilized a transwell system, whereby cells in the upper chambers could produce soluble mediators to affect luciferase production in cells in the lower chambers (Fig. 6). The upper chambers contained either uninfected or L. major-infected RAWp40LUC cells and the lower chambers contained RAWp40LUC cells either unstimulated or stimulated with IFN-γ + LPS. As a positive control for inhibition, we infected cells in the lower chamber with L. major parasites. Lower-chamber cells from wells containing uninfected cells in the upper chambers exhibited the typical pattern of luciferase production; that is, high amounts of luciferase from uninfected cells stimulated with IFN-γ and LPS and an inhibition of luciferase production from L. major-infected cells. The same pattern is found in lower-chamber cells from wells containing infected cells in the upper chambers, indicating that L. major infection is not inducing MP autocrine/paracrine regulation of IL-12 promoter activity.

**Inhibitors of phagocytosis block Leishmania spp.-induced IL-12p40 inhibition**

We investigated the role phagocytosis plays in Leishmania spp.-induced inhibition of IL-12 in response to IFN-γ and LPS stimulation by using 2 different phagocytosis inhibitors, cytochalasin D and the PLC inhibitor, U73122. Because both of
Inhibition of IL-12p40Luc by Leishmania does not involve autocrine MP factors; 1 × 10⁶ RAW264.7 cells stably transfected with human IL-12p40-LUC construct were plated in lower wells and 1 × 10⁶ were plated in upper wells of a transwell system. Cells were infected with L. major at a 5:1 ratio for 2 hr followed by IFN-γ (100 U/ml) stimulation for 16 hr and LPS (1 μg/ml) stimulation for 8 hr. Luciferase values were detected from cells in lower chambers. Samples were run in triplicate and mean relative luciferase units ± standard deviation is reported. *Infected values were significantly different from uninfected, stimulated controls (P < 0.05). † Uninfected, stimulated values were different from uninfected, untreated controls (P < 0.05). One representative of 3 independent experiments is shown.

**DISCUSSION**

Leishmaniasis is a chronic infection; even in the case of self-healing L. major infection, disease resolution takes several weeks to months. One mechanism Leishmania spp. parasites use to establish and persist in a host is the inhibition of IL-12 production and a delay in the ensuing cell-mediated response (Melby, 1991). MP are not the only cell type capable of producing IL-12 and certainly it has been demonstrated that the in vivo source of IL-12 during Leishmania spp. infection is likely dendritic cells (Gorak et al., 1998; Marovich et al., 2000; McDowell et al., 2002). However, Leishmania spp.-infected dendritic cells require CD40L costimulation for IL-12 production (Marovich et al., 2000; McDowell et al., 2002), suggesting that during infection, dendritic cells initiate a protective response after interaction with antigen-specific T-cells in lymphoid tissue. For parasites that normally target MP for survival, the avoidance of IL-12 early during infection would serve as an adaptive strategy to establish an infection. Although it is known that Leishmania spp. parasites impair IL-12 production in host MP, defining the mechanisms by which these pathogens inhibit IL-12 has been difficult. In the present study, we demonstrate that IL-12 suppression by Leishmania spp. is transcriptionally mediated and requires cell signaling and cytoskeletal rearrangement associated with phagocytosis.

Using a luciferase reporter assay, we demonstrate that Leishmania spp.-mediated downregulation of IL-12p40 occurs at the level of transcription. Consistent with previous reports assessing steady-state IL-12p40 mRNA levels (Carrera et al., 1996), our data indicate that L. major, L. donovani, and L. chagasi infection suppresses IFN-γ- and LPS-induced IL-12p40 production and extend this finding to CD40L cross-linking.

Contrary to L. mexicana amastigote-infected MP, in which LPS- and CD40L-induced IL-12p40 mRNA levels are not altered, L. major and L. donovani infection inhibits stimuli-induced activation of the IL-12p40 promoter. One possible explanation, other than species differences, to explain this discrepancy is that the previous study investigated axenic amastigotes; our study used all life-cycle stages and utilized lesion-derived amastigotes. Amastigotes obtained from lesions are covered with immunoglobulin that binds Fc receptors (FcR) (Guy and Belosevic, 1993; Dominguez and Torano, 1999) and infectious-stage metacyclic promastigotes are readily coated with endogenous ligands (C3b and iC3b) for complement receptor 3 (CR3) (Guy and Belosevic, 1993). Axenic amastigotes, on the other hand, are cultured in vitro and, therefore, do not have any of these endogenous host proteins on their surface. It previously has been suggested that Leishmania spp.-induced
Figure 7. Inhibition of IL-12p40Luc by Leishmania requires phagocytosis. RAW264.7 cells stably transfected with human IL-12p40-LUC construct were untreated (A, C) or pretreated with 1 μM cytochalasin D (B, D), then infected with L. major strain V1 and subsequently treated with 1 μg/ml LPS ± 100 U/ml IFN-γ. Samples were run in triplicate, treated with IFN-γ + LPS, and assessed for luciferase production (A, B) or endogenous levels of IL-12p40 by quantitative RT-PCR (C, D). Mean percentage expression ± standard deviation of is reported. * Infected values were significantly different from uninfected, stimulated controls (P < 0.05). One representative of 2 independent experiments is presented.

Suppression of IL-12 production is initiated by the binding of specific receptors on the host cell surface that send a negative signal (McDowell and Sacks, 1999). Activation of CR3, FcR, and scavenger receptor (SR) by antibodies or opsonized erythrocytes leads to the downregulation of LPS-induced IL-12 in murine MP (Sutterwala et al., 1997). Furthermore, FcR, CR3, and mannose receptor (MR) ligation of human phagocytes inhibits IL-12 production (Berger et al., 1997; Marth and Kelsall, 1997; Nigou et al., 2001), whereas the amastigotes used in our studies likely engaged the FcR–receptor interaction for pro-mastigotes is less clear. Our studies indicate that opsonization of Leishmania spp. parasites with normal mouse serum is not necessary for inhibition (data not shown), suggesting that CR3 ligation is not mediating the interaction. However, MP are able to synthesize complement components (McPhaden and Whaley, 1993) that opsonize parasites even in serum-free conditions (Wozencraft et al., 1986) and parasite components have been suggested to bind CR3 directly (Russell and Wright, 1988; Talhamas-Rohana et al., 1990; Kedzierski et al., 2004). Furthermore, the repetitive structure and glycan modifications associated with many Leishmania spp. cell-surface molecules suggest that these parasites also interact with MR and SR (Turco and Descoteaux, 1992), which have been shown to send a negative signal in other systems.

Interestingly, it previously has been reported that L. major lesion-derived amastigotes (Reiner et al., 1994; Piedrafita et al., 1999) and log-phase promastigotes (Sartori et al., 1997) stimulate IL-12p40 production in murine MP and human PBMC, respectively. We were unable to confirm this observation using the RAW 264.7 MP cell line, as procyclic promastigotes or amastigotes did not induce any luciferase expression in the presence or absence of LPS stimulation. The reason for these discrepancies is unclear; however, our data are in agreement with others that have shown that lesion-derived L. major amastigotes inhibit, at least indirectly, IFN-γ + LPS-induced IL-12 production (Kane and Mosser, 2001). Further, it is possible that the IL-12 detected in human PBMC infected with procyclic promastigotes may have been produced by cell types other than MP.

Here, we utilized a murine MP cell line stably expressing a human IL-12p40 promoter construct to assess the effect of Leishmania spp. infection on IL-12 production. Although this system certainly is not identical to human MP, it previously has been shown to mirror endogenous murine and human IL-12p40 production in terms of cell type specificity and stimulus responsiveness (Aste-Amezaga et al., 1998). We demonstrate that endogenous murine IL-12p40 mRNA is inhibited by L. major infection in this system. We cannot rule out, however, that the cell-surface receptors that engage Leishmania spp. or the signaling pathways activated by such engagement may differ between murine and human MP; thus altering the response in human cells. Although the effect of Leishmania spp. infection on human MP remains to be determined, supporting our conclusion...
is the observation that *L. major* promastigotes inhibit SAC-induced IL-12 production in PBMC (Sartori et al., 1996).

Although the predominant surface molecule of *Leishmania* spp. promastigotes, LPG, has been implicated in inhibiting macrophage signal transduction pathways (Descoteaux and Turco, 2002), this glycoconjugate is not required for IL-12 inhibition by *Leishmania* infection in murine MP (Carrera et al., 1996) or human PBMC (Sartori et al., 1997). High concentrations of PG, on the other hand, have been shown to inhibit LPS-induced IL-12p40 production (Piedrafita et al., 1999). Here, we utilized *Leishmania* spp. parasites that lack LPG or PG expression to test the necessity of these moieties in suppressing IL-12p40 responses. We confirmed previous observations (Carrera et al., 1996) that LPG is not necessary and discovered that in a natural context PG also is not essential for *Leishmania* spp.-induced inhibition. Either the physiological concentrations of PG that were presented in our experiments do not inhibit IL-12p40 promoter activity or *Leishmania* spp. parasites use redundant mechanisms to impair IL-12 production (or both).

The repeating PG domain is a characteristic feature of other molecules of the leishmania glycoalxyl, including proteo-PG (Ilg, Overath et al., 1994; Ilg, Stierhof et al., 1994; Ilg et al., 1996), secreted PG, and acid phosphatase (Shakarian and Dwyer, 2000). The PG domain contains species and strain-specific substitutions (Tolson et al., 1989; McConville et al., 1990; Ilg et al., 1992) and developmentally regulated polymorphisms (Glaser et al., 1991; Sacks, 1992; Moody et al., 1993). Furthermore, it recently has become apparent that *L. major* strains differ in their PG structure (Dobson, Mengeling et al., 2003; Dobson, Scholtes et al., 2003). Our data indicate that these polymorphisms likely do not influence *Leishmania* spp.-induced inhibition because all of the species, strains, and life-cycle stages that we tested were able to block IL-12p40 promoter activity, implicating some nonpolymorphic parasite factor(s) or redundant mechanisms.

LPS-induced IL-12p40 promoter activity was inhibited by treatment of MP with heat-killed parasites, parasite lysates, or supernatants from in vitro *L. major* cultures, indicating that the factor(s) responsible were likely present on the cell surface and secreted. The most abundant surface glycoprotein, MSP, recently has been shown to be released into the extracellular medium (Ellis et al., 2002; McGwire et al., 2002; Yao et al., 2002). Our data using *L. chagasi* promastigotes that lack MSP surface expression (*L. chagasi* L5) indicate that MSPs are likely not involved in the inhibition of IL-12p40 production. However, the L5 strain is not a specific gene knockout and, therefore, trace amounts of MSP are possibly expressed. Glycosolphosphatidylinositol (GPI)-anchored molecules, including LPG, PG, and MSP, glycosylinositolphospholipids, and gp46, dominate the *Leishmania* spp. surface. Studies have demonstrated that GPI anchors from *Leishmania* spp. and other protozoan parasites are able to modulate MP signaling cascades (Tachado et al., 1999), suggesting that *Leishmania* spp. GPI anchors are responsible for inhibiting MP functions. Recent studies indicate that the lipid moiety of *Leishmania* spp. GPI anchors is not required for inhibiting MP activation (Zufferey et al., 2003); however, inhibition of IL-12 production was not assessed in these studies. As uncontrolled pro-inflammatory cytokine production is potentially dangerous, negative feedback loops exist to downmodulate IL-12. Both IL-10 (Ma et al., 1998) and TGF-α (D’Andrea et al., 1995) are potent inhibitors of IL-12 production. In addition to these anti-inflammatory cytokines, prostaglandin-E2 downregulates this molecule (van der Pouw Kraan et al., 1995, 1996). One possible mechanism by which *Leishmania* spp. could regulate IL-12 production would be to activate infected MP to produce 1 or more of these downregulatory modulators. The fact that we observed that IL-10 is upregulated in MP following *Leishmania* spp. infection makes it particularly appealing to implicate IL-10 as the mechanism for downregulating IL-12 expression; however, it previously has been demonstrated that inhibition still occurs in the absence of IL-10 (Carrera et al., 1996; Weinheber et al., 1998). Using a transwell system, we show here that no autocrine- or paracrine-acting MP factors are involved in suppression of IL-12 by *Leishmania* spp. infection, lending support for a direct interaction of these parasites with host signaling machinery or the downregulatory receptor hypothesis.

The role of phagocytosis in IL-12 production and inhibition is unclear. Phagocytosis of inert polystyrene beads greater than 2 μm in diameter upregulates IL-12p40 mRNA in human monocytes (Fulton et al., 1996) and 3.1-μm latex beads induce IL-12p40 production in bone-marrow-derived MP, whereas 1.2-μm beads do not (Ladel et al., 1997). In a conflicting study, phagocytosis of 3.2-μm latex beads led to a 50% suppression of IFN-γ + CD40L-induced IL-12 production (Weinheber et al., 1998). However, phagocytosis is not required for IL-12 suppression by apoptotic cells (Kim et al., 2004). *Leishmania* spp. amastigotes are only 1–2 μm in size and still suppress MP IL-12 production, suggesting that at least for *Leishmania* spp., size is likely not a factor in the inhibitory process.

We utilized cytochalasin D to block actin polymerization and the PLC inhibitor U73122 to prevent phagocytosis of *Leishmania* spp. parasites to determine if only cell contact was required for IL-12 inhibition. In both cases, the inhibitors alone suppressed LPS-induced luciferase expression and endogenous IL-12 transcription, suggesting that contrary to previous reports (Poussin et al., 1998) LPS-dependent activation requires LPS internalization. The inhibition was selective, however, as TNF-α and IL-10 production were enhanced in the presence of the phagocytosis inhibitors. Regardless of this suppression, we were able to detect significant IL-12 mRNA and luciferase expression in response to IFN-γ + LPS stimulation over untreated controls. This upregulation was not inhibited by *Leishmania* spp. infection in the presence of either phagocytosis inhibitor. There are 2 possible explanations to explain this result. First, these data could indicate that phagocytosis is necessary to inhibit the signal that leads to IL-12 transcription. Consistent with this explanation is a kinetic analysis that revealed that 16 hr of infection was required for the inhibition of MP activation to be detected (Nandan and Reiner, 1995). In contrast, another study investigating the same responses indicates that inhibition actually occurs before the parasites have been internalized, suggesting that the suppression is mediated via cell-surface receptor binding (Blanchette et al., 1999). Alternatively, our results may indicate that phagocytosis per se is not necessary for inhibition but that the downregulatory signaling pathways require actin polymerization and PLC activation. Recent work has highlighted the role of the cytoskeleton in mediating cell-signaling events and suggests that endocytosis/phagocytosis machinery may play a role in signaling not directly related to the uptake.
of exogenous particles (Liu and Shapiro, 2003). It is likely that Leishmania spp. factors, perhaps redundant molecules, interact with downregulatory phagocytic receptors to mediate IL-12 inhibition. As it is extremely difficult to uncouple the signaling and phagocytosis associated with these receptor interactions, whether Leishmania spp. uptake is essential remains to be determined.

ACKNOWLEDGMENTS

We are grateful to Dr. Salvatore Turco (University of Kentucky) for the use of the LPG Leishmania donovani mutants and to Jesmin Eblers for his help with the luciferase assays. We are also thankful to Dr. Jeff Schorey for his helpful suggestions regarding our work and thoughtful review of the manuscript. Our gratitude also goes to Dr. Mary Wilson for the generous gift of the L. chagasi strains and for her insightful suggestions regarding our manuscript. This work was supported by a Scientist Development grant from the American Heart Association (#0435332).

LITERATURE CITED


of Leishmania donovani deficient in lipophosphoglycan. Molecular and Biochemical Parasitology 101: 285-293.


leads to a sustained suppression of IL-12 production. European Journal of Immunology 28: 2467–2477.


DETECTION OF CRYPTOSPORIDIUM PARVUM OOCYSTS BY DOT-BLOTTING USING MONOCLONAL ANTIBODIES TO CRYPTOSPORIDIUM PARVUM VIRUS
40-kDa CAPSID PROTEIN

Mark C. Jenkins, Celia N. O’Brien, and James M. Trout*  
Animal Parasitic Diseases Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705.  
e-mail: mark.jenkins@ars.usda.gov

ABSTRACT: Monoclonal antibodies (MAb) were prepared against the 40-kDa capsid protein of Cryptosporidium parvum virus (CPV) by immunizing mice with purified recombinant CPV-40 protein. In immunoblotting analysis, MAbCPV40-1 bound to a 40-kDa protein in extracts of C. parvum oocysts. This 40-kDa protein was localized in the sporozoite cytoplasm by immunofluorescence (IFA) staining with MAbCPV40-1. In a dot-blot assay, MAbCPV40-1 was capable of detecting 10e6 non-bleach-treated and 102–103 bleach-treated C. parvum oocysts. MAbCPV40-1 was capable of detecting CPV40 antigen in both soluble and total C. parvum oocyst protein extracts, indicating a potential use for detecting this parasite in environmental samples.

Preventing cryptosporidiosis in humans depends in part on identifying Cryptosporidium spp. oocysts in surface waters prior to their entry into a water treatment facility. The standard technique (EPA method 1623) for detecting Cryptosporidium parvum (and Giardia lambia) involves cartridge filtration followed by immunomagnetic separation (IMS) and epifluorescence staining using antibodies specific for C. parvum or G. lambia surface antigens. Over the last several years, research has been directed at modifying the concentration protocol and other assay steps to improve the recovery and detection of C. parvum oocysts (Simmons et al., 2001; DiGiorgio et al., 2002; Ware et al., 2003; Hu et al., 2004; Zuckerman and Tzipori, 2006). Incorporation of the polymerase chain reaction (PCR) has proven useful for more sensitive detection and species determination of C. parvum oocysts (Di Giovanni et al., 1999; Hallier-Souli and Guillot, 2000; LeChevallier et al., 2003; Xiao et al., 2004). Although many laboratories have embraced PCR as a method for detecting C. parvum (reviewed in Morgan and Thompson, 1998; Quintero-Betancourt et al., 2002), I report suggests that widely used primers directed at the 18S-rDNA may not be specific for C. parvum (Sturbaum et al., 2002). Also, because of simplicity, most water quality laboratories continue to rely on fluorescence-based methods such as IFA, fluorescence in situ hybridization (FISH), or flow cytometry (FC) (Ferrari et al., 2000; Lindquist et al., 2001; Myoda and Huang, 2001; Smith et al., 2002; Kuczynska et al., 2003; Lemos et al., 2005; Taguchi et al., 2007). Each of these methods relies in part on staining with MAbs that bind to a surface protein of C. parvum oocysts (Garcia et al., 1992; Ferrari et al., 2000; Weir et al., 2000; Kuczynska et al., 2003). Of some concern is a study demonstrating that most commercial MAbs reagents bind to an immunodominant oocyst wall protein epitope, which is damaged by chlorine treatment, leading to false negative reactions (Moore et al., 1998). Previous studies in our laboratory (Kniel et al., 2004) and others (Kozwich et al., 2000) found that reagents directed to the C. parvum viral symbiont CPV could detect oocysts in environmental samples. The purpose of the present study was to evaluate a MAb directed against the CPV capsid protein for its ability to identify C. parvum oocysts in a dot-blot assay.

MATERIALS AND METHODS

Cryptosporidium parvum oocysts

Recombinant CPV40 antigen was expressed in Escherichia coli BL21 cells (Novagen, San Diego, California) and purified by NINATA affinity chromatography as described (Kniel et al., 2004). Under a BAACUC-approved protocol, BALB/c mice were immunized by intraperitoneal (i.p.) injection with 40 µg purified rCPV40 antigen emulsified in ImmunoMax SR adjuvant (Repros Therapeutics Inc., The Woodlands, Texas). The mice received a booster i.p. injection with rCPV40-ImmunoMax SR adjuvant 1 mo later, and then a final tail vein injection with phosphate-buffered saline (PBS)-dialyzed rCPV40 antigen 2 mo after the booster immunization. After 3 days, the mice were necropsied and spleens were processed for fusion to SP2/AG14 myeloma cells (ATCC CRL-1581, Manassas, Virginia) using standard protocols (Yokoyama, 1994).

Screening monoclonal antibodies and establishment of hybridomas

 Supernatants from hybridoma cultures were assayed for the presence of monoclonal antibodies to both rCPV40 and native CPV protein. In the former, purified rCPV40 protein was adsorbed to the surface of Immulon 2 ELISA plates (Dynex Technologies, Chantilly, Virginia) for 1 hr at 37°C, then overnight at 4°C. The ELISA plates were washed to remove excess rCPV40 protein, blocked for 1 hr at RT with 5% normal chicken serum (Sigma-Aldrich, St. Louis, Missouri), incubated for 1 hr at 37°C with 100 µl supernatant from culture wells containing viable hybridoma cells, and then for 1 hr at 37°C with 100 µl alkaline phosphatase-labeled goat anti-mouse IgG (1:1,000 dilution, H+L chain specific, Kirkegaard and Perry, Gaithersburg, Maryland). Between each step, antibodies were removed by washing the plate 3 times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-TW). Binding of monoclonal antibodies was assessed by the addition of 1 mg/ml substrate (p-nitrophenyl phosphate disodium, Sigma-Aldrich) and measurement at 405 nm on a multiwell plate reader (Biotek, Winooski, Vermont). Recognition of native CPV antigen was performed using aqueous-soluble and 3 M urea-soluble whole C. parvum oocyst protein by ELISA and vacuum blotting. Antigen for both native CPV assays was prepared by freeze thawing in a dry ice–ethanol bath sodium hypochlorite–treated C. parvum oocysts in 10 mM Tris-HCl (pH 7.3) 1 mM MgCl2, containing PMSF protease inhibitor (Sigma-Aldrich). Aqueous-soluble C. parvum protein was separated from insoluble protein by centrifugation at 13,500 g for 15 min. The insoluble pellet was extracted.

Received 3 May 2007; revised 18 July 2007; accepted 18 July 2007.

* Environmental Microbial Safety Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705.
with 3 M urea for 30 min at RT and then centrifuged at 13,500 g for 15 min to recover urea-soluble supernatant. Aqueous-soluble and urea-soluble protein equivalents to 10^9 C. parvum oocysts were adsorbed to the surface of ELISA microtiter plates or adhered onto Immobilon membrane (Millipore, Billerica, Massachusetts) under vacuum using a dot-blot apparatus (BioRad, Hercules, California). ELISA of hybridoma supernatants against native CPV protein was performed in a manner identical to that described above for rCPV40 antigen. Dot-blot assay was carried out by cutting adjacent wells containing either aqueous- or urea-soluble C. parvum protein and incubating the membrane section in a 1.5-ml microcentrifuge tube containing hybridoma supernatant. After 1 hr incubation at RT, the strips were incubated with biotinylated goat anti-mouse IgG (H+L), followed by alkaline phosphatase-labeled avidin (Sigma-Aldrich). Primary and secondary antibodies were removed by washing the blots 3 times with PBS-Two. Binding of MAb to native CPV protein was assessed by the addition of BCP/NBT alkaline phosphatase substrate (Pierce Chemical Co., Rockford, Illinois). Hybridoma cultures containing supernatants that produced a strong signal to recombinant and/or native CPV antigen were further subcloned by limiting dilution. Subcloning was repeated 3 times for each hybridoma to ensure clonality of the cell line. Ig isotyping and light chain determination was carried out using a commercial isotyping test kit (Biomedical, Burlingame, California).

Immunoblot analysis

Hybridoma cultures established using the above screening method were expanded into 25-cm² and eventually into 75-cm² tissue culture flasks (Corning, Lowell, Massachusetts). Supernatants were harvested and assayed for binding to native CPV protein by immunoblotting. In brief, C. parvum protein extracts were solubilized with sample buffer (Laemmli, 1970) in the presence or absence of 2-mercaptoethanol (Sigma-Aldrich), followed by heating on a boiling water bath for 1 min. Insoluble protein was removed by centrifugation at 13,500 g for 5 min. Soluble protein (equivalent to 10^9 C. parvum oocysts) was subjected to preparative sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon membrane in a semi-dry gel blotter (BioRad). Molecular weight standards (low range, BioRad) were electrophoresed in a separate well of the SDS-PAGE to allow for Mr estimation. The membrane section containing SDS-PAGE-fractionated C. parvum protein was treated for 30 min at RT with 2% nonfat dry milk in PBS. Vertical strips were excised from the blot and were incubated overnight at 4 C followed by 1 hr at RT with monoclonal anti-CPV supernatant or HT media containing an irrelevant MAb at equal IgG concentration. Binding of anti-CPV monoclonal antibodies was assessed as described above for dot blots.

Immunofluorescence staining of C. parvum sporozoites

Cryptosporidium parvum oocysts were excysted using a modified rapid excystation method (O’Brien and Jenkins, 2007). The released sporozoites and excysted/unexcysted oocysts were dried onto individual wells of multiwell microscope slides (Eric Scientific, Portsmouth, New Hampshire). The slides were left untreated, or were treated with ice-cold methanol for 1 min, or were treated with 4% buffered formalin for 10 min at RT followed by a 10-min exposure to 0.1% saponin (Willingham, 1994). All slides were washed with PBS, allowed to air dry, and then either left untreated or were treated for 5 min with 3 M urea in PBS. All wells were washed with PBS, allowed to air dry, and then incubated for 30 min in PBS containing 2% bovine serum albumin. The wells were washed, dried, and then incubated for 1 hr with anti-CPV MAb supernatant or with HT medium containing an irrelevant MAb at equal IgG concentration. Binding of anti-CPV MAb was assessed by incubating each well with fluorescein isothiocyanate–labeled goat antimouse IgG (H+L, Sigma-Aldrich) for 1 hr at RT. All wells were washed between steps by gently pipetting PBS over the slide surface and then 2 brief immersions in PBS. After drying, several drops of antibleaching mounting medium (Vectorshield, Vector Laboratories, Burlingame, California) was pipetted onto the slide surface, which was then overlaid with a coverslip and examined by epifluorescence microscopy.

Sensitivity testing of monoclonal anti-CPV40 antibodies

Cryptosporidium parvum oocysts were pelleted by centrifugation, suspended in 3 M urea, and extracted by freeze-thawing 3 times in a dry ice–ethanol bath, followed by incubation on a rotating rocker for 30 min at RT. Urea-soluble protein was collected by harvesting the supernatant after centrifugation at 13,500 g for 5 min. Urea-soluble and total protein was serially diluted in 3 M urea to achieve oocyst concentrations from 10^9 to 10^1 oocysts/ml. An aliquot (100 μl) of each suspension (10^1 to 10^6 oocysts/well) was adhered to the surface of Immobilon membrane in the multwell dot-blot apparatus as described above. Immobilon membrane strips containing serial dilutions of C. parvum oocyst urea-soluble or total oocyst protein were incubated with either CPV40 MAb supernatant or HT media containing an irrelevant MAb at equal IgG concentration. Binding to C. parvum oocyst protein was assessed as described above for dot-blot screening of hybridoma supernatants.

RESULTS

SDS-PAGE/immunoblot findings

In a primary screen, 20 hybridoma supernatants exhibited reactivity to either rCPV40 antigen or native C. parvum protein. Of these, 5 were chosen for further study because of their strong reactivity to recombinant or native protein. Only 1 MAb designated, MAbCPV40-1 (IgG1, kappa), displayed consistent recognition of native C. parvum 40-kDa protein (Fig. 1, left blot strip). Under nonreducing SDS-PAGE, MAbCPV40-1 appeared to recognize 2 higher Mr bands (~77 and 125 kDa, Fig. 1, right blot strip).

IFA staining of C. parvum sporozoites with MAbCPV40-1

Only sporozoites treated with 3 M urea displayed internal fluorescence after labeling with MAbCPV40-1 (Fig. 2). Although detectable staining was observed with methanol-treated and, to a lesser extent, with untreated sporozoites, the most intense binding was observed with C. parvum sporozoites that had been pretreated with buffered formalin and saponin detergent. IFA staining of C. parvum sporozoites was negligible with control MAb (data not shown).

Cryptosporidium parvum oocyst detection sensitivity

MAbCPV40-1 showed a detection limit of 10^2 non–bleach-treated C. parvum oocysts (Fig. 3, upper blot strip, +). The

![Figure 1. Immunoblot analysis of SDS-PAGE-fractionated Cryptosporidium parvum oocyst protein labeled with MAbCPV40-1. +, C. parvum protein treated with sample buffer containing 2-mercaptoethanol; -, C. parvum protein treated with sample buffer not containing 2-mercaptoethanol, MrS, molecular mass standards (kDa).](image-url)
sensitivity decreased about 10-fold after bleach treatment (Fig. 3, upper blot strip, +), which may be due to nonspecific binding of contaminating material in the oocyst preparation. This hypothesis is supported by the low level binding of non-bleach-treated C. parvum oocysts by the control MAb (Fig. 3, lower blot strip, −), which is not apparent in the bleach-treated oocysts (Fig. 3, lower blot strip, +).

**DISCUSSION**

In the present study, MAbCPV40-1 was produced by immunizing mice with a recombinant 40-kDa capsid protein of C. parvum virus. Monoclonal antibodies to the viral symbiont of C. parvum were pursued for several reasons. One was that MAb would represent an unlimited source of detection reagent. A
second was to increase sensitivity of *C. parvum* detection. The rationale for this approach was that higher sensitivity would be afforded by a MAb directed to a target that is present at higher concentrations in oocysts (~2,000 CPV particles/oocyst) compared with an oocyst wall reactive protein. Although MAbCPV40-1 displayed specific detection of the 40-kDa protein, the level of sensitivity was not appreciably greater than that reported by others using oocyst wall reactive MAb (Lee et al., 2001; Kuczynska et al., 2003). Increasing the detection sensitivity of MAbCPV40-1 may require ascites production and direct labeling of MAb with reporter enzyme, e.g., alkaline phosphatase.

The native CPV40 epitope recognized by MAbCPV40-1 is probably marked by the natural 3-dimensional structure of the native protein because MAb recognition was only observed in the presence of denaturants such as 3 M urea or SDS. For this reason, all extractions were done in the presence of 3 M urea. This requirement for urea treatment of sporozoites to obtain reducing conditions. There is precedent for this with other viral capsid proteins, which appear to exist in multimeric form.

This requirement for urea treatment of sporozoites to obtain reducing conditions. There is precedent for this with other viral capsid proteins, which appear to exist in multimeric form. The rationale for this approach was that higher sensitivity would be afforded by a MAb directed to a target that is present at higher concentrations in oocysts (~2,000 CPV particles/oocyst) compared with an oocyst wall reactive protein. Although MAbCPV40-1 displayed specific detection of the 40-kDa protein, the level of sensitivity was not appreciably greater than that reported by others using oocyst wall reactive MAb (Lee et al., 2001; Kuczynska et al., 2003). Increasing the detection sensitivity of MAbCPV40-1 may require ascites production and direct labeling of MAb with reporter enzyme, e.g., alkaline phosphatase.

The native CPV40 epitope recognized by MAbCPV40-1 is probably marked by the natural 3-dimensional structure of the native protein because MAb recognition was only observed in the presence of denaturants such as 3 M urea or SDS. For this reason, all extractions were done in the presence of 3 M urea. This requirement for urea treatment of sporozoites to obtain binding of MAbCPV40-1 in IFA corroborates the immunoblotting results. This phenomenon is probably due to the nature of *E. coli* expression of CPV40. It is possible that rCPV40 is not expressed in the same configuration as native CPV40, and thus antibodies to the recombinant would only recognize denatured (unfolded native) protein. An interesting phenomenon was the recognition by MAbCPV40-1 of higher Mr proteins under non-reducing conditions. There is precedent for this with other viral capsid proteins, which appear to exist in multimeric form, usually as dimers or tetramers (Martinez-Costas et al., 1997; Grande et al., 2002; Bender et al., 2005). It is possible that the observed 77- and 125-kDa proteins represent dimeric and tetrameric forms of CPV40.

MAbCPV40-1 showed sensitive detection of *C. parvum* oocysts in a dot-blot format, exhibiting measurable binding at 10⁵ oocysts. A fraction of this signal may have been due to the presence of contaminants in the oocyst preparation because the signal against an equal number of bleach-treated *C. parvum* oocysts was detectably less. The increased binding of MAbCPV40-1 to non-bleach-treated oocysts is probably due to Ab binding by Fc receptors in bacterial cell membranes. A measurable difference in signal intensity was observed between MAbCPV40-1 and MAbControl with purified *C. parvum* oocysts (Fig. 3). In general, there was a higher level of binding by CPV40-reactive MAb to insoluble compared with soluble *C. parvum* protein. These findings suggest that a portion of CPV may remain associated with insoluble oocyst material even after freeze–thawing to rupture the oocyst wall. Future research will focus on producing higher concentrations of MAbCPV40-1 in ascites to increase sensitivity and to obtain sufficient amounts of MAb for preparing FAb fragments to eliminate Fc binding by contaminants, i.e., bacteria that are naturally present in oocyst samples from calf feces and in surface waters.

**LITERATURE CITED**


Identification and determination of the viability of Giardia lamblia cysts and Cryptosporidium parvum and Cryptosporidium hominis oocysts in human fecal and water supply samples by fluorescent in situ hybridization (FISH) and monoclonal antibodies. Parasitology Research 98: 48–53.


Viable Cryptosporidium parvum oocysts exposed to chlorine or other oxidising conditions may lack identifying epitopes. International Journal for Parasitology 28: 1205–1212.


Detection of Cryptosporidium parvum oocysts using a microfluidic device equipped with the SUS micromesh and FITC-labeled antibody. Biotechnology and Bioengineering 96: 272–280.


Portable continuous flow centrifugation and method 1623 for monitoring of waterborne protozoa from large volumes of various water matrices. Journal of Applied Microbiology 100: 1220–1227.
INHIBITION OF INTERLEUKIN-12 PRODUCTION BY TRYPANOSOMA BRUCEI IN RAT MACROPHAGES

Kazuhiro Nishimura, Shinsuke Sakakibara, Kououe Mitani, Jyoji Yamate*, Yoshihiro Ohnishi, and Shinji Yamasaki

Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan
E-mail: nisimura@vet.osakafu-u.ac.jp

ABSTRACT: The immune response of a host infected with Trypanosoma brucei is modulated by trypomastigotes. We examined the changes in cytokine production in T. brucei gambiense (Wellcome strain; WS) infected rats and the influence on production of interleukin (IL)-12 by macrophages. The blood concentration of interferon-γ (IFN-γ), tumor necrosis factor-α, and IL-10 increased beginning the second day after infection. However, an increase in IL-12p40 was not observed until 4 days after infection. When spleen macrophages and Kupffer cells harvested from uninfected rats and HS-P cells (a rat macrophagelike cell line) were cocultured with WS, IL-12p40 production did not change. When HS-P cells were cultured with WS, transport of nuclear factor-kb into the nucleus increased. Levels of macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor mRNA in the spleens and livers of WS-infected rats were high in comparison with uninfected rats, suggesting that the WS promotes macrophage proliferation. The level of IL-12p40 mRNA in HS-P cells cocultured with WS increased in response to transfection with a small interfering RNA against M-CSF and addition of anti-M-CSF antibody. These results suggest that the WS inhibits IL-12p40 mRNA production by promoting production of macrophage colony-stimulating factor by macrophages.

African trypanosomiasis is a zoonosis caused by Trypanosoma brucei, a parasitic organism that lives within the bloodstream of its mammalian hosts. It is thought that trypanomastigotes in the infected host evade or inhibit host immune processes via characteristics that most other infectious diseases do not possess, i.e., changes in antigenicity via variant surface glycoproteins (Sternberg, 1998); production of Trypanosoma-derived lymphocyte triggering factor (TLTF), which promotes interferon-γ (IFN-γ) production (Bakhiet et al., 1991, 1996; Hamadien et al., 1999); or immunosuppression (Ayub et al., 1993; Baetselier et al., 2001). Many studies have shown that cytokines play an important role in the infection defense mechanism of trypanosomes. Uzonna et al. (1998) found that trypanosome-resistant mice produced higher levels of interleukin (IL)-12 in comparison with sensitive mice, and that trypanosome-resistant mice produced low levels of IL-10 and IFN-γ. Like IL-12, tumor necrosis factor-α (TNF-α) is produced by macrophages, and TNF-α and IFN-γ are reported to have a trypanocidal effect (Hertz et al., 1998; Magez et al., 1999). TNF-α and IFN-γ can be cytotoxic to the host itself, causing symptoms such as cachexy (Magez et al., 1999) and reducing the survival of the trypanosome-infected mouse (Uzonna et al., 1998; Shi et al., 2003). On the other hand, IFN-γ expression, which is promoted by TLTF, stimulates an increase in trypanosomes (Bakhiet et al., 1991, 1996; Hamadien et al., 1999), a phenomenon that was confirmed in our laboratory (Nishimura et al., 2004). It is thought that production of cytokines in response to trypanosome infection affects the proliferation of trypanomastigotes closely, but opinions vary on the action of cytokines. In contrast, IL-12 promotes cell-mediated immunity and is a cytokine that is indispensable for defense against trypanosome infection; macrophages play an important role in this response (Baetselier et al., 2001; Duleu et al., 2004). In a signal cascade that takes place in macrophages, nuclear factor-κB (NF-κB) (a transcription factor) is transported to the nucleus, where NF-κB promotes the transcription of IL-12 mRNA, thus increasing IL-12 production (Xiong et al., 2004; Yue et al., 2005). IL-12 production is regulated by nitric oxide (NO) synthetized by inducible nitric oxide synthase (iNOS) in macrophages at the time of infection (Xiong et al., 2004). In addition, macrophage colony-stimulating factor (M-CSF) is produced not only by macrophages, but also lymphocytes, blood vessel endothelium, fibroblasts, and hepatocytes; it is also involved in IL-12 production (Ji et al., 2004). The mechanism by which IL-12 production is regulated during trypanosome infection is unclear, and the influence trypanosomes have on macrophages is also unknown. Therefore, in the present study, changes in cytokine production in trypanosome-infected rats were examined, and the influence of trypanosome infection on IL-12 production by the macrophage was analyzed.

MATERIALS AND METHODS

Materials

Iscove’s modified Dulbecco’s medium (IMDM) was purchased from Invitrogen Corp. (Carlsbad, California), and lipopolysaccharide (LPS) from Wako Pure Medicine (Osaka, Japan). Rat recombinant IFN-γ (specific activity of ≥1 × 10^7 units/mg) was obtained from PeproTech EC Ltd. (London, UK), and anti-rat IFN-γ from PeproTech Inc. (Rocky Hill, New Jersey). Anti-M-CSF was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California), while primers for real time reverse transcription–PCR (RT-PCR) came from Genedesign Co. (Osaka, Japan). Standard laboratory chemicals and reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). To amplify mRNA using real time RT-PCR, we designed pairs of primers for cytokines and other factors (Table I).

Preparation of trypanosomes

Noncloned, monomorphic trypomastigotes of the Trypanosoma brucei gambiense Wellcome strain (WS) were used. Trypanosomes were prepared as described previously (Nishimura et al., 2001). Trypomastigotes stored in liquid nitrogen were thawed and subcultured in vitro in IMDM containing 100 μM hypoxanthine, 30 μM thymidine, 40 μM adenosine, 1 mM sodium pyruvate, 50 μM l-glutamine, 100 μM 2-mercaptoethanol, 200 μM l-alanine, 100 μM glycine, 20 μM l-ornithine-HCl, 10 μM l-citrulline, and 5% fetal bovine serum (FBS) using the method of Yabu et al. (1998). Trypomastigotes were cultured in modified IMDM in 95% O2, and 5% CO2 at 37°C. Trypanosomes were counted every 24 hr using light microscopy.
Table I. Primers used for RT-PCR analysis.

<table>
<thead>
<tr>
<th>Product</th>
<th>Sequence</th>
<th>Region</th>
<th>Reference or accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GCACCAACCAACTGCTTAGCAC-3'</td>
<td>342-442</td>
<td>Tajima et al., 1999</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-CTGAGTGGCGAGTATGCGATCT-3'</td>
<td>5-105</td>
<td>Goodman et al., 1992</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>5'-AGACCCTGAGCTATTGACTG-3'</td>
<td>621-721</td>
<td>Gulko et al., 1998</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-TTTGTGAGATCTGAGCT-3'</td>
<td>288-388</td>
<td>Visse et al., 1999</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-AGGAGTGTGAACTGGCAGCA-3'</td>
<td>22-122</td>
<td>Estler et al., 1992</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-GACACATTCCTGCTGTTGAA-3'</td>
<td>25-125</td>
<td>Stasiulek et al., 2000</td>
</tr>
<tr>
<td>M-CSF</td>
<td>5'-AGACACAAACAAAATCAGAGG-3'</td>
<td>947-1049</td>
<td>Yang et al., 2006</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5'-CATGTTTCTGACACCCACCCC-3'</td>
<td>42-142</td>
<td>XM.340799</td>
</tr>
<tr>
<td>i-KBb</td>
<td>5'-TGTTGAGATTGGACGCTGCA-3'</td>
<td>771-879</td>
<td>Yue et al., 2005</td>
</tr>
</tbody>
</table>

Animals

Experiments were carried out using 10-wk-old male Wistar rats that were supplied with food and water ad libitum (Nippon SLC Co., Shizuoka, Japan). Animals were kept at 25°C with a 12:12 hr light:dark cycle starting at 0600 hr. After subcutaneous inoculation of 10^8 trypomastigotes per rat, blood was collected daily from each rat’s tail vein at 0800 hr and the number of parasites was counted. Blood samples were collected from the abdominal aorta of 4 rats using a heparinized syringe every day until 5 days postinfection (PI). Blood samples were immediately cooled to 4°C, and plasma was prepared by centrifugation at 900 g for 10 min. Sera were stored at -80°C for determination of cytokine concentration.

Immunohistochemistry

Four days PI, the rats were killed. The rat spleens and livers were extracted, fixed in 10% neutral buffered formalin (pH 7.4), and embedded in paraffin. Sections were immunohistochemically stained using monoclonal ED1 antibody (Chemicon International Inc., Temecula, California), which recognizes rat-infiltrated macrophages, using an ABC kit (Vector Laboratories Inc., Burlingame, California) (Yamate et al., 2001). The number of macrophages was counted using light microscopy. An increase in macrophages of infected rats was calculated relative to uninfected rats.

Splenocyte and spleen macrophage preparation

Splenocytes were prepared as described previously (Nishimura et al., 2004). Spleens were removed aseptically from infected or uninfected rats and dispersed into Hank’s solution (HBSS) using a 180-μm stainless steel mesh. Cell suspensions were centrifuged at 300 g for 5 min. Spleenocytes were washed twice with HBSS then stored at -80°C until RT-PCR, or were cocultured. Spleenocytes were resuspended in trypomastigote culture medium and incubated for 1 hr at 37°C to permit monocyte adherence to occur. After incubation, the floating and adherent cells were separated, then the adherent cells were harvested and resuspended at a density of 10^6 cells/ml in IMDM for trypomastigotes. To confirm that >90% of the cells were adherent, we checked for phagocytosis of carbon powder while culturing in 5% CO₂ at 37°C for 1 hr.

Preparation of Kupffer cells

Rat Kupffer cells were collected using the collagenase method (Sugano et al., 1988). Rat liver was perfused with Ringer’s solution containing 5% collagenase (Sigma-Aldrich Inc., St. Louis, Missouri) at 37°C for 15 min. After perfusion, liver cells were suspended in HBSS and centrifuged at 50 g at 4°C for 1 min. The pellet was removed, and the remaining cells were resuspended and centrifuged at 800 g at 4°C for 10 min. After centrifugation, the pellet was resuspended in 6 ml of 17% Ficoll (Sigma-Aldrich Inc.) containing PBS, then 2 ml of HBSS was added. The mixture was centrifuged at 1,300 g at 4°C for 30 min. After centrifugation, the middle zone was harvested and washed with PBS 3 times.

Kupffer-rich cells were suspended in HBSS containing 5% FBS and incubated in 5% CO₂ at 37°C on tissue culture plates. After 2 hr, adherent cells (Kupffer cells) were harvested, their viability was measured using trypan blue, and their purity was measured by phagocytosis of carbon powder. Cells of >95% viability and >90% purification were used for experiments.

Preparation of HS-P cells

HS-P cells, a rat macrophagelike cell line, and similar to macrophages with respect to features such as cytokine production, etc. (Yamate et al., 2001), were cultured in modified IMDM containing 5% FBS. WS was resuspended at 2.0 × 10^6 cells/ml in the same medium. Equal amounts of suspensions were mixed and cultured in 5% CO₂ at 37°C for 24 hr. Twenty-four hours later, LPS (final concentration: 10 μg/ml) or rat recombinant IFN-γ (final concentration: 20 U/ml) was added. Then, all cells were collected after culture for 8 hr for determination of mRNA concentrations. In addition, 24 hr after addition of the reagents, the culture supernatant was collected and the concentrations of cytokines and other factors were measured. In another experiment, anti-M-CSF (final concentration: 2 μg/ml) was added at the start of the coculture, and 24 hr later all cells and supernatants were collected.

Twenty-four hours after addition of reagents, cell viability was measured by trypan blue dyeing in each experiment. Splenocytes, spleen macrophages, and Kupffer cells collected from a rat and HS-P cells were used for duplication of the coculture experiment.

Inhibition of M-CSF and GM-CSF mRNA by RNA interference

A small interfering RNA (siRNA) for M-CSF and GM-CSF mRNA was prepared using the siRNA cocktail kit (Takara Bio Inc., Shiga, Japan). To amplify double-stranded (ds) DNA, including the partial arrangement of the M-CSF or GM-CSF mRNA with the T7 promoter...
arrangement at both ends, RT-PCR was performed using SuperScript 1-step RT-PCR with platinum Taq (Invitrogen Corp.) and spleen macrophage RNA.

We designed the following pairs of primers: 5'-GGCTAATACGAC TCATTAGGAGAATCCAGCACAGCAATGGAA-3' and 5'-GGCT ATACGACTCTAGTAGGAGACCCGTCTTCTAGGAGTA-3' for M-CSF mRNA, which spanned the region from position 336 to 1,282 and 5'-GGCTAATACGACTCTAGTAGGAGACCCGTCTTCTAGGAGTA-3' for GM-CSF mRNA, which spanned the region from position 96 to 591. The following PCR program was used: 40 cycles of 94°C for 30 sec, 63°C for 45 sec, and 72°C for 90 sec. dsDNA was transcribed into dsRNA and refined using the Gen Elute mammalian total RNA kit (Sigma-Aldrich, Inc.). The dsRNA was treated with ColdShock-Dicer and 5' -GCGTAATACGACTCACTATAGGGAGAAAGCAC-CAGAG GCAAAAG-3' and 5'-GGCTAATACGACTCTACTATAA-GGAGAGGT GGTGACTCTATTTT-3' for GM-CSF mRNA, which spanned the region from position 96 to 591. The following PCR program was used: 40 cycles of 94°C for 30 sec, 63°C for 45 sec, and 72°C for 90 sec. dsDNA was transcribed into dsRNA and refined using the Gen Elute mammalian total RNA kit (Sigma-Aldrich, Inc.). The dsRNA was treated with ColdShock-Dicer and 100 μM siRNA for M-CSF, and GM-CSF mRNA was prepared. The TransIT-TKO transfection reagent (3 μl/ml, Mirus Bio Co., Madison, Wisconsin) and the siRNA (final concentration: 1.0 X 10⁵ cells/ml) were added to a 1.0 X 10⁶ cells/ml suspension of HP-1 cells in modified IMDM containing 5% FBS; cells were cultured in 5% CO₂ at 37°C. After 24 hr, WS (final concentration: 1.0 X 10⁴ cells/ml) was added and co-cultured with the HP-1 cells.

In addition, HP-1 cells were preincubated with WS or LPS for 24 hr, after which siRNA for M-CSF and GM-CSF was added and cultured for 48 hr.

**Determination of concentrations of cytokines and other factors**

Concentrations of IL-12p40, IL-10, TNF-α, and IFN-γ in serum and culture media were determined by using an ELISA kit (Biosource International, Camarillo, California). Nuclei of HP-1 and Kupffer cells were harvested using a Nuclear Extraction Kit (Marligen Biosciences Inc., Ijamsville, Maryland). Glyceraldehyde phosphate dehydrogenase (GAPDH) activity in extracted nuclei was measured to confirm contamination of the cytoplasm using a GAPDH assay kit (Applied Biosystems). The following program was used: 50°C for 3 min, 95°C for 5 min, 50 cycles of 95°C for 15 sec, 60°C for 1 sec, then 40°C for 1 min. After PCR, a dissociation curve was constructed to confirm a uniform product. Quantification mRNA was used with the comparative delta CT method. GAPDH mRNA was used as an expression control, and for each mRNA a relative ratio to GAPDH mRNA was calculated. Each RNA sample was measured twice; cytokines and GAPDH were measured with a same sample.

**Statistical analysis**

Student's t-test was used to determine significant differences.

**RESULTS**

Changes in the concentration of cytokine in the serum and cytokine mRNA in the spleens of WS-infected rats are shown in Figure 1. Concentrations of IFN-γ and TNF-α increased beginning 1 day PI, and concentrations of IL-10 increased beginning the second day PI (Fig. 1A). However, concentrations of IL-12p40 did not begin to increase until day 4 PI (Fig. 1B).}

When splenocytes harvested from uninfected rats were co-cultured with WS, the levels of IFN-γ, TNF-α, and IL-10 mRNA increased within 48 hr, but the level of IL-12p40 mRNA did not change (Fig. 2A). After 48 hr, changes in the concentration of each cytokine were parallel to the changes in mRNA level (data not shown). Because IL-12 is produced mainly by macrophages, levels of IL-12p40 mRNA (Fig. 2B) and IL-12p40 concentration (Fig. 2C) in the spleen macrophages, Kupffer cells, and HP-1 cells cultured with WS were measured. The addition of LPS to each cell type increased the level of IL-12p40 mRNA and the concentration of IL-12p40 in the culture media, but promotion of IL-12 production by LPS was reduced in cells cultured with WS. Coculture with WS and addition of LPS did not influence cell viability (data not shown).
Because IFN-γ promotes IL-12 production in macrophages, the influence of WS on the action of IFN-γ was examined using HS-P cells (Fig. 3). The addition of IFN-γ led to an increase in the level of IL-12p40 mRNA in HS-P cells, and the stimulating effect of IFN-γ weakened when WS was cultured with HS-P cells.

Transport of NF-κB, a transcription factor to the nucleus, is necessary for increasing IL-12 mRNA expression in macrophages (Xiong et al., 2004; Yue et al., 2005). When HS-P cells were cultured with WS, the quantity of NF-κB in the nucleus increased and accured at the same level as when LPS was added to HS-P cells (data not shown).

In addition to having a trypanocidal effect (Sternberg and Mabbot 1996; Villalta et al., 1998; Duleu et al., 2004), NO appears to control IL-12 production (Xiong et al., 2004). Figure 4A shows the NO₂ concentration in culture media of HS-P cells cultured with WS. NO production was promoted not only by stimulation with LPS, but also in HS-P cells cultured with WS; NO production was inhibited by the addition of nitro-L-arginine methyl ester (l-NAME), an NOS inhibitor (Fig. 4A). However, there was no difference in IL-12p40 mRNA expression with and without l-NAME (Fig. 4B). These findings suggest that the NO produced by coculture with WS did not influence IL-12 production. When Kupffer cells and spleen macrophages were used, similar results were obtained (data not shown).

Increases in M-CSF have been observed to decrease IL-12 production (Ji et al., 2004). Figure 5 shows the results of immunohistochemical staining with ED1 antibody, indicating the distribution of macrophages in the spleen and liver of a WS-infected rat. In WS-infected rats on day 4 PI, the spleen and liver macrophages increased in comparison with uninfected rats. The macrophages increased to 3.0 ± 0.7 times (mean ± SE) in spleens and 3.4 ± 0.1 times in livers by WS infection. Since the levels of M-CSF and GM-CSF mRNA in the spleens and the livers of infected rats were high in comparison with uninfected rats, an increase in macrophage multiplication is indicated (Fig. 6A). The level of M-CSF mRNA expression increased in HS-P cells cocultured with WS (Fig. 6B). Therefore, to suppress the effect of M-CSF and GM-CSF mRNA, siRNA for both was made and transfected into HS-P cells. Thereafter, the effect of this treatment on IL-12p40 mRNA expression in HS-P cells cocultured with WS was examined (Fig. 7). HS-P...
cells pretreated with the siRNA did not increase the level of M-CSF mRNA when LPS or WS was added (Fig. 7A). The level of IL-12p40 mRNA in HS-P cells cocultured with WS increased after treatment with the siRNA (Fig. 7B), suggesting that WS controlled IL-12p40 mRNA expression through an increase in M-CSF production. Addition of siRNA for HS-P cells cultured with WS or LPS for 24 hr decreased the levels of M-CSF mRNA at 24 hr after siRNA addition (data not shown). When only siRNA for M-CSF mRNA was transfected, a similar result was obtained (data not shown).

Figure 8 shows the changes in the level of IL-12p40 mRNA when M-CSF antibody was added to HS-P cells cocultured with WS; the M-CSF was neutralized. The M-CSF antibody did not influence M-CSF mRNA expression in HS-P cells cocultured with WS (Fig. 8A), suggesting that M-CSF production was not influenced. M-CSF antibody did not influence levels of IL-12 mRNA alone, whereas in HS-P cells cocultured with WS, addition of antibody increased the level of IL-12p40 mRNA (Fig. 8B). When Kupffer cells and spleen macrophages were used, similar results were obtained (data not shown).

In the present study, we observed that, characteristically, IL-12 production does not increase in rats during the early period of WS infection. Although the blood concentration of IFN-γ increased beginning 1 day PI, and normally promotes IL-12 production, the latter did not occur. Furthermore, in cocultures of macrophages and WS, TNF-α production increased. In rats infected with the *T. brucei brucei* IL-tat 1.4 strain, the proliferation of trypomastigotes was slower in comparison with WS (Nishimura et al., 2006), and inhibition of IL-12 production was not observed (data not shown). It is thought that a rapid increase of WS in the infected rat was related to the restraint of IL-12 production.

Inhibition of cell-mediated immunity, which is an effective infection defense mechanism for trypomastigotes, promotes an increase in parasite numbers in infected hosts (Ayub et al., 1993; Baetselier et al., 2001). It is thought that the inhibition of IL-12 production leads to the inhibition of cell-mediated immunity in the early period of infection, but details of how suppression of IL-12 production relates to an increase in WS are not clear.

Because IL-12 stimulates IFN-γ production, it is commonly considered that the restraint of IL-12 output by WS infection cannot promote IFN-γ production. However, since IFN-γ production is stimulated in WS-infected rats, TLTF released by WS promoted IFN-γ output (Bakhiet et al., 1991, 1996; Hamadien et al., 1999). Because IL-12 production induced by IFN-γ is inhibited by WS in cocultures with macrophages, it seems that WS inhibits the effect of IFN-γ at the same time it is promoting IFN-γ production in WS-infected rats.

Trypanosomes reportedly release factors other than TLTF that act on macrophages (Baetselier et al., 2001; Nishimura et al., 2001). In cocultures of WS and splenocytes, even if spleen cells are separated from WS using a filter with a diameter of 0.2 μm, WS inhibits IL-12 production (data not shown). There-
fore, we conclude that a water-soluble factor inhibits IL-12 output, but the details are unclear.

Liu et al. (1999) reported that IL-12 mRNA is up-regulated in response to T. brucei infection in rats. Furthermore, purified T. brucei components induce IL-12p40 mRNA in macrophages (Magez et al., 1998; Kaushik et al. 2000; Paulnock and Coller, 2001). Increase of WS in infected rats is very rapid, the symptoms are acute, and the rat dies in about 1 wk. Therefore, it is thought that the difference with these reports depends on a characteristic of WS.

Expression of IL-12 mRNA reportedly increases through transport of NF-κB into the nucleus (Cameron et al., 2004; Xiong et al., 2004; Yue et al., 2005); we have confirmed that the addition of LPS promoted transport of NF-κB into the nucleus in HS-P cells. However, transport of NF-κB was not inhibited in cocultures with WS; in fact, it was promoted to the same extent as by LPS. This finding suggests that transport into the nucleus of NF-κB does not influence inhibition of IL-12 production by WS. However, TNF-α mRNA expression depends on NF-κB (Clarke et al., 1998), and, in cocultures of WS and macrophages, TNF-α mRNA expression increased; therefore, it seems that WS affects a stage of transcription of the IL-12 mRNA via another transcription factor. Trypomastigotes have also been shown to affect the toll-like receptor (TLR) (Drennan et al., 2005), but the details of the mechanism are not clear; the interaction with the signal cascade through TLR-4 (the receptor of LPS) and the IFN-γ receptor also remains unclear.

NO and M-CSF are both factors that are considered to control IL-12 production (Ji et al., 2004; Xiong et al., 2004), but the detailed mechanism of their action is not clear. There have been some reports that NO production is promoted by trypanosome infection (Sternberg and Mabbot, 1996; Villalta et al., 1998; Drennan et al., 2005). We confirmed that there were increases in NO in the spleen cells of WS-infected rats and in cocultures of Kupffer cells or spleen macrophages with WS. When LPS was added to macrophages, the NOS inhibitor caused expression of IL-12 mRNA to increase slightly; this result agrees with that of Xiong et al. (2004). However, even if an NOS inhibitor was added to cocultures of WS and macrophages, IL-12 production was still inhibited, suggesting that NO does not participate in inhibition of IL-12 production by WS.

**Figure 6.** Effect of WS on the expression of M-CSF and GM-CSF mRNA. M-CSF and GM-CSF mRNA levels in the spleens and livers of WS-infected rats (A). Spleens and livers were harvested from WS-infected rats 4 days PI, and the level of mRNA was determined. Each value and bar represents the mean ± SE of results from 4 rats. Asterisks indicate significant differences from values for uninfected rats (P < 0.05). M-CSF and GM-CSF mRNA levels in HS-P cells cocultured with WS (B). WS or LPS was added to HS-P cells, and all cells were collected after culture for 8 hr. None corresponds to cultures with no WS and no LPS. Each value and bar represents the mean ± SD of results from 4 replicates. Asterisks indicate significant differences from values for no reagents (P < 0.05).

**Figure 7.** Effect of siRNA on expression of IL-12 mRNA in HS-P cells. HS-P cells were cultured with or without siRNA and a transfection reagent for 24 hr. After 24 hr, WS was added, and the cells were cocultured for 24 hr. Forty-eight hours after the start of the experiment, LPS was added, and all cells were collected after culture for a further 8 hr. Levels of M-CSF mRNA (A) and IL-12 mRNA (B) were determined at this time. None corresponds to cultures without siRNA, WS, and LPS. Each value and bar represents the mean ± SE of results from 4 replicates. Asterisks indicate significant differences from values for no reagents (P < 0.05). Crosses indicate significant differences between with and without the siRNA (P < 0.05).
Expression of M-CSF and GM-CSF mRNA increased in WS-infected rats, suggesting that increases in M-CSF and GM-CSF production influenced the increase in the number of macrophages in the spleens and livers of WS-infected rats. It has been shown that M-CSF suppresses IL-12 production (Ji et al., 2004), whereas the influence on IL-12 production of GM-CSF is not clear. Expression of M-CSF mRNA markedly increased in cocultures of WS-infected rats, suggesting that increases in M-CSF and GM-CSF levels of M-CSF mRNA (A) and IL-12 mRNA (B) were measured. None corresponds to cultures with no WS and no antibody. Each value and bar represents the mean ± SE of results from 4 replicates. Asterisks indicate significant differences from values for no reagents (P < 0.05). Crosses indicate significant differences between with and without antibody (P < 0.05).

**FIGURE 8.** Effect of Anti–M-CSF antibody on expression of IL-12 mRNA in HS-P cells. HS-P cells and WS were cultured with or without anti M-CSF antibody for 24 hr. After 24 hr, all cells were collected and levels of M-CSF mRNA (A) and IL-12 mRNA (B) were measured. None corresponds to cultures with no WS and no antibody. Each value and bar represents the mean ± SE of results from 4 replicates. Asterisks indicate significant differences from values for no reagents (P < 0.05). Crosses indicate significant differences between with and without antibody (P < 0.05).

It is unclear whether WS produces a factor promoting the production of M-CSF; the possibility that WS promotes the increase in macrophages directly cannot be ruled out. In addition, WS promotes M-CSF production by macrophages, and transport of NF-kB into the nucleus, but it is not clear whether these actions depend on the same factor. Elucidation of the mechanism by which IL-12 production is suppressed may lead to the development of new methods for regulating immune responses.

**LITERATURE CITED**


KAUSHIK, R. S., J. E. UZONNA, Y. ZHANG, J. R. GORDON, AND H. TABEL. 2000. Inmate parasite resistance to experimental African trypanosomiasis: Differences in cytokine (TNF-alpha, IL-6, IL-10 and IL-12) production by bone marrow-derived macrophages from resistant and susceptible mice. Cytokine 12: 1024–1034.


CYTOKINE AND NITRIC OXIDE PRODUCTION BY *TRYPANOSOMA BRUCEI* INFECTION IN RATS FED POLYAMINE-DEFICIENT CHOW

Kazuhiko Nishimura, Michiko Yagi, Yoshihiro Ohnishi, and Shinji Yamasaki

Laboratory of Infectious Diseases Control, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan. e-mail: nisimura@vet.osakafu-u.ac.jp

**ABSTRACT:** Feeding polyamine-deficient chow (PDC) to rats decreases blood polyamines, increases the activity of ornithine decarboxylase as an index of polyamine production, and increases resistance to *Trypanosoma brucei gambiense* (Wellcome strain) (WS) infection. In this study, we investigated the influence on cytokine and nitric oxide (NO) production of feeding PDC to rats infected with WS. At 4 days postinfection with WS, serum concentration of interleukin (IL)-12, tumor necrosis factor-α, interferon-γ IL-10, and NO increased in PDC-fed rats; however, IL-12 concentration in normal chow (NC)-fed rats did not increase. In spleen cells cocultured with WS, levels of IL-12 and inducible NO synthase (NOS) mRNA expression were higher in PDC-fed rats than in NC-fed rats. Proliferation of WS in coculture with spleen cells from PDC-fed rats was inhibited, but inhibition of WS proliferation was not observed when an NOS inhibitor was added into the culture media. Ornithine decarboxylase (ODC) activity increased in NC-fed rats after WS infection, but decreased in PDC-fed rats. These results show that feeding WS-infected rats PDC influences the production of cytokines such as IL-12 and the regulation of NO and polyamine production, and also leads to an increase in resistance against WS.

Polymamines play an important role in DNA, RNA, and protein synthesis, and in the stabilization of cellular membranes (Igarashi and Kashiwagi, 2000). Polymamines are synthesized intracellularly, but polymamines present in food and synthesized by microflora in the intestinal tract can be absorbed via the intestinal tract. In fact, reabsorption of polymamines originating from an individual’s own intestinal cells is an important source of blood polymamines (Bardocz, 1993). *Trypanosoma brucei* is a parasite that resides in its host’s circulatory system; it depends on the polymamines putrescine and spermidine for proliferation (Bacchi et al., 1980), and synthesizes trypanothione (an antioxidant specific to trypanosomes) from spermidine. Trypanosomes also utilize extracellular polymamines via uptake from the bloodstream (Mutomba et al., 1999; Nishimura et al., 2006).

The amount of polymamines ingested in the diet can be reduced by feeding an animal polyamine-deficient chow (PDC). In a previous study, we reported that proliferation of *Trypanosoma brucei gambiense* (Wellcome strain) (WS) in PDC-fed rats was lower than that in rats fed normal chow (NC) (Nishimura et al., 2001). This demonstrates that limiting the polyamine content in the host’s blood can inhibit trypanosome proliferation.

The immunoresponse of the host plays an important role in inhibition of trypanosome proliferation (Hasko et al., 2000; Kaushik et al., 2000). For infection defense against trypanosomes, cytokines such as interleukin (IL)-12, IL-10, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) are important (Hasko et al., 2000; Kaushik et al., 2000). Nitric oxide (NO) produced by immunocytes reportedly has a trypanocidal effect in vitro (Gobert et al., 2000; Duleu et al., 2004). Recently, several studies have demonstrated that polymamines influence the regulation of cytokine production (Hasko et al., 2000; Moreira et al., 2004), and it has become clear that polymamines participate in the immunoresponse. Therefore, changes in internal polyamine metabolism due to feeding with PDC influence cytokine and NO production, and proliferation of trypanosomes may also be affected. Therefore, in the present study, we investigated whether feeding WS-infected rats PDC influences cytokine and NO production, and whether feeding rats PDC inhibits WS proliferation.

**MATERIALS AND METHODS**

**Materials**

PDC was purchased from the Oriental Yeast Co., Ltd. (Osaka, Japan). The preparation and composition of PDC have been described previously (Nishimura et al., 2001). Commercially available solid rat chow (Oriental Yeast Co., Ltd.) was used as the NC. The PDC contained 14 nmol/g putrescine, 14 nmol/g spermidine, 3.2 nmol/g ornithine, and 7.2 mg/g arginine, whereas standard chow contained 115 nmol/g putrescine, 84 nmol/g spermidine, 17 nmol/g spermine, 2.4 mg/g ornithine, and 8.6 mg/g arginine. Iscove’s modified Dulbecco’s medium (IMDM) was purchased from Invitrogen Corp. (Carlsbad, California). Primers for real-time reverse transcription–polymerase chain reaction (RT-PCR) were obtained from GeneDesign Co. (Osaka, Japan). To amplify rat mRNA, we designed the following pairs of primers: 5’-TTTGGGATTCGCC ACTGCA-3’ and 5’-GCTTTTTGCGCTTCAAA-3’ for ornithine decarboxylase (ODC) mRNA, spanning positions 614 to 718 (Hill and Pegg, 2003); 5’-CCTGCTCAAGCACCTGCTATG-3’ and 5’-ACTGGG AAGTGGGTGACGT-3’ for IL-10 mRNA, spanning positions 5 to 105 (Goodman et al., 1992); 5’-AGACCTGCCCATTTAGACCTG-3’ and 5’-TGGTTCGGTTGTGTGATGTG-3’ for IL-12 mRNA, spanning positions 621 to 721 (Gulk et al., 1998); 5’-TGTCATCAAGATCG ACCTGA-3’ and 5’-TCTTGGTTGGTGTCACTCCTG-3’ for IFN-γ mRNA, spanning positions 288 to 388 (Visse et al., 1999); 5’-CGAG ATGTGGAACTGACAGCA-3’ and 5’-ACGAGCGGGAATGAGAAG AG-3’ for NOS mRNA, spanning positions 22 to 122 (Estler et al., 1992); and 5’-GACACAGTGGCIGTGGTGGTGA-3’ and 5’-AGGCT ACAAGACCCAAGCCT-3’ for inducible nitric oxide synthase (iNOS) mRNA, spanning positions 25 to 125 (Sastiole et al., 2000). The primers 5’-GACACCAAACCTGTTAGCC-3’ and 5’-CTGAGGT GCAGTGATGGCCT-3’ were selected for amplification of rat glycoldehyde phosphate dehydrogenase (GAPDH) mRNA (positions 342 to 442 based on the report of Tajima et al. [1999]). Recombinant rat IL-12 was obtained from R&D Systems, Inc. (Minneapolis, Minnesota). Lipopolysaccharide (LPS), as well as standard laboratory chemicals and reagents, were purchased from Wako Pure Chemical Co. (Osaka, Japan).

**Preparation of trypanosomes**

Trypanosomes were prepared as described previously (Nishimura et al., 2001, 2004). Trypanosomes stored in liquid nitrogen were thawed and subcultured in vitro using the method of Yabu et al. (1998); the culture medium contained 100 μM hypoxanthine, 30 μM thymidine, 40 μM adenosine, 1 mM sodium pyruvate, 50 μM L-glutamine, 100 μM 2-mercaptoethanol, 200 μM L-alanine, 100 μM glycine, 20 μM L-ornithine-HCl, 10 μM L-citrulline, and 5% fetal bovine serum. Trypanosomes were cultured in modified IMDM in 95% O2 and 5% CO2 at 37°C. When trypanosomes reached a concentration of 107 cells/ml, 25 μl of the trypanosome suspension was subcultured to 500 μl in new medium. The number of trypanosomes was determined every 24 hr using light microscopy.

Received 21 March 2007; revised 16 June 2007; accepted 9 July 2007.
Animals

Experiments were carried out using 8-wk-old male Wistar rats (Nippon SLC Co., Shizuoka, Japan). Each experimental group comprised 4 rats, which were supplied with food and water ad lib. Rats were maintained at 25 C under a 12-hr light cycle starting at 0600 hr. Rats were fed PDC beginning 2 wk before injection of WS. After injection by subcutaneous inoculation of 10^6 trypanosomes in phosphate-buffered saline (PBS) per rat, blood was collected from the tail vein at 0800 hr every day. Using these blood samples, the number of trypanosomes was counted using light microscopy. In control experiments, rats were inoculated with the same volume of PBS. Blood samples were also collected from the abdominal aorta using a heparinized syringe at 4 days postinfection (PI), after which the rats were killed. Four days PI is the first day when the WS can be confirmed in blood of both PDC- and NC-fed rats with a microscope. These blood samples were immediately cooled to 4 C and heparinized plasma was prepared by centrifugation at 900 g for 10 min. Samples of heparinized plasma were also stored at ~80 C for determination of polyamine and cytokine concentrations.

Spleen cell preparation

Spleen cells were prepared as described previously (Nishimura et al., 2004). Spleens were removed aseptically from infected or uninfected rats after they were killed. Spleens were dispersed into Hank’s solution (HBSS) using a stainless steel mesh (180-μm diameter). Erythrocytes and WS in the blood were hypotonically lysed using 10 ml lysis buffer (150 mm NaCl, 10 mm KHCO3, 0.5 mm EDTA, pH 7.3) for 10 min. Cell suspensions were centrifuged at 300 g for 5 min. Spleen cells were resuspended twice with HBSS, then resuspended to 2.0 × 10^6 cells/ml and used for RT-PCR, polyamine contents, or measurement of ODC activity, or they were cultured for the coculture experiment. Five milliliters of spleen cell suspensions were centrifuged at 300 g for 5 min and pellets were stored at ~80 C until RT-PCR was performed. Contamination with WS in spleen cell suspensions of 2.0 × 10^6 cells/ml was less than 7.0 × 10^3 cells/ml for NC-fed rats and less than 1.4 × 10^3 cells/ml in PDC-fed rats.

Coculture of spleen cells and WS

Spleen cells from uninfected rats were resuspended to 2.0 × 10^6 cells/ml in modified IMDM containing 5% fetal bovine serum (FBS). WS cells were resuspended to 2.0 × 10^6 cells/ml in the same media. The spleen cell and WS cell suspensions (0.5 ml of each) were mixed in 24-well microplates and cultured in 5% CO2 at 37 C for 24 hr. Twenty-four hours later, LPS (final concentration, 10 μg/ml) was added. After a further 8 hr, all cells were collected for determination of mRNA concentration. In addition, 24 hr after the LPS was added, culture supernatants and cells were separated by centrifugation, then supernatants were collected and the concentrations of cytokines and NO were measured. The cell pellets were measured for mRNA level and ODC activity. For measurement of ODC activity, the cell pellets were hypotonically lysed using 10 ml lysis buffer for 10 min. Cell suspensions were centrifuged at 300 g for 5 min. Spleen cells were washed twice with HBSS, then the cell pellets were collected. Contamination with WS in a spleen cell suspension of 1.0 × 10^6 cells/ml was less than 3.2 × 10^3 cells/ml for NC-fed rats and less than 1.1 × 10^4 cells/ml in PDC-fed rats. The numbers of trypanosomes in culture suspension were enumerated using light microscopy. Spleen cells collected from a rat were used for use in triplicate of the coculture experiment.

Determination of concentrations of cytokines and NO2

Concentrations of IL-12, IL-10, TNF-α, and IFN-γ in heparinized plasma and culture media were determined using an ELISA kit (Bio-source International, Camarillo, California). The concentration of NO2 in the culture media was determined spectrophotometrically at 540 nm after reaction with Griess reagent (Hageman and Reed, 1980). Heparinized plasma of each rat and culture media of each hole was measured twice.

Quantification of mRNA expression

RNA was extracted from pellets of spleen cells harvested from rats or each well of the microplate in coculture experiments using the Gen Elute Mammalian total RNA kit (Sigma-Aldrich, Inc., St. Louis, Missouri). Concentrations of extracted RNA were measured by absorbance (260 nm) for preparation of the PCR mixture. mRNA was determined by real-time RT-PCR using SuperScript Cyber Green One-Step RT-PCR with Platinum Taq (Invitrogen Corp., Carlsbad, California) and an ABI Prism 7000 (Applied Biosystems, Foster City, California). The PCR program was as follows: 50 C for 3 min, then 95 C for 5 min, 50 cycles of 95 C for 15 sec and 60 C for 30 sec, then 40 C for 1 min. After PCR, a dissociation curve was constructed to confirm that the product was unimeric. GAPDH mRNA was used as an expression control, and the level of each mRNA was calculated relative to GAPDH mRNA. Finally, mRNA expressions were compared with those in the untreated group for each experiment. Each RNA sample was measured twice, and mRNA of cytokines, iNOS, ODC, and GAPDH was measured with the same sample.

Quantification of polyamine

The high-pressure liquid chromatography (HPLC) method used for quantifying polyamine has been described previously (Nishimura et al., 2001). 0.3 ml of heparinized plasma and 0.3 ml of spleen cell suspensions were mixed with 3 volumes of ice-cold 1.66 N HClO4. Polyamine content was determined by separation of ion pairs formed with n-octanesulfonic acid on a reversed-phase column attached to a HPLC system (LC-6AD; Shimadzu, Kyoto, Japan). A precolumn (STR ODS-2; 10 mm × 4.6 mm inside diameter; Shimadzu) was included in the system and separation was carried out using a STR ODS-M column (150 mm × 4.6 mm inside diameter; Shimadzu). Elution was performed at 40 C using a gradient of acetonitrile in buffer solution (5 mM NaH2PO4·2H2O; 5 mM 1-octanesulfonic acid, sodium salt; 100 mM sodium perchlorate monohydrate; 3.5 mM phosphoric acid; pH 2.6) at a flow rate of 1 ml/min. Eluent from the column and the o-phenaldehyde-2-mercaptoethanol reagent (384 mM Na2CO3, 219 mM H3BO3, 108 mM NaNO3, 1.86 mM o-phenaldehyde, 0.056% [w/v] 2-mercaptoethanol) were mixed at a ratio of 1:0.03 and the mixture was read at 455 nm with excitation at 345 nm on a spectrophuorometer (RF-535; Shimadzu). Limits of detection were as follows: 0.1 nmol/ml for putrescine and 0.05 nmol/ml for both spermidine and spermine. Retention times were 12.2 min for putrescine, 22.9 min for spermidine, and 25.5 min for spermine. Each sample was measured twice.

Determination of ODC activity

ODC activity was measured using a method described previously (Nishimura et al., 2001). Pellets of spleen cells were dissolved in 20 μl distilled water. The disrupted samples and 20 μl of heparinized plasma were mixed with 70 μl of ODC buffer (2.5 mM dithiothreitol, 20 mM pyridoxal-5-phosphate, 20 mM NaH2PO4, and L-ornithine·HCl, pH 7.2) and 10 μl of 0.5 mM dichlorohexylamine, an inhibitor of spermidine synthetase, and incubated for 2 hr at 37 C. The reaction was terminated by the addition of 300 μl of 0.5 N HClO4, and solutions were centrifuged at 6,400 g for 5 min. The quantity of putrescine generated in the supernatant was measured by HPLC as an index of ODC activity. The protein content of the samples was measured using the Lowry method (Fountoulakis et al., 1992). Each sample was measured twice.

Statistical analysis

ANOVA was used to assess statistical significance. Student’s t-test was used to determine the level of significance for the NC rats, uninfected rats, and no treatment in coculture experiments.

RESULTS

The daily gain in body weight did not differ between PDC- and NC-fed rats, and rats in both groups remained healthy. However, after 2 wk of the PDC diet, the blood polyamine concentration of PDC-fed rats fell. The spermidine concentration of heparinized plasma spermidine concentration was 2.1 ± 0.2 (mean ± SD) nmol/ml in NC-fed rats, but 0.3 ± 0.05 nmol/ml plasma in PDC-fed rats, which conformed to the findings of a previous study (Nishimura et al., 2001). Figure 1 shows the cytokine mRNA expression levels in spleen cells and cytokine...
had higher levels of mRNA expression and higher blood concentrations of IL-12 and TNF-α, and IL-10 mRNA increased to the same level in spleen cells from both NC- and PDC-fed rats when cocultured with WS, whereas IL-12 mRNA expression levels increased only in PDC-fed rats. Addition of LPS to spleen cells from both NC- and PDC-fed rats induced an increase in the levels of all 4 cytokine mRNAs, but PDC-fed rats had a greater increase in IL-12 mRNA expression than NC-fed rats. Cytokine concentrations in culture media after 48 hr mirrored the mRNA expression data, i.e., IL-12 concentrations in the culture media of spleen cells cocultured with WS in the presence of LPS were higher for PDC-fed rats than for NC-fed rats (data not shown).

Figure 2 shows the cytokine mRNA expression levels when spleen cells of uninfected rats were cocultured with WS cells. Concentrations of TNF-α, IFN-γ, and IL-10 mRNA increased in the blood of WS-infected rats on the fourth day after infection. The cytokine mRNA expression levels of the spleen cells before infection were low for both PDC- and NC-fed rats (Fig. 1A), and the concentration of each cytokine in blood was less than the detection limit for both NC- and PDC-fed rats (data not shown). Four days after WS infection, mRNA expression levels and serum concentrations of TNF-α, IFN-γ, and IL-10, but not IL-12, had increased in NC-fed rats. In contrast, in PDC-fed rats, mRNA expression levels and blood concentrations of all 4 cytokines had increased. PDC-fed rats had higher levels of mRNA expression and higher blood concentrations of IL-12 and TNF-α than NC-fed rats, but NC-fed rats had higher levels of mRNA expression and higher blood concentrations of IFN-γ and IL-10. The number of WS cells present in the blood at 4 days PI was $4.9 \times 10^5 \pm 0.2 \times 10^4$ (mean ± SD) cells/ml in PDC-fed rats, but $7.6 \times 10^4 \pm 0.6 \times 10^4$ cells/ml blood in NC-fed rats. Thus, WS proliferation was inhibited in PDC-fed rats, with the number of WS cells in PDC-fed rats being less than one-fifteenth of the number in NC-fed rats.

Figure 2 shows the cytokine mRNA expression levels when spleen cells of uninfected rats were cocultured with WS cells. Concentrations of TNF-α, IFN-γ, and IL-10 mRNA increased to the same level in spleen cells from both NC- and PDC-fed rats when cocultured with WS, whereas IL-12 mRNA expression levels increased only in PDC-fed rats. Addition of LPS to spleen cells from both NC- and PDC-fed rats induced an increase in the levels of all 4 cytokine mRNAs, but PDC-fed rats had a greater increase in IL-12 mRNA expression than NC-fed rats. Cytokine concentrations in culture media after 48 hr mirrored the mRNA expression data, i.e., IL-12 concentrations in the culture media of spleen cells cocultured with WS in the presence of LPS were higher for PDC-fed rats than for NC-fed rats (data not shown).

Figure 3 shows the levels of NO2 in spleen cells cocultured with WS. When spleen cells were cocultured with WS, or LPS was added, the iNOS mRNA expression levels of spleen cells (Fig. 3A) and the NO2 concentration in culture media (Fig. 3B) were higher for PDC-fed than for NC-fed rats. NO reportedly has a trypanocidal effect (Gobert et al., 2000; Duleu et al., 2004). Therefore, changes in the number of WS cells cocultured with spleen cells were examined (Fig. 4). For the spleen cells of NC-fed rats cocultured with WS, the number of WS cells increased over time, and this increase was not influenced by addition of LPS. However, in cocultures of spleen cells from PDC-fed rats and WS, the increase in the number of WS cells was already inhibited at 8 hr, and the number of WS cells decreased further after addition of LPS. When nitro-L-arginine methyl ester (L-NAME) was added to the culture media, the number of WS cells in coculture with spleen cells from PDC-fed rats increased, and no inhibition of WS proliferation was observed (Fig. 5B). Spleen cells from infected PDC-fed rats had higher levels of iNOS mRNA than did those from infected NC-fed rats (Fig. 6), indicating that NO was produced in higher amounts in PDC-fed rats than in NC-fed rats.

Arginine is a substrate for both NO and polyamine synthesis and, therefore, NO synthesis competes, and interacts, with polyamine (Gobert et al., 2000; Satriano, 2004). In spleen cells from uninfected PDC-fed rats, mRNA expression levels of ODC (Fig. 7A) and levels of putrescine production (Fig. 7B) were high in comparison with those in NC-fed rats. The extent of ODC mRNA expression and putrescine production increased in spleen cells harvested from infected NC-fed rats, but decreased in spleen cells harvested infected from PDC-fed rats. There was no difference in the polyamine content of spleen cells from PDC-fed and NC-fed rats (data not shown), and uninfected PDC-fed rats clearly actively synthesized polyamines. Furthermore, levels of ODC mRNA expression (Fig. 7C) and putrescine production (Fig. 7D) in spleen cells cocultured with WS also decreased in PDC-fed rats, whereas concentrations in NC-fed rats increased. These results suggest that regulation of polyamine and NO synthesis differs between PDC- and NC-fed rats.
FIGURE 2. Effect of a PDC diet on cytokine production in spleen cells cocultured with WS. Expression of cytokine mRNA (A) and cytokine concentration in culture media (B) in spleen cells cocultured with WS were determined. Spleen cells harvested from uninfected rats were cultured with WS. Twenty-four hours later, LPS was added, and all cells were collected after culture for a further 8 hr to determine mRNA concentration and after a further 24 hr to determine cytokine concentration. None corresponds to cultures with no WS and no LPS. Each value and bar represents the mean ± SD of results from 4 replicates. Asterisks indicate significant differences from values for NC-fed rats (P < 0.05).

DISCUSSION

In the present study, we showed that feeding WS-infected rats PDC induces changes in cytokine production and promotion of NO synthesis. The number of WS cells present 4 days PI in PDC-fed rats was markedly lower than that present in NC-fed rats, possibly indicating that PDC-fed rats are resistant to WS. High levels of IL-12 production have been reported in trypanosome-resistant mice (Uzonna et al., 1998), and the results of the present study conformed to this finding. The observed high levels of IL-12 and TNF-α production in infected
PDC-fed rats suggest that the function of macrophages, the main sources of these cytokines, changes during feeding with PDC. IFN-γ and IL-10 production were lower in infected PDC-fed rats than in infected NC-fed rats, but in spleen cells cocultured with WS, there was no difference between cells from PDC- and NC-fed rats. The WS-dependent increase in IFN-γ production depends on the number of WS cells (Nishimura et al., 2004). Furthermore, the increase in IFN-γ production promotes IL-10 production. It seems likely that in the present study, the small number of WS caused low IFN-γ and IL-10 production in WS-infected PDC-fed rats. Trypanosomiasis is worse in NC-fed rats, which have a greater number of WS cells relative to PDC-fed rats, and TNF-α production increases with progress of the infection (Sternberg, 1998; Kaushik et al., 2000). There was no difference in the TNF-α production levels of spleen cells cocultured with WS between NC- and PDC-fed rats. However, WS-infected PDC-fed rats produced higher concentrations of TNF-α than did WS-infected NC-fed rats, although the number of WS cells in the blood of PDC-fed rats was low in comparison with the NC-fed rats. These findings indicate that TNF-α production is promoted to a greater degree in PDC-fed rats than in NC-fed rats. TNF-α reportedly has a trypanocidal effect (Magez et al., 1999), and it is possible that promotion of TNF-α production in PDC-fed rats infected with WS acted to inhibit the proliferation of WS. There are, however, no data to support the view that IL-12 inhibits trypanosome proliferation directly, and even when IL-12 (final concentration 10 ng/ml) was added to cultured WS, no inhibition of WS proliferation was observed (data not shown). Because IL-12 promotes the cell-mediated immunity that defends against trypanosomiasis, it is possible that IL-12 indirectly influences the inhibition of WS proliferation. However, it is not clear how decrease of polyamine supply to the host by the PDC feeding has influenced IL-12 and TNF-α production by WS infection.

When cocultured with WS, spleen cells harvested from PDC-fed rats had inhibited growth of WS and, relative to NC-fed rats (with the exception of IL-12), there were no differences in the concentrations of the studied cytokines in the culture supernatant. Because IL-12 did not inhibit WS proliferation directly, it seems possible that spleen cells from PDC-fed rats secreted other factors that inhibited the proliferation of WS. Furthermore, it is thought that the action of those factors increased with addition of LPS.

NO plays an important role in an organism’s immunoreponse, and is known to have a trypanocidal effect (Gobert et al., 2000). NO production of spleen cells cocultured with WS was higher in spleen cells from PDC-fed rats than in spleen cells from NC-fed rats. WS proliferation in cocultures with spleen cells of PDC-fed rats was restrained, however, when a NOS inhibitor (l-NAME) was added; there was no inhibition of WS proliferation in cocultures with spleen cells of PDC-fed rats.
rats. These data suggest that NO produced by spleen cells from PDC-fed rats influences the inhibition of WS proliferation. In WS-infected rats, the level of iNOS mRNA expression in the spleen was higher in PDC-fed rats than in NC-fed rats, indicating that NO production had increased. In PDC-fed rats, a decrease in blood polyamine concentration led to a decrease in trypanothione production by WS, which decreased tolerance of oxidative stress (Nishimura et al., 2004). Therefore, it is probable that oxidative stress via NO against WS was larger in PDC-fed rats than NC-fed rats. However, it has been reported that NO does not have a trypanocidal effect in the host mice (Sternberg et al., 1994). Because the action of NO as determined in various experiments varies, it seems that an increase in NO production influences WS proliferation indirectly.

NO synthesis competes with polyamine synthesis for arginine (a substrate in both synthetic pathways), so an increase in polyamine production inhibits NO production (Hillary and Pegg, 2003; Gobert et al., 2004; Moreira et al., 2004). However, in the spleen cells of NC-fed infected rats and the spleen cells of NC-fed rats cocultured with WS, WS induced an increase in expression of both NO and polyamine production. The supply of arginine is an important regulating factor for these pathways (Moreira et al., 2004), and an increase in the supply of NO and polyamines results in the activation of and increase in the number of immunocytes available for infection defense. However, although the spleen cells of PDC-fed rats had higher ODC activity than the spleen cells of NC-fed rats, NO production by spleen cells of PDC-fed rats when cocultured with WS was higher than that in the spleen cells of NC-fed rats, and ODC activity was lower. These results suggest that regulation of ODC activity was changed by feeding with PDC. It is possible that the ODC activity of PDC-fed rats is controlled by WS infection, and that NO production increased because polyamine production decreased. The ODC activity of WS-infected rats and their spleen cells cocultured with WS did not differ in PDC-fed and NC-fed rats. However, it is unclear whether there was a difference in arginine supply to spleen cells in PDC-fed rats and NC-fed rats; the mechanisms causing the decrease in ODC also remain unclear. Moreover, the details of the interaction between NO and polyamine production are not fully understood. It is well established that NO inhibits IL-12 production (Yao et al., 2001; Bodupalli et al., 2007). Nonetheless, in the present study, IL-12 production increased in PDC-fed rats in concurrence with an increase of NO production. The interaction between NO and IL-12 in PDC-fed rats thus also remains unclear.

Feeding with PDC not only induced a decrease in blood polyamine concentration, it also increased polyamine production by the host’s cells. The influence of a PDC diet on the production of cytokines (such as IL-12) and regulation of NO and polyamine production is thought to lead to increased resistance to
WS. However, it is unknown how the increase in polyaniline production by PDC feeding has influenced IL-12, TNF-α, and NO production by WS infection. It is necessary to examine details of function changing of the macrophages, and the influence of other cytokine in WS-infected PDC-fed rats.

**ACKNOWLEDGMENTS**

The authors thank Dr. Susan Bardocz for advice regarding polyamine-deficient diets.

**LITERATURE CITED**


ANTIBODIES IN COLD STRESSED MICE RECOGNIZE A SURFACE PROTEIN IN TOXOPLASMA GONDII TACHYZOITES

Eric G. Thompson, Hernan O. Aviles*, and Fernando P. Monroy†

Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona 87011. e-mail: fernando.monroy@nau.edu

ABSTRACT: Physical or psychological stressors are known to have significant consequences for immune function and the outcome of disease in human and animal models. In mice, cold water stress (CWS) has been shown to delay control of acute infection and reactivation of latent infections. Increased levels of parasite-specific IgG and IgM antibodies are observed when CWS is applied in the chronic phase. The present study examined the effects of a physical stressor, CWS, on tachyzoites antigens of Toxoplasma gondii, with particular emphasis on a low molecular weight antigen, 5 kDa, which seems to be recognized by antibodies from mice subjected to CWS in the chronic phase. This antigen is not recognized by antibodies from infected mice not subjected to CWS. Sera obtained from stressed and infected (CWS + INF) mice subjected to CWS during the chronic phase (CWS + INF + CWS) were used to harvest anti-5-kDa antibodies for immunolocalization studies. Tachyzoites lysate preparations were electrophoretically separated and transferred to nitrocellulose membranes. Strips of nitrocellulose containing tachyzoite antigens in the 4-10-kDa range were used to select for anti-5-kDa antibodies. Harvested anti-5-kDa localized this antigen on the surface of tachyzoites. This antigen was not present in bradyzoites. Treatment with phosphatidylinositol-specific phospholipase C showed this antigen was not anchored to the cell membrane through glycosyl-phosphatidylinositol (GPI) structures (Tomavo, 1996). Early antibody responses seem to be directed against membrane parasite antigens. Antigens of 30, 22, and 5 kDa are recognized by human sera from acute infections (Erlich et al., 1983; Sharma et al., 1983; Potasman et al., 1986), and the 5-kDa antigen has been proposed to be a good marker of acute toxoplasmosis (Sharma et al., 1983; Tomavo et al., 1994). The 5-kDa antigen was localized and GPI anchored to the parasite membrane (Tomavo et al., 1994). Although the 5-kDa antigen seems to be recognized by both IgG and IgM, we reported that it was only recognized by sera from infected mice subjected to CWS (Aviles and Monroy, 2001b). In the present study, we harvested anti-5-kDa antibodies to immunolocalize this antigen to the parasite membrane of tachyzoites; it was absent in bradyzoites, and it was not anchored via GPI to the cell membrane.

MATERIALS AND METHODS

Animals

Six- to 8-wk-old female BALB/c mice (Jackson Laboratory, Bar Harbor, Massachusetts) were housed in pathogen-free conditions under controlled conditions of temperature and light cycle with free access to food and water.

Cold water stress (CWS) paradigm and infection

CWS procedure, infection, and antigen preparation procedures were previously described (Ben-Nathan et al., 1990; Banerjee et al., 1999; Monroy et al., 1999). Briefly, mice were placed in shallow, clear glass chambers (5 min each day for 8 days) filled with 2 cm of cold water (1 ± 0.5 C), deep enough to cover their backs. Control animals were handled in the same way as the stress group, but they were not subjected to CWS. For infection, groups of mice were orally infected on the last day of stress (day 8) with 30 cysts of the low virulent ME49 T. gondii strain. Tachyzoites of the virulent T. gondii RH strain maintained in vitro by infection of human foreskin fibroblasts were used for T. gondii lysate antigen preparation (Monroy et al., 1999).

Experimental design

Mice were randomly distributed in 3 groups, i.e., control (CON, n = 5), T. gondii infected (INF, n = 15), and cold stressed and infected mice.
CWS + INF, n = 15). After 60 days postinfection (PI), animals in the CWS + INF group were subjected to a second CWS in the chronic phase and serum samples from all groups were obtained 1 day later. This collection time has been shown to contain anti-5-kDa antibodies in this group, and no differences were observed in anti-5-kDa concentrations when samples were collected days 1, 15, or 25 PI (Aviles and Monroy, 2001b).

Parasite antigen preparation

Tachyzoite lyse antigen (TLA) was prepared using tachyzoites of the virulent T. gondii RH strain maintained in vitro by infection of human foreskin fibroblasts. Parasites were collected and purified using a 3-μm polycarbonate membrane filter (Millipore, Bedford, Massachusetts) (Monroy et al., 1999; Aviles and Monroy, 2001a). In addition, purified tachyzoites were also used for transmission electron microscopy studies and phosphatidylinositol-specific phospholipase C treatment. Cysts and antigen preparation was performed by continuous density gradient centrifugation according to Cornellsen et al. (1981).

Electrophoretic separation of proteins

Toxoplasma gondii proteins in tachyzoites and bradyzoites were separated by discontinuous SDS-PAGE under denaturing conditions (Laemmli, 1970). Parasite homogenates (30 μg) obtained by sonication were dissolved in sample buffer containing denaturing agents (SDS and 2-mercaptoethanol). After being boiled for 3 min, parasite antigens were electrophoresed in a 15% SDS-PAGE gel using a modular mini-Protein II system (Bio-Rad, Richmond, California). Protein samples were run at 180 V for 50 min in 1X of Tris/Glycine/SDS buffer. In each run, 5 μl of prestained low range standards (14.4-97 kDa; Bio-Rad) were included in the same gel to estimate the molecular weights of the sample proteins.

Western blot

Electrophoresed proteins were transferred to nitrocellulose membrane in a Bio-Rad Protein II transblot apparatus (Bio-Rad) at 80 V for 1 hr and then at 100 V for additional 15 min in Tris/Glycine transfer buffer. The membrane was incubated in blocking buffer (PBS containing 2% BSA and 0.05% Tween 20) for 2 hr. One strip was cut from the membrane and incubated with a 1:50 dilution of CWS + INF + CWS sera in blocking buffer, and then with a 1:400 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (HRP). The 5-kDa band was visualized with HRP substrate (4-chloro-l-naphthol) and used as reference to cut horizontally an area in the membrane containing the 5 kDa. These strips were used to harvest anti-5-kDa antibodies from CWS + INF + CWS sera using 0.1 M glycine-HCl, pH 2.9 (Rybicki, 1986). This process was performed at room temperature, and 200 μl of pooled sera from each group was diluted to 4 ml in blocking buffer. After 15 min incubation with agitation, strips were washed in PBS before elution with 0.1 M glycine-HCl, pH 2.9. This process was repeated 5 times and collected antibodies were neutralized with 0.5 M Tris-HCl, pH 10.

Absorbance at 280 nm indicated that little or no antibody was present after the fourth incubation. A different nitrocellulose strip was used to elute antibodies from the infected mice not subjected to CWS. Absorbance at 280 nm indicated that few or no antibodies eluted from the nitrocellulose membrane. Pooled samples were concentrated in a Microsep 10 (Pall Life Sciences, Ann Arbor, Michigan), protein concentration was determined, and samples were stored at 0°C until used. This experiment was repeated 3 times, and we consistently collected between 0.5 and 0.7 μg/ml antibodies in the CWS + INF group.

Transmission electron microscopy

Purified tachyzoites were washed in PBS and fixed for 5 min in PBS containing 2% paraformaldehyde and 0.1% gluteraldehyde. After washing with PBS 2% fetal calf serum (FCS), parasites were incubated for 15 min in blocking buffer (PBS 10% FCS), followed by a 1:20 dilution of anti-5-kDa antibody for 60 min. One sample was first treated with phosphatidylinositol-specific phospholipase C (PI-PLC) for 20 min at room temperature. After washing, parasites were fixed in PBS containing 2% paraformaldehyde and 0.1% gluteraldehyde and then incubated in 1:15 dilution of 30 nm colloidal gold conjugated to goat anti-mouse IgG antibodies (EY Laboratories, San Mateo, California) in PBS 10% FCS. Parasites were also incubated with a 1:20 dilution of the concentrated fraction eluted from the infection sera without stress, and another group was incubated in PBS only as a substitute for the primary antibody. After washing, parasites were incubated with 2% osmium tetroxide and embedded in Formvar resin (EMS, Fort Washington, Pennsylvania) for thin sectioning. Seventy-nanometer sections were stained with uranyl acetate and observed with a JEOL 1200EX II electron microscope.

RESULTS

Effects of cold water stress (CWS) during the chronic phase of infection

Antigens in tachyzoites and bradyzoites were recognized by serum IgG during the chronic phase of T. gondii infection (Fig. 1). We collected sera during the chronic phase from infected mice (INF) or from mice subjected to CWS in the chronic phase, which had been previously stressed and infected (INF + CWS). This group is now described as CWS + INF + CWS. Antigen recognition pattern in tachyzoites was very similar to that reported by Aviles and Monroy (2001b), who used a similar protocol to identify stress-induced proteins. Antibodies in sera from INF mice recognized unique antigens of 70 and 43 kDa in tachyzoites and cross-reacted with an antigen of 21 kDa in bradyzoite preparations (Fig. 1A). Sera from stressed and infected mice undergoing stress in the chronic phase (CWS + INF + CWS) uniquely recognized a 5-kDa antigen that did not cross-react with bradyzoite antigens (Fig. 1A). Anti-5-kDa antibodies were collected by their reactivity to the 5 kDa, which had been separated by SDS-PAGE and electro-blotted to nitrocellulose membrane. These antibodies did not react against any other tachyzoite antigens (Fig. 1B).

Immunolocalization and phosphatidylinositol-specific phospholipase C (PI-PLC) sensitivity of the 5-kDa antigen

Purified anti-5-kDa antibodies were used to immunolocalize this antigen in tachyzoites collected from in vitro cell cultures. Figure 2 shows the reactivity of this antibody toward the parasite surface, suggesting its localization on the cell membrane. A 5-kDa molecule has been previously described in tachyzoites being anchored to the tachyzoite cell membrane through GPI structures (Tomavo et al., 1994). In the present study, tachyzoites were incubated with PI-PLC, which did not abolish the reactivity of anti-5-kDa antigen, suggesting this antigen is not GPI anchored to the parasite surface (Fig. 2). Figure 3 shows further evidence that anti-5-kDa antibodies are only present in sera from CWS mice. When strips of nitrocellulose containing tachyzoites antigen preparations in the <10-kDa area were used to harvest anti-5-kDa antibodies, only CWS + INF + CWS mice were able to produce anti-5-kDa antibodies. Anti-5-kDa antibodies were absent in sera from untreated but infected mice (Fig. 3).

DISCUSSION

Parasite antigens recognized by human sera soon after infection have been well characterized (Erlich et al., 1983; Sharma et al., 1983; Pitasman et al., 1986; Smith et al., 1996). Among these, a low molecular weight antigen that migrates at 5 kDa is recognized by IgM in sera from acute human infections, but it is absent in sera from individuals with chronic infections (Erlich et al., 1983; Sharma et al., 1983). The 5 kDa has been
proposed to be a good marker of acute infections in adults, but not congenital infections (Erlich et al., 1983; Sharma et al., 1983).

Aviles and Monroy (2001b) studied the effects of CWS on antibody responses during the acute and chronic phases of infection. In the acute phase, there was a stronger recognition of the commonly reported antigens in tachyzoites (Kasper, 1989; Darcy et al., 1990; Makioka et al., 1991), but antigens, specifically the 30 kDa, were more strongly recognized by serum samples obtained from the CWS + INF group than those obtained from INF mice. Delay in parasite replication by CWS has been previously reported (Banel'jee et al., 1999), and this delay could result in increased antibody titers observed in the CWS + INF mice (Aviles and Monroy, 2001b). Interestingly, the 5-kDa antigen was only recognized by IgM from the CWS + INF group after 1 wk of infection; however, during the chronic phase, this antigen was detected by both IgG and IgM antibodies from CWS + INF mice (Aviles and Monroy, 2001b).

Tomavo et al. (1994), using 2 monoclonal antibodies to this antigen, were able to localize it to the parasite surface, where it was anchored to the membrane through a GPI structure. Some discrepancies have been reported regarding the nature and location of this antigen. Others have reported the lack of a GPI anchor structure, presence of glycosylation, and sensitivity to sodium periodate treatment. It was also resistant to trypsin, proteinase K, and lipase digestion (Erlich et al., 1983; Sharma et al., 1983). Our results are in agreement with those of Erlich et al. (1983), since we were unable to remove this antigen from the parasite surface after enzyme treatment with PI-PLC. Molecular heterogeneity within this antigenic region has been described with up to 3 bands detected in SDS-PAGE after silver staining (Erlich et al., 1983; Sharma et al., 1983). Therefore, it is not unlikely that the data and antigenic characteristics described by the previous authors may relate to different antigenic molecules in this molecular weight range.

Still puzzling is the fact that mice previously stressed and infected with *T. gondii* (CWS + INF) when subjected once more to CWS in the chronic phase (CWS + INF + CWS) were able to produce antibodies that strongly recognize the 5-kDa antigen (Aviles and Monroy, 2001b). An explanation may be that CWS in the chronic phase induces reactivation of latent infection in which parasites are available to immune cells, but they are quickly controlled by a strong Th1 host response (Yap and Sher, 1999; Aviles and Monroy, 2001b). In addition, increased antibody production is observed in stressed and infected (CWS + INF) mice as soon as CWS is applied a second time in the chronic phase. Furthermore, epitope variability of the 5 kDa has been described (Tomavo et al., 1994) in which this antigen is lost in parasites or parasite preparations produced in vitro, while it is retained in parasites obtained from in vivo
infections. Similarly, recognition of our “stress protein” in sera from CWS + INF + CWS could provide “in vivo” parasites to the immune system due to the CWS-induced reactivation of latent infections. This would be very similar to the environment described by Tomavo et al. (1994), which led to epitope variability and enable antibodies in sera from CWS + INF + CWS mice to recognize the 5-kDa antigen.

Whatever the molecular weight or heterogeneity of this antigen, or antigens, in this molecular weight range, the 5-kDa antigen constitutes a unique immunogenic component of T. gondii, with significant diagnostic potential for identifying reactivation of latent infections. Because of its immunogenicity...
ACKNOWLEDGMENTS

We thank Marilee Sellers for valuable assistance with EM procedures. These studies were supported by a grant from NIAID-NIH, 5 R21 AI060401-02 to EM.

LITERATURE CITED


HEAT SHOCK RESPONSE OF BABESIA GIBSONI HEAT SHOCK PROTEIN 70

Masahiro Yamasaki, Mototsu Tajima †, Osamu Yamato †, Shiang-Jyi Hwang, Hiroshi Ohta, and Yoshimitsu Maede

Laboratory of Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan. e-mail: masayama@vetmed.hokudai.ac.jp

ABSTRACT: mRNA and protein expression profiles for heat shock protein 70 (Hsp70) of Babesia gibsoni (BgHsp70) exposed to either high or low temperatures were examined by quantitative real-time reverse transcription-polymerase chain reaction and Western blotting. In the present study, a commercially available anti-human Hsp70 antibody that could recognize recombinant BgHsp70 was used. BgHsp70 was detected in the parasites cultured under standard conditions at 37 °C by Western blotting and immunostaining of thin smears of infected erythrocytes. These results suggested that BgHsp70 was expressed constitutively at the erythrocyte stage. BgHsp70 levels were elevated when the parasites were incubated at 42 °C for 1 hr. In contrast, its mRNA amount was decreased and its protein amount was unchanged when the parasites were incubated at 32 °C for 1 hr. Moreover, the level of parasitemia of B. gibsoni incubated at either 42 °C or 32 °C was almost the same as that at 37 °C. These results indicated that the exposure of B. gibsoni to elevated temperatures might result in increased expression of BgHsp70 and that the exposure of the intracellular parasites to decreased temperatures might not induce the overexpression of BgHsp70.

Babesia gibsoni is a protozoan parasite that infects dogs and causes canine babesiosis. Previously (Yamasaki et al., 2002), we analyzed the sequence of the heat shock protein 70 (Hsp70) gene of B. gibsoni and compared it with the corresponding sequences from Babesia bovis, Babesia microti, Theileria annulata, and Theileria sergenti (renamed T. orientalis). The nucleotide sequences and the predicted amino acid sequences of the genes from those parasites were well conserved (Yamasaki et al., 2002). However, the role and function of Hsp70 from Babesia parasites remain largely unknown.

Hsp70, a 70-kDa Hsp, acts as a protein chaperone (Heike et al., 1996). In general, Hsp70 plays important roles in cell proliferation and the control of cellular functions (Lindquist, 1986). Moreover, the major physiological functions of Hsp70 are of greater importance for survival when cells are exposed to increased temperatures or a number of other noxious insults (Kauffman, 1990). It is considered that the Hsp70 of pathogens, such as schistosomes, malaria plasmodia, trypanosomes, and leishmanias, might play important roles in survival and proliferation within the host (Lindquist, 1986). These pathogens face sudden shifts in temperature during transmission from their respective insect vectors to their mammalian hosts. A temperature increase from 25 °C to 37 °C induces a significant heat shock response in certain Leishmania sp. promastigotes in vitro, resulting in stage differentiation (Van der Ploeg et al., 1985; Shapira et al., 1988). Moreover, it has been reported that the expression of Hsp70 in Babesia divergens (Carty et al., 1991) and Plasmodium falciparum (Biswas and Sharma, 1994) was enhanced at a higher temperature. In Babesia spp. parasites, however, the role and function of Hsp70 have not been well elucidated. In the present study, therefore, we investigated the change in gene transcription and protein synthesis for Hsp70 of B. gibsoni that is exposed to either high or low temperatures.

MATERIALS AND METHODS

Exposure of cultured B. gibsoni to either high or low temperature

The B. gibsoni used in the present study had been maintained in cultures for several years (Yamasaki et al., 2003). The parasites were usually incubated at 37 °C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ in a culture medium consisting of RPMI 1640 (Invitrogen, Carlsbad, California), 20% dog serum, and numbers of dog red blood cells (RBCs) sufficient to yield a packed cell volume of 5%. Every 7 days, an equal volume of fresh dog RBC suspension was added to the cultured RBC suspension.

For exposure to either high or low temperatures, the parasites cultured under normal conditions were divided into 3 groups, and they were incubated at 32 °C, 37 °C, and 42 °C, respectively, for 1 hr. A thin smear sample was made after the incubation, and percentage of parasitemia was calculated by counting the number of parasitized cells per 2,000 RBCs. Those incubated RBCs infected with B. gibsoni were used to prepare samples for Western blotting and quantitative real-time reverse transcription-polymerase chain reaction (real-time qRT-PCR).

Expression and purification of recombinant B. gibsoni Hsp70

The entire B. gibsoni heat shock protein 70 (BgHsp70) gene was amplified by using the PCR as described previously (Yamasaki et al., 2002). PCR was performed with the extracted genomic DNA as a template and specific oligonucleotide primers (BgHsp70F: 5'-ATGDCWGGSHCCMGTATYGGWATTGACTTGGG-3'; BgHsp70R, 5'-TTAGTCACATCCTCTACAGTGG-3'). The entire BgHsp70 gene was ligated into the bacterial expression vector pCR® T7/N-TOPO® (Invitrogen) and then expressed as a HisGI epitope fusion construct in E. coli (DE3)pLysS chemically competent Escherichia coli (Invitrogen) according to the protocol supplied (pCR T7 TOPO TA Expression kits, Invitrogen). During the expression procedure, the 0 hr (preinduction) and 3 hr (postinduction) time point samples of E. coli culture after the induction were collected and frozen at −20 °C for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The insoluble recombinant BgHsp70 (rBgHsp70) was purified by using nickel-nitrilo­triacetic acid (Ni-NTA) agarose (Ni-NTA Purification System, Invitrogen). The purified rBgHsp70 protein was used as a sample for SDS-PAGE and Western blotting.

Analysis of the expression of the recombinant protein in E. coli, and the synthesis of B. gibsoni Hsp70 by Western blotting

To prepare the parasite sample, the infected RBCs described above were lysed by the addition of 5 mM phosphate buffer, pH 7.4, and centrifuged at 15,000 g for 10 min at 4 °C. After removal of the erythrocyte ghosts and lysate, the pelleted parasites were resuspended in M-PER® Mammalian Protein Extraction Reagent (Pierce Chemical, Rockford, Illinois), and the protein concentration of the suspension was measured with a commercial kit (Bio-Rad Protein Assay, Bio-Rad, Hercules, California) using the method of Bradford (1976). The suspension was mixed with the sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 0.02% bromphenol blue, pH 6.8) to yield a final protein concentration of 18 μg/μL, and boiled at 95 °C for 5 min. Both pre- and postinduction samples of E. coli culture were resuspended in the sample buffer, and boiled at 95 °C for 5 min. The solution of purified rBgHsp70 was also boiled at 95 °C for 5 min in the sample buffer and used as the expression sample.

Aliquots of 5 μL of the parasite samples prepared, which included 90
μg of protein, pre- and postinduction samples, and expression samples, were subjected to electrophoresis in a 7.5% polyacrylamide gel according to the method of Laemmli (1970). After SDS-PAGE, the proteins were transferred electrophoretically onto a nitrocellulose membrane (Trans-Blot® Transfer medium, Bio-Rad) using the method of Towbin et al. (1979), or stained with Coomassie blue. For Western blotting, after being immersed in a 1% blocking solution (Roche Diagnostics GmbH, Mannheim, Germany), the membranes were incubated with diluted mouse anti-human Hsp70 antibody (SPA-810, Nventa Biopharmaceuticals, San Diego, California) (1:1,000) in a 1% block solution for 1 hr at room temperature and washed twice with Tris-buffered saline/Tween 20 (TBST) (10 mM Tris/HCl, 150 mM NaCl, and 0.05% Tween 20 [MP Biomedicals, Irvine, California], pH 7.5). The membrane was incubated with diluted alkaline phosphatase-conjugated goat anti-mouse IgG antibody (SA-B-101, Stressgen Biotechnologies) (1:500) in TBST for 30 min at room temperature and washed twice with TBST. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used for the color development. We calculated and compared the relative intensity of BgHsp70 protein in the parasite samples by using a GS-800 calibrated densitometer (Bio-Rad) and Quantity One Version 4.4.0. (Bio-Rad). This experiment was conducted 3 times.

**Immunostaining of B. gibsoni Hsp70 on thin smear**

Thin smears of B. gibsoni-infected RBCs and uninfected RBCs were made and fixed with 6% paraformaldehyde (Nakarai Chemicals Ltd., Kyoto, Japan) in 10 mM phosphate-buffered saline (PBS), pH 7.2, for 20 min. The smears were rinsed 3 times in PBS for 5 min, pretreated for 30 min in 1% Triton X-100 in PBS, washed 3 times, and blocked for 30 min in 3% bovine serum albumin (BSA) in PBS. After another 3 washes, the smears were incubated with diluted mouse anti-human Hsp70 monoclonal antibody (1:200) in 3% BSA for 1 hr, washed 3 times, incubated for 30 min with diluted alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:200) in 3% BSA, and again washed 3 times. NBT and BCIP were used for color development. Immunostaining without first and/or second antibodies was also conducted as negative controls.

**Analysis of gene transcription of B. gibsoni Hsp70 by qRT-PCR**

The infected RBCs described above were harvested and exposed to lysis them. The liberated parasites were pelleted for RNA extraction using an RNAeasy mini kit (QIAGEN, Valencia, California). The DNA contamination in extracted RNA was checked by PCR with extracted RNA as a template. cDNA was synthesized from the total RNA according to the method of Tajima et al. (1995). The quantity of BgHsp70 gene in a cDNA sample was measured by qRT-PCR. PCR was performed with the resulting cDNA as a template and specific oligonucleotide primers (BgHsp70-1, 5' ACGTACACACTGAG-3'; BgHsp70-2, 5' GTGCTTGGCTTCGACACAGC-3'). Reaction mixtures with 50 ng of cDNA as template were amplified with an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Tokyo, Japan). After an initial incubation for 10 sec at 95 C, the cDNA was subjected to 40 cycles of amplification. The program used was as follows: denaturation at 95 C for 5 sec and reannealing and extension at 60 C for 31 sec. Continuous observation of amplifying DNA was done with SYBR® Premix Ex Taq® (TAKARA BIO Inc., Otsu, Japan). To confirm the specificity of the amplification product, a melting curve analysis was performed. Also, to check for DNA contamination in the water, primers and enzymes for PCR, PCR with water as a template was performed. The Ct value, representing the PCR-cycle number at which the fluorescence emission passes a fixed threshold within the logarithmic phase of the PCR, was measured. The copy number of the BgHsp70 gene in each sample was calculated from a standard curve, obtained by plotting known input concentrations of plasmid ligated with the BgHsp70 gene at log dilutions against the PCR-cycle number at which the detected fluorescence emission reaches a fixed threshold. Values were given as raw copy numbers (per microliter of cDNA). The quantity of 18S rRNA was also measured by qRT-PCR with the same method. The primers used for the amplification of 18S RNA were Bg18S-1, 5' -TC GTATTTAACCTGTCAGAGG-3' and Bg18S-2, 5' -AAGGTTAACCTGAT CGCTCTTCG-3'. The copy number of 18S RNA was also calculated from a standard curve, obtained by plotting known input concentrations of plasmid ligated with 18S RNA. To correct for differences in the amount of RNA, the calculated copy numbers of the BgHsp70 gene were adjusted according to the copy numbers of B. gibsoni 18S rRNA. Thus, values were also given as relative amounts, i.e., the copy number of BgHsp70 mRNA adjusted to that of 18S rRNA. This experiment was conducted 3 times.

**Statistical analysis**

Data on the relative intensity of BgHsp70 protein, the relative amount or copy numbers of BgHsp70 genes, and copy numbers of 18S rRNA at 42 C or 32 C were compared with those at 37 C. Each data point was expressed as the mean ± SD (n = 3). The statistical analysis was performed using a Student's t-test. The difference between data was considered to be significant if P < 0.05.

**RESULTS**

**Western blot analysis of recombinant BgHsp70 protein with mouse anti-human Hsp70 antibody**

The BgHsp70 gene was inserted into the bacterial expression vector pCR® T7/NT-TOPO and then expressed as a HisG epitope fusion construct in E. coli. The recombinant BgHsp70 protein was produced for 3 hr (Fig. 1A) and purified with Ni-NTA agarose (Fig. 1B). Western blotting showed that the mouse anti-human Hsp70 antibody (SPA-810) recognized the purified rBgHsp70 protein (Fig. 1C). The molecular mass of the rBgHsp70 protein was estimated as approximately 70 kDa.

**Expression of BgHsp70 protein in the cultured parasites**

Western blotting of the parasites cultured under standard conditions showed that the protein, the molecular mass of which was estimated as approximately 70 kDa, could be detected with the mouse anti-human Hsp70 antibody (Fig. 2). Moreover, B.
**Change of BgHsp70 gene transcription with a temperature shift**

To examine the change in the transcription of the BgHsp70 gene in the cultured *B. gibsoni* after a shift in temperature, we performed qRT-PCR. Before using 18S rRNA as an internal control, the changes in the copy number of 18S rRNA were examined. The copy number of 18S rRNA at 42°C was almost the same as that at 37°C, although the number at 32°C was significantly (*P* < 0.05) lower than that at 37°C (Table I). This result suggested that 18S rRNA might be inadequate for use as an internal control for the analysis of gene transcription of *B. gibsoni*, especially at low temperature. We found that the copy number of the BgHsp70 gene was significantly (*P* < 0.05) increased when the temperature was shifted from 37°C to 42°C for 1 hr, although the amount of 18S rRNA was not changed (Table I). Therefore, the relative amount of the BgHsp70 gene was also significantly (*P* < 0.05) enhanced. In contrast, the copy number of the BgHsp70 gene decreased when the temperature was shifted from 37°C to 32°C for 1 hr (Table I). Because the amount of 18S rRNA was also decreased as described above, the relative amount of the BgHsp70 gene did not change. When temperatures were changed to either 32°C or 42°C, the level of parasitemia at either 32°C (4.2 ± 1.2%) or 42°C (4.5 ± 1.7%) was almost the same as that at 37°C (4.7 ± 1.4%). This result indicated that the exposure of *B. gibsoni* to elevated temperatures might result in increased transcription of the BgHsp70 gene and that the decreased temperature might lead to a decrease of RNAs in the parasites.

**DISCUSSION**

In the present study, mRNA and protein expression profiles for Hsp70 of *B. gibsoni* were examined by real-time qRT-PCR and Western blotting. Initially, the cross-reactivity of a commercially available anti-human Hsp70 antibody with BgHsp70 was estimated. The homology between the deduced amino acid sequence of BgHsp70 and the sequence of human MHC class III HSP70-1 (GenBank M59828) was 71.1%. The epitope region (amino acids 436–503) of human HSP70-1 in particular was highly (82.1%) homologous with the corresponding region of BgHsp70. From these results, we expected the anti-human Hsp70 antibody to cross-react with BgHsp70. Indeed, the antibody did recognize the recombinant BgHsp70 protein. Moreover, it detected a 70-kDa protein in the cultured parasites, sug-

### Table I. Transcription of BgHsp70 gene in Babesia gibsoni at 32°C, 37°C, or 42°C. The real-time qRT-PCR was performed with 50 ng of cDNA. The copy number of the BgHsp70 gene and 18S rRNA was calculated in the same RT-PCR run. The relative amount of the BgHsp70 gene was also calculated by adjusting the copy number of the gene to that of 18S rRNA. Data are expressed as the mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>Incubation temp.</th>
<th>32°C</th>
<th>37°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Copy no. (μl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BgHsp70</td>
<td>42,681.60 ± 11,477.6*</td>
<td>274,184.20 ± 109,056.3</td>
<td>907,575.80 ± 28,962.6*</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>10,997,961.63 ± 665,500.2*</td>
<td>66,133,618.60 ± 25,388,629.9</td>
<td>81,064,914.30 ± 19,690,081.0</td>
</tr>
<tr>
<td>Relative amount (BgHsp70/18S rRNA)</td>
<td>0.0039 ± 0.0008</td>
<td>0.0041 ± 0.0007</td>
<td>0.0116 ± 0.0027*</td>
</tr>
</tbody>
</table>

* Significantly (*P* < 0.05) different from the value for the incubation at 37°C.
Figure 3. Immunostaining of *Babesia gibsoni*-infected erythrocytes (A) and uninfected canine erythrocytes (B) with mouse anti-human Hsp70 antibody. Arrows show the *B. gibsoni* parasites harbored in erythrocytes. Arrowheads show the canine Hsp70 on the erythrocyte membrane.
Western blot analysis of BgHsp70 in Babesia gibsoni incubated at 32 C, 37 C, or 42 C. (A) Western blot analysis of BgHsp70 in B. gibsoni. The parasites were incubated at either 32 C (lane 1), 37 C (lane 2), or 42 C (lane 3). The arrowhead shows BgHsp70. Arrows indicate the smaller proteins (approximately 42 kDa, respectively). These bands might be products of the degradation of BgHsp70. (B) Relative intensity of BgHsp70 protein in B. gibsoni incubated at either 32 C, 37 C, or 42 C. The relative intensity of BgHsp70 was calculated by a GS-800 calibrated densitometer (Bio-Rad). Data are expressed as the mean ± SD (n = 3).

Hsp70 antibody used in the present study recognizes the canine Hsp70 protein. Indeed, the Hsp70 on the canine RBC membrane was stained with this antibody. Although this antibody could not distinguish BgHsp70 protein from canine Hsp70 protein, the stained protein in the cytoplasm of B. gibsoni might be BgHsp70. Moreover, it is reported that the membrane of mature canine erythrocytes included little Hsp70 protein (Jeong et al., 2005), suggesting the contamination by canine erythrocyte ghosts of the parasite sample for Western blotting to be negligible. Therefore, the present results revealed that BgHsp70 protein was expressed constitutively in the parasites at the erythrocytic stage under standard culture conditions. It is speculated that BgHsp70 plays a housekeeping role at the erythrocyte stage. However, this housekeeping role remains unclear.

Additionally, when the temperature was elevated from 37 C to 42 C, both the gene transcription and protein synthesis of BgHsp70 increased, suggesting that the expression of BgHsp70 was enhanced. At that time, the level of parasitemia at high temperature was almost the same as that under normal conditions, indicating that this enhanced expression of BgHsp70 was independent from the multiplication of the parasite. This result indicated that BgHsp70 is heat inducible and that its amount in the parasite is increased. Based on expression patterns, the hsp70 family can be divided into 3 groups: constitutively expressed (heat shock cognate protein or hsc70), heat-inducible (hsp70), and glucose-regulated (grp78) proteins (Jie et al., 1996). Heat-inducible forms of the hsp70 family have been reported to be intronless (Gunther and Walter, 1994). Functionally, intronless hsp70 genes may serve as more rapid responders to stress than intron-containing hsp70 genes because RNA splicing is unnecessary. We, too, had reported that the BgHsp70 gene has no introns (Yamasaki et al., 2002). These reports also indicate that BgHsp70 would be heat-inducible. In dogs with canine babesiosis caused by B. gibsoni, fever is a normal immune reaction. Therefore, BgHsp70 might show elevated levels of expression at high temperature within infected dogs having a fever. However, the relationship between the elevated expression of BgHsp70 and the survival of the parasites under high temperature is still unclear. Additionally, it is reported that a temperature increase from 25 C to 37 C induces a significant heat shock response in certain Leishmania sp. promastigotes in vitro, resulting in stage differentiation (Van der Ploeg et al., 1985; Shapira et al., 1988). Because in the present study we did not expose B. gibsoni to the same temperature shift from 25 C to 37 C, further efforts are necessary to clear the correlation between the expression of BgHsp70 and stage differentiation of B. gibsoni.

The amount of BgHsp70 protein, and the level of parasitemia did not change when the temperature was lowered from 37 C to 32 C. The amount of the BgHsp70 gene and 18S rRNA was decreased, although the cDNA content of each sample was equalized. These results indicated that a decrease in temperature of 5 C might induce a decrease in the levels of those RNAs in B. gibsoni and that it might not lead to either the degradation or the production of the BgHsp70 protein. Moreover, no morphological changes of the parasite were observed at 32 C (data not shown). This response of the parasite was likely to be different from the heat shock response, suggesting that a temperature decrease from 37 C to 32 C might not induce a heat shock
Quantitative real-time RT-PCR was used to analyze the gene transcription of *B. gibsoni* in this study. Because the cDNA content of each sample was equalized, the differences in the copy number of the BgHsp70 gene are expected to well represent the change in the transcription of the gene. Additionally, the amount of 18S rRNA has been used to adjust the expression of several genes measured by qRT-PCR in *P. falciparum* (Blair et al., 2002; Nirmalan et al., 2002; Yano et al., 2005). The method proved to be a very successful way to compare the relative temporal dynamics of transcriptional regulation for members of certain gene families (Blair et al., 2002). Quantitative real-time RT-PCR will greatly improve the speed and accuracy with which the transcription of individual genes can be characterized (Blair et al., 2002). Accordingly, we tried to use the amount of 18S rRNA as an internal control. In the present study, because the copy number of 18S rRNA at 42 C was almost the same as that at 37 C, it is considered that 18S rRNA could be used as an internal control in this instance. Therefore, the transcription of the BgHsp70 gene might be elevated by a temperature increase from 37 C to 42 C according to the copy number and the relative amount of the BgHsp70 gene. However, the copy number of 18S rRNA was decreased at 32 C. This suggested that 18S rRNA might not be suitable as an internal control for the comparison of the gene expression in *B. gibsoni*, especially after a temperature decrease. It is necessary to find a suitable internal control for comparison of the gene expression in the parasite. Moreover, Western blotting was used for the analysis of protein synthesis. Because there was no suitable internal control mechanism for adjusting the amount of the protein, we equalized the protein concentration of each sample. Therefore, the relative intensity of BgHsp70 might not be suitable for a comparison of the synthesis of the protein in a precise sense. However, the results of the Western blot analysis were well correlated with those of qRT-PCR. Recently, an actin gene from *B. gibsoni* has been reported (Zhou et al., 2006). Actin is thought to be useful as an internal control for the comparison of protein synthesis by *B. gibsoni*. Nevertheless, BgHsp70 showed elevated levels of expression at high temperature. Because the mRNA and protein expression profiles for BgHsp70 overlap to the same degree at high temperature, the gene expression is likely regulated at the transcriptional level under heat shock. Further detailed study might be necessary to elucidate the roles and functions of BgHsp70 in the survival and proliferation of *B. gibsoni* within its host.

**ACKNOWLEDGMENTS**

This work was supported in part by a grant from the Science Research Fund of the Ministry of Education, Cultures, Sports, Science and Technology of Japan.

**LITERATURE CITED**


NUMERICAL QUANTIFICATION OF PERKINSUS MARINUS IN THE AMERICAN OYSTER CRASSOSTREA VIRGINICA (GMELIN, 1791) (MOLLUSCA: BIVALVIA) BY MODERN STEREOMETRY

Antonio Remacha-Triviño, Doranne Borsay-Horowitz*, Christopher Dungant, Ximo Gual-Arnau†, Javier Gómez-Leon, Luisa Villamil, and Marta Gómez-Chiarri

Department of Fisheries, Animal and Veterinary Sciences, University of Rhode Island, 20A Woodward Hall, Kingston, Rhode Island 02881. e-mail: tonirem@gmail.com

ABSTRACT: Species of Perkinsus are responsible for high mortalities of bivalve molluscs worldwide. Techniques to accurately estimate parasites in tissues are required to improve understanding of Perkinsosida. This study quantifies the number and tissue distribution of Perkinsus marinus in Crassostrea virginica by modern stereology and immunohistochemistry. Mean total number of trophozoites were (mean ± SE) 11.80 ± 3.91 million and 11.55 ± 3.88 million for the optical dissector and optical fractionator methods, respectively. The mean empirical error between both stereological approaches was 3.8 ± 1.0%. Trophozoites were detected intracellularly in the following tissues: intestine (30.1%), Leydig tissue (21.3%), hemocytes (14.9%), digestive gland (11.4%), gills (6.1%), connective tissues (5.7%), gonads (4.1%), palps (2.2%), muscle (1.9%), mantle connective (0.8%), pericardium (0.7%), mantle epithelium (0.1%), and heart (0.1%). The remaining 0.6% were found extracellularly. Percentages of trophozoite stages were (mean ± SE): large, log-phase trophonts, i.e., signet rings, 97.0 ± 1.2%; meronts, 2.0 ± 0.9%; clusters of small, log-phase trophonts, i.e., merozoites, 1.0 ± 0.5%. Levels of infection in hemocytes and Leydig tissue were representative of total parasite intensity. These techniques are a powerful tool to follow parasite distribution and invasion, and to further explore mechanisms of Perkinsus spp. pathogenesis in bivalves.

Species of Perkinsus are responsible for high mortalities of bivalve molluscs worldwide (Villalba et al., 2004). The pathology caused by Perkinsus spp. is characterised by infiltration of hemocytes into infected tissues, followed by chronic wasting, impairment of growth, and eventual mortality of the shellfish by emaciation (Ford and Tripp, 1996). To improve the knowledge of the pathogenesis of Perkinsosida, accurate techniques to estimate the number of parasitised tissues are required (Romestand et al., 2001). Accurate quantification of Perkinsus marinus infections in tissues is essential for understanding the biological interaction between the parasite and its host, including elucidation of portals of entry and mechanisms of disease progression. Present techniques to quantify Perkinsus spp. include (1) visual detection of parasite stages in tissue smears (Stein and Mackin, 1957) and tissue sections (Dungan and Roberson, 1993); (2) assays based on counting hynospores in Ray’s fluid thioglycollate medium (RFTM)-incubated tissues from the whole animal (Choi et al. 1989; Bushek et al., 1994; Fisher and Oliver, 1996), tissue subsamples (Ray, 1952), and hemolymph sampling (Gauthier and Fisher, 1990; Nickens et al., 2002); and (3) molecular biological techniques based on detection of DNA or RNA by polymerase chain reaction (PCR; Marsh et al., 1995; Robledo et al., 1998; Yarnall et al., 2000; Audemard et al., 2004; Elandalloussi et al., 2004). However, no current procedures are able to perform accurate quantifications for the number and tissue distribution of total parasites or parasite stages in tissue sections.

Modern stereology is a discipline of wide application in areas of biology and medicine like neurosciences and oncology, among others (Pakkenberg and Gundersen, 1988; Cruz-Orive and Roberts, 1993; Mattfeldt et al., 1993; Roberts et al., 1994; McNulty et al., 2000; Garcia-Finana et al., 2003). Modern stereological methods can be defined as a collection of strongly based mathematical methods aimed to quantify geometrical properties of target objects without assumptions concerning inherent characteristics of these objects, like size, shape, or distribution. These techniques stem from the concept of accuracy as an additive property of unbiasedness and precision. The first modern stereological approaches were developed through the 1980s (Cruz-Orive, 1980; Sterio, 1984). Precedent stereological techniques known as classical methods are assumption-dependent (e.g., Saltykov, 1958).

One of the goals of design-based modern stereology has been the development of new techniques oriented to the unbiased and highly efficient quantification of particles in tissue sections. Among these, the optical dissector (OD) (Gundersen, 1986) and the optical fractionator (OF) (West et al., 1991) are considered some of the most reliable approaches.

The present study aims to quantify the number and tissue distribution of different stages of the protozoan parasite P. marinus in a natural population of Crassostrea virginica by the application of modern stereology and immunohistochemistry. Immunohistochemistry was used to aid in the detection and quantification of P. marinus, since the parasite is difficult to resolve efficiently in tissue sections, particularly in areas of heavy hemocytic infiltration (Choi et al., 1989; Dungan and Roberson, 1993). The information provided by these techniques will be useful to follow parasite distribution and progression in experimental and natural infections, and to further explore mechanisms of pathogenesis.

MATERIALS AND METHODS

Initial processing of samples

Eight specimens of C. virginica of mean shell height (mean ± SD) of 98.6 ± 8.5 mm were harvested intertidally from Cedar Tree Point (Wickford, Rhode Island) in July 2005. Oysters were maintained for 24 hr in a 50-L filtered tank filled with artificial seawater at 25 °C and 27% salinity.
Extraction of the meat

Stereological approaches applied here require maintenance of the original conformation and perfect condition of the whole tissues. Therefore, oyster meats, including the boundaries between mantle and adductor muscle and the edges of the latter, were extracted intact by gently wedging the valves to open a narrow access without affecting the soft tissues, and detaching the edges of the adductor muscle with a spatula.

Estimation of the volume of the meat

Gravimetric estimations: A container large enough to prevent over­fl ows was placed on a precision balance, filled partially with filtered artificial seawater, and weighed to the closest milligram before performing the immersion of each oyster meat mass. Meats were drained on filter paper for 2 min, held by a fine thread attached to the adductor muscle, and submerged into the container. After a complete immersion without meat contact with container walls, container and meats were weighed to the closest milligram. The density of the immersion fluid was estimated to the closest milligram from the average weight of 10 replicates of 100 ml. Meat mass (mtm) volumes were estimated gravimetrically according to Scherle’s (1970) method.

\[
V(\text{mtm}) = \frac{W_s - W_i}{\rho}
\]

where \(V(\text{mtm})\) is the meat mass volume (note that symbol "-" is used to distinguish the estimator of the parameter \(V(\text{mtm})\) from the parameter \(V(\text{mtm})\) itself), \(W_s\) is the weight after the immersion of the meat, \(W_i\) is the weight before the immersion of the meat, and \(\rho\) is the density of the fluid. Next, specimens were preserved in Davidson’s AFA fixative (Dungan and Roberson, 1993) for 24 hr and dehydrated in 70% ethanol for 48 hr.

Cavalieri estimations: Six-millimeter serial thick sections, or slabs, were obtained by positioning each animal at random within a stereological cutting machine (Michel and Cruz-Orive, 1988; Paché et al., 1993), enrobed in 2% agar, and sliced exhaustively (Figs. 1, 2). Cavalieri areas were estimated by superimposing a stereological grid of points of \(a/p = 14.06 \text{ mm}^2\) at random on a fixed side of each slab. Cavalieri volumes were obtained according to the point-counting variant of Cavalieri’s Principle (Cavalieri, 1915):

\[
V(\text{mtm}) = T \cdot \frac{M^2}{p} \cdot \sum P_i
\]

where \(V(\text{mtm})\) is the mean thickness of the tissue slabs, \(a/p\) is the area per point of the points grid, \(M\) is the magnification, and \(P_i\) are the number of points counted in the \(i\)th slab.

Embedding, immunostaining, and routine staining

A systematic subsample of \(k = 2\) slabs was randomly selected from each animal (Fig. 2a), dehydrated in S-29 reagent (Technicon, Chauncey, New York) for 5 hr, cleared in UC-670 clearing reagent (Technicon) for 4 hr, infiltrated in Parafl ast X and Parafl ast Plus (Fisher Scientific, Pittsburgh, Pennsylvania) (50% each) for 2 hr, embedded in the previous Parafl ast mixture, and sectioned with a microtome. Forty-micrometer Cavalieri serial sections were mounted on silanized slides, equilibrated in 0.15 M phosphate-buffereal saline (PBS), blocked for 75 min, incubated for 75 min in 12 μg/ml rabbit anti-P. marinus IgG (Dungan and Roberson, 1993), washed in PBS plus 0.05% (v/v) Tween-20 (PBST), incubated for 75 min in 4 μg/ml anti-rabbit IgG horseradish peroxidase conjugate (Pierce, Rockford, Illinois), washed in PBST and PBS, stained for 20 min with HistoMark BLACK DAB-cohitl reagent (KPL, Gaithersburg, Maryland), and counterstained for 30 sec with 1% (w/v) metanil yellow in 1% (v/v) glacial acetic acid. For a parallel monitoring of the pathology by histology, 7-μm serial sections were similarly immunostained and counterstained with eosin Y–phloxine B (Fisher Scientific).

Estimation of the total number of trophozoites

Parasite trophozoites were counted with the aid of computer-aided stereological toolbox (CAST) grid system (Olympus, Copenhagen, Denmark). This system includes a monitored microscope stage that allows performance of systematic sampling in the \(x-y\) plane, avoiding repetitive counts of the same parasite cells within sections. Displacements along the \(z\) coordinate are measured with a microcator. The magnification of the CAST grid system, i.e., \(X=4,000\), was high enough to prevent mis­detection of trophozoites or to confuse them with histological elements with endogenous peroxidase activity, e.g., mucous cells, or with pig­mented oyster cells that may resemble immunostained P. marinus cells at lower magnification, e.g., brown cells. Quantification of P. marinus was performed by the application of the OD and OF.

OD: The numerical density of trophozoites within the reference space was estimated by the OD, whose expression is:

\[
N_V(tpz, mtm) = \frac{\sum Q(tpz)}{\sum [a'f(s) - h(s) - P]}
\]

where \(\Sigma Q(tpz)\) is the total number of trophozoites counted in the disectors, \(a'f(s)\) is the disector area, \(h(s)\) is the disector height corrected for slice thickness, and \(P\) are the number of disectors that intersected the meat mass volume \(V(\text{mtm})\).

The disector height was corrected for slice thickness shrinkage in each specimen by means of the identity (Cruz-Orive et al., 2002):

\[
h(s) = h - t_l r^l
\]

where \(h\) is the theoretical disector height, \(t\) is the nominal slice thickness, and \(r\) is the final slice thickness after shrinkage (Figs. 2i, j). Finally, the total number of trophozoites was estimated by:

\[
N(tpz) = N_V(tpz, mtm) \cdot V(\text{mtm})
\]

Estimation of trophozoite stages within different tissues

Percentage of trophozoite stages, i.e., log-phase trophonts, including signet rings, meronts, and clusters of small, log-phase trophonts, i.e., merozoites, and percentages of total trophozoites within the following tissues: intestine, Leydig tissue, hemocytes, digestive gland, gills, connective tissues, gonads, palps, muscle, mantle connective, pericardium, mantle epithelium, and heart, were estimated by applying Eq. (6) to each corresponding subsample.

Practical implementation

Systematic slicing: Oysters meats were sliced into 6-mm slabs (Figs. 1a, b). Systematic sampling can be performed bidirectionally and excludes the first slab, i.e., edge of the oyster, as it lies before the random start (Fig. 2a). All slabs were used for Cavalieri, except the first one.

Cavalieri approach: A points grid was positioned and rotated at random over a fixed face of each slab, i.e., Cavalieri surface, and relevant areas were estimated by point counting. The total number of points counted in a single slab was \(X P_i\) (Fig. 2b). Cavalieri areas were estimated according to Eq. (2).

Systematic subsampling and embedding: One of the 2 possible \(k = \ldots\
Figure 1. (a) Cavalieri sections of C. virginica. (b) Detail of C6 displaying the boundaries of the reference area chosen for the approach of Cavalieri. Boundaries were selected to provide the closest reference space to the gravimetric probe, i.e., Scherle's approach. In consequence, the reference space was bounded by the outer surfaces of mantle, gills, and palps.
FIGURE 2. Scheme of the stereological design. (a) Random selection of $k = 2$ systematic sample after serial sectioning. (b) Estimating Cavalieri areas by point counting. (c) Sampling fraction $T/t$. (d) Estimating the areal shrinkage for the OF by point counting. (e) Sampling fraction $a/p$. (f) Counting trophozoites using a systematic grid of disectors. (g) Sampling fraction $a(f)/a'(f)$. (h) Tridimensional view of the optical disector probe within a cube of tissue. (i) Microtome thickness $t$ before shrinkage. (j) Section thickness $t'$ after shrinkage. Three objects marked with "×" are counted in the example. $h$, theoretical disector height. $\Sigma P_i$, points counted in the $i$th slab; $\Sigma P_j$, points counted in the $j$th thick section; $\Sigma Q'(tpz)$, trophozoites counted in the $j$th thick section.
FIGURE 3. Succession of consecutive focal planes in Leydig tissue displaying how the quantifications of trophozoites of *P. marinus* were performed. Countings were made in the central 25 μm (theoretical disector height) of the 40-μm thick section; the region where the unbiased probe, i.e., OD, appears (c and d). Two trophozoites were counted in this example. Arrows show the trophozoites in focus. Arrow points show the unbiased frame.
2 systematic samples \((r = 1 \text{ or } r = 2)\) was subsampled at random (Fig. 2a). Slabs were embedded, cut into 40-μm thick sections, and immunostained.

Concept of sampling fraction: A sampling fraction is a dimensionless ratio derived from dividing an n-dimensional predefined total sampling space, e.g., slab thickness \(T\), by its space sampled, e.g., slice thickness \(t\) (Fig. 2c). The ratio \(T/t\) was the first sampling fraction of the stereological design for OD and OF.

Estimating the areal shrinkage by point counting (used in OF exclusive): The 40-μm Cavalieri section and its estimated area were obtained from the same slab and grid used in Figure 2b. The slab and points grid of Figure 2b were used, respectively, to get the corresponding 40-μm section and to estimate its area. The total number of points counted in a single thick section was \(\Sigma P_i\). The section profile of Figure 2d is smaller than Figure 2b because of the shrinkage effect. The sampling fraction of each points grid \(a/P\) was derived from dividing the total sampling space or fundamental tile area \(a\) by the point p or sample event (Fig. 2e).

Counting trophozoites: CAST grid system overlaid a systematic grid of disectors within the central 25 μm of the thick sections (Fig. 2f). The sampling fraction \(a/f'\) for the OD and OF stemmed from dividing the fundamental tile area \(a\) by the unbiased frame area \(a/f\) (Fig. 2g).

Estimating the thickness shrinkage: The section thickness \(t\) before shrinkage was given by the micrometre thickness. The section thickness \(t'\) after shrinkage was measured with CAST grid between the first and last focal planes of the mounted thick section, and \(h\) was the theoretical disector height (25 μm, Fig. 2i, j).

Numerical example

In this section, we present a numerical example on the calculations involved in the different stereological approaches used for 1 oyster. Our objectives are to estimate: (1) the meat volume by Cavalieri’s Principle; (2) the total number of trophozoites by the OD; and (3) the total number of trophozoites by the OF.

1. The number of points counted in the 10 sections were, respectively: \(P_i = \{5, 9, 9, 16, 20, 23, 25, 23, 22, 13\}\), the sample total being \(\Sigma P_i = 167\). \(\hat{V}_{\text{mm}}^3 = 6.15 \times 14.06 \times 1.2 \times 167 = 14,440.32\) mm³. The magnification was \(M = 1\) as point countings were performed at the real scale. Therefore, applying Eq. (2), the meats volume was \(\hat{V}_{\text{mm}}^3 = 6.15 \times 14.06 \times 1.2 \times 167 = 14,440.32\) mm³.

2. The total number of trophozoites counted was \(\Sigma Q\) (tpz) = 140, using \(\Sigma P = 2,159\) dissectors. The area of the sampling frame was \(a/f = 3.246\) mm² and the height of the disector corrected for the shrinkage was \(h(s) = 29.34\) μm. \(h(s)\) was stemmed from a theoretical disector height \(h = 25\) μm, a nominal slice thickness \(t = 40\) μm, and a final slice thickness after shrinkage \(t' = 34.08\). By applying Eq. (3), the numerical density of trophozoites was \(N_{\text{tpz}} (\text{tpz, mmt}) = 140 \times (2,159 \times 29.34 \times 3.246) = 680.87\) cells · mm⁻³. Finally, using Eq. (5) we estimated a total number of 9,831,981 trophozoites in the tissues of this oyster.

3. The number of trophozoites counted were \(\Sigma Q\) (tpz) = 140. The sampling fraction was \(a/f'\) = 156.25. The systematic subsample of \(r, k = (2, 2)\), where \(r \in (1, k)\) is a random number and \(k\) is the sampling period, yielded the point counts \(P_i = \{9, 16, 23, 25, 13\}\), the sample total being \(\Sigma P_i = 86\); point counts in the 40-μm sections were, respectively, \(P_j = \{10, 17, 21, 20, 9\}\), the sample total being \(\Sigma P_j = 77\). The area shrinkage was \(a/s = \Sigma P_i/\Sigma P_j = 1.12\) and \(\hat{T}' = \hat{T} = 12,300\) μm. \(\hat{T}'\) doubles \(\hat{T}\) as the Cavalieri period \(K = 1\) halves the systematic subsample period \(K = 2\). Therefore, by Eq. (6), the estimate of the total number of parasites was \(\hat{N}_{\text{tpz}} = 140 \times 1.12 \times (156.25 \times 12,300 \times 29.34) = 10,270,961\) trophozoites. The empirical error between these 2 estimates for the total number of trophozoites was 4.27%.

Correlations and allometric models

The following relationships: (1) total number of parasites versus number of parasites in the most infected tissues, i.e., intestine, hemocytes, and Leydig; (2) number of parasites between these most infected tissues; and (3) number of parasites between the most representative parasites stages, i.e., signet rings, tomons, and meronts, were investigated by applying least-squares linear regression models or by alternative common least-squares linear transformations of \(x, y\) and \((x + 1, y + 1)\), e.g., logarithmic, logarithmic, semiarc tangent, and arc tangent. Significant correlations and regressions were investigated. Significant regressions were established as allometric models.

RESULTS

Comparisons between Scherle’s method and Cavalieri’s Principle

Paired comparisons corresponding to the oyster meat volumes obtained by Scherle’s and Cavalieri’s approaches are presented in Figure 4. Scherle’s valuations were higher than Cavalieri’s estimations in all the specimens. The mean empirical error between both techniques expressed in percentages was (mean ± SD) 10.2 ± 3.7%.

Number of parasites and parasite stages

Mean numbers of trophozoites for the 8 oysters analyzed in this study were (mean ± SE) 11.80 ± 3.91 million and 11.55 ± 3.88 million for the OD and OF methods, respectively (Fig. 5). The mean empirical error between both stereological approaches was 3.8 ± 1.0%. Trophozoites were detected intra-
cellularly in the following tissues: intestine (30.1%) (Fig. 6a); Leydig tissue (21.3%) (Figs. 6b, c); hemocytes in sinuses, vessels, and tissues (14.9%) (Fig. 6d); digestive gland (11.4%) (Fig. 6e); gills (6.1%); connective tissues (except Leydig and mantle) (5.7%); gonads (4.1%) (Fig. 6f); pulps (2.2%); muscle (1.9%); mantle connective (0.8%); pericardium (0.7%); mantle epithelium (0.1%); and heart (0.1%). The remaining 0.6% of the trophozoites were found extracellularly throughout different tissues (Fig. 6g). Brown cells were clearly distinguished from parasites by their different appearance and the endogenous pigmentation of the ceroid bodies (Fig. 6h).

Percentages of trophozoite stages were (mean ± SE): large, log-phase trophonts, i.e., signet rings, 97.0 ± 1.2%; meronts, 2.0 ± 0.9%; clusters of small, log-phase trophonts, i.e., merozoites, 1.0 ± 0.5%. We were unable to count the individual merozoite cells within clusters because of their small size. Furthermore, because of the low numbers of meronts and merozoites compared with trophozoites, we did not evaluate the relative abundance of these stages in different tissues.

Correlations and allometric models

The following significant correlations were detected: (1) log_{10}(total number of trophozoites) versus log_{10}(trophozoites in Leydig tissue) ($P = 0.027$, $r^2 = 0.67$) (Fig. 7a); and (2) total number of trophozoites versus trophozoites in hemocytes ($P = 0.016$, $r^2 = 0.64$) (Fig. 7b). In contrast, no significant regressions ($P > 0.05$) or contradictions to regression model assumptions were found between (1) total number of trophozoites versus trophozoites in intestine; (2) trophozoites in intestine versus trophozoites in Leydig tissue; (3) trophozoites in intestine versus trophozoites in hemocytes; (4) trophozoites in Leydig tissue versus trophozoites in hemocytes; (5) total number of toomonts versus total number of signet rings; (6) total number of clusters of merozoites versus total number of signet rings; and (7) total number of clusters of merozoites versus total number of toomonts. The total number of toomonts and clusters of merozoites showed a positive trend (with the exception of the outlier 20.4 × 10^6 signet rings, 0 toomonts) with respect to the total number of signet rings.

As a result, the subsequent allometric models were found:

$$P = 0.83L + 4.25$$

where $P$ and $L$ are the napierian (by 1) logarithms of the total number of trophozoites and trophozoites in Leydig tissue, respectively.

$$T = 5.83H + 10^{6.54}$$

where $T$ is the total number of trophozoites and $H$ is the number of trophozoites in hemolymph.

DISCUSSION

We have shown here that the application of modern stereological techniques in combination with immunohistochemistry allows for the quantification and assessment of the tissue distribution of the trophozoites of *Perkinsus marinus* in naturally infected oysters. The use of an antibody-based differential staining to detect the parasite in tissue sections has proven to be decisive for the accurate detection of the parasite. Nevertheless, modern stereological approaches undoubtedly played the main role in the process of quantification by applying unbiased probes combined with unbiased, efficient, rational, and objective protocols of sampling in which the researcher has complete control of each step of the sampling scheme. The perspective of techniques of modern stereology is radically different from computer methods of image analysis oriented to perform counts of objects automatically by complex mathematical algorithms (Young et al., 1998; Malpica et al., 2002). These methods of image analysis generally fail in common histological situations, like cases of cellular superposition, variations in the staining intensity or affinity among specimens or tissue portions, gradient staining, etc. In modern stereology, the counting is performed by the human eye, the best tool for identifying target objects accurately. In addition, errors derived from the estimations can be predicted mathematically, as the sampling process is unbiased (e.g., Howard and Reed, 1998). Likewise, the accuracy of the modern stereological methods applied here was shown for the total number of parasites, whereas the mean empirical error between the OD and the OF was negligible, i.e., less than a 4%.

When comparing the 2 methods that we used to estimate the meat volumes of the oysters, Scherle’s valuations were consistently higher than Cavalieri estimations in all the specimens. Scherle’s (1970) method has been thoroughly proved to be the most accurate gravimetric approach to determine the volume of bounded objects (Scherle, 1970; Weibelson, 1979; Roberts et al., 1993). However, since this gravimetric probe is a function of the difference in weight of the object submerged in a constant volume of liquid with respect to the aforementioned fluid volume, inaccuracies are expected when objects to be evaluated release or absorb fluids during the process of immersion in the valuation fluid. Although meats of oysters were blotted on filter paper for short intervals to capture excess liquid, a flow of fluid from meats to the peripheral gravimetric liquid was observed from all the different soft parts while immersed, as an obvious consequence of the faculty of bivalve tissues to absorb and release fluids, as well as from bleeding precipitated by even the most careful excision of oyster adductor muscles from their anchoring valve myostraca. These observations may explain why Scherle’s valuations were higher than Cavalieri estimations. Regardless of the differences in the estimates for oyster volumes between the 2 methods, those differences were con-

![Figure 5. Total number of trophozoites of *P. marinus* in C. virginica estimated by the OD and the OF. Means for each method are shown by horizontal bars. Estimates for the same oyster are connected by a line.](image-url)
FIGURE 6. Detection of *P. marinus* in tissues of *C. virginica*. (a) Advanced infection in intestine. (b) *Perkinsus marinus* signet ring in vascular vessel wall. (c) Trophozoite adjacent to nucleus of Leydig cell. (d) Trophozoite inside hemocyte within the Leydig tissue. (e) Extracellular signet-ring trophozoite in digestive gland. (f) Trophozoite inside oocyte. (g) Free signet-ring trophozoites in pericardial cavity. (h) Brown ceroid cells with multiple ceroid bodies.
sistent between individual oysters, suggesting that the application of Scherle’s approach for performing rapid and approximated valuations of meat volumes in oysters is appropriate.

A relevant aspect of the stereological design concerns the correspondence of reference spaces in the estimation of the total number of trophozoites in Eq. (5). For the estimations to be correct, the reference spaces from 40-μm sections analyzed with CAST grid have to be bounded using the same criteria applied to the 6-mm Cavaliere sections. To do so, all the internal spaces of meats with or without *Perkinsus* are included in both reference spaces. Because Cavaliere volumes were estimated before the embedding, the areal shrinkage must not be corrected in OD. Inclusion of the internal spaces devoid of *P. marinus* cells is irrelevant because both reference volumes are equal in Eq. (5). However, empirical differences between both reference volumes (including the empty cavities) must be contrasted. The correspondence between both Cavaliere and density volumes is demonstrated by comparing the results from the OD and OF, because the latter approach lacks of reference volumes. In this account, the relatively small empirical error between these 2 methods for each oyster validates our results.

Stereological methods have been widely applied to bivalve mollusc research, especially in studies that estimate fecundity by counting the number of oocytes (e.g., Bayne et al., 1978; Lowe et al., 1982; Newell et al., 1982; Sundet and Lee, 1984; Pipe, 1985; Beninger, 1987; Morvan and Ansell, 1988; Pau1et al., 1992; Pazos et al., 1996). However, most of these techniques are not modern stereological procedures and none of them is an optical method. These classical approaches (e.g., Saltykov, 1958), commonly known as unfolding techniques, are not recommended because they are subjected to errors derived from assumptions concerning the size, shape, or distribution of the target particles, which were demonstrated when applied to cells to give rise to results too unrealistic to be scientifically accepted (see Mayhew and Gundersen, 1996). In bivalves, Cavaliere’s Principle, OD, OF, and their respective formulas for predicting theoretical errors were applied to *Solen marginatus* (Remacha-Triviño, 2002) to estimate hemocyte numbers in kidney tissues. Expressions of these theoretical errors were updated for Cavaliere’s Principle to estimate oocyte numbers by the physical disector in a single specimen of *Tapes pulastra* (Alvarez and Gual-Arnau, 2006). The main advantage of the optical procedures used in our work (OD and OF) with respect to precedent physical methods (Sterio, 1984; Gundersen, 1986) is that particles are counted in a succession of focal planes without losing any information between the 2 disector physical planes, as well as an optimal preservation of the target tissues. To ensure optimal preservation of the tissues, quantifications performed by the OD and the OF require leaving a region free of counting (7.5 μm in our case) at both sides of the 40-μm thick sections. This gives rise to quantifications free of errors related to losses of tissue removed from the edges of the sections, a well-known phenomenon derived from the combined action of the microtome blade and the different chemical agents supplied for sampling, processing, and embedding (see Andersen and Gundersen, 1999).

One of the additional requirements of the OD and OF is that the target objects to be counted must be small enough to be assumed as points, or alternatively, to associate a point to each target object to be counted. Otherwise, quantifications would depend on the size of particles. Therefore, the practical rule to quantify populations of cells is generally based on counting a fixed part of the cell, e.g., upper part of the nucleolus or nucleus, which can be identified in every cell, at 1 unit per cell, and small enough to be counted as it comes into focus. In the case of *P. marinus*, the protozoan stages of this study matched the maximum size of 16 μm in cellular diameter described for cultured *P. marinus* (Sunila et al., 2001), dimensions small enough to be clearly identified by their first focal plane as they come into focus.

In general, optical methods can be applied to convex objects, as is the case of the spheroid trophozoites investigated here, but excludes particles whose conformation could give rise to errors in their identification, e.g., ramified cells like neurons. However, nonconvex and ramified objects can also be counted by OD and OF if they are small enough to be observed sequentially in all of their focal planes within the limits of the screen in whichever position they intersect the OD probe. This second variant resembles serial sectioning as it requires scanning all the different...
parts of the objects to guarantee their right quantification; whereas it is not restricted to particular geometries.

Another challenge to the use of stereology to detect parasites in tissue sections stemmed from the fact that *P. marinus* is distributed in aggregates (Chintala et al., 2002). The heterogeneous presence of the protozoan within tissues and oysters required a sampling design focused on increasing the sampling intensity at the level of the sections to give rise to a sampling fraction \( a'(f) \alpha(a(f) of 0.64\% in oysters with less than 20 million parasites, and 0.32\% for the rest of the specimens.

In addition to applying modern stereological methods for the quantification of *Perkinsus* spp. parasites in oysters, this work provides useful information regarding perkinsiosis in American oysters. Our observations showing the highest densities of the parasite in the intestine and Leydig tissue of naturally infected oysters contrast with the findings of previous researchers who observed the highest densities of *P. marinus* in the digestive gland (Mackin, 1951; Choi et al. 1989; Fisher and Oliver et al. 1996). However, they are consistent with results from several experimental infections that point to the important role of the connective tissue and the hemocytes in the proliferation and transport of *P. marinus* in oysters (Chintala et al. 2002).

Interestingly, the lack of correlation between the number of parasites in the intestine and the total number of parasites in the oyster indicated that the degree of infection in intestine is not representative of the overall levels of infection in oysters. On the contrary, the degree of infection in Leydig tissue and hemocytes was shown to be more representative of perkinsiosis intensity in *C. virginica*. This indicates that the infection in intestine is asynchronous with respect to the spread of the parasite in the rest of the tissues, suggesting that results of RFTM-based tissue assays from hemolymph and Leydig tissue may be more accurate than assays using intestinal tissue. In this regard, it is important to mention that Leydig tissue and hemolymph are some of the most frequent and homogeneously distributed tissues in the oyster.

When compared with the modern stereological methods applied here, procedures traditionally used to quantify *P. marinus* show several disadvantages. First, observations from histological, immunohistochemical, tissue RFTM, or molecular assays are based on partial samplings. Therefore, extrapolations of subsample results to the whole animal are subject to error. Second, RFTM methods are focused on enumerations of a unique cell type, the hypnospore, after enlargement of different stages of *P. marinus* in culture media (Ray, 1954), and do not provide information on the relative abundance of the different parasitic stages. Third, RFTM methods applied to the whole animal, i.e., whole body burdens (Choi et al., 1989; Bushek et al., 1994; Fisher and Oliver, 1996), are based on the assumption of considering a 100% enlargement and survival of the hypnospores in the culture media. However, an unknown rate of mortality or lysis of weaker trophozoites and hypnospores during their enlargement in RFTM may occur. In this sense, Nickens et al. (2002) reported that simple modifications of the RFTM medium, including the addition of 5% lipid concentrate, resulted in larger hypnospores, indicating that the enlargement of *P. marinus* in RFTM may not be optimal. Fourth, molecular biology approaches on the basis of PCR amplification of extracted DNA subsamples to detect or quantify *P. marinus* DNA (Marsh et al., 1995; Robledo et al., 1998; Yarnall et al., 2000; Penna et al., 2001; Audemard et al., 2004; Elandalloussi et al., 2004; Gauthier et al., 2006) have both sampling errors, as well as potential detection of DNA from nonviable cells (MDTAO, 2003). Moreover, the unknown effects of the procedures for DNA isolation and purification on yield and the presence of contaminants or PCR inhibitors in the tissue samples must be considered and mitigated.

The stereological methods applied here, however, showed some limitations with the OD and the OF failed in counting the following parasite stages: (1) trophozoites located in hemocytes lost during phases of processing and embedding; (2) extracellular trophozoites lost similarly due to processing and embedding; (3) merozoites within clusters, as these cells were too small to be counted separately and had to be necessarily classified as clusters; and (4) zoospores and free single extracellular or intracellular merozoites, which had to be ignored because they were too small. Nevertheless, all the precedent biases are not due to limitations of the stereological techniques applied here, but to the peculiarities of the life cycle of *P. marinus*.

In summary, the combination of modern stereological techniques with immunohistochemistry has proven to be a useful method to quantify parasites in the tissues of naturally infected oysters. The information provided by these techniques could be useful to follow parasite distribution and progressive invasion in experimental infections, and to further explore mechanisms of pathogenesis. Further combination of modern stereology techniques with immunohistochemistry and histologically based assays of cell viability and apoptosis (Sunila and LaBlanca, 2003) would provide an extremely valuable tool in the investigation of host-parasite interactions.

**ACKNOWLEDGMENTS**

This research was supported by Alfonso’s Martín Escudero Foundation (Spain) under Grant 2004-2005 for the Specialization in International Universities or Research Centers and USDA CSREES Award 2004-34438-15041.

**LITERATURE CITED**


**Chintala, M. M., D. Bushek, and S. Ford.** 2002. Comparison of in
vitro-cultured and wild-type Perkinsus marinus. II. Dosing methods


ULTRASTRUCTURAL DEVELOPMENTAL CYCLE OF HAPLOSPORIDIUM MONTFORTI (PHYLUM HAPLOSPORIDIA) IN ITS FARmed ABALONE HOST, HALIOTIS TUBERCULATA (GASTROPODADA)

Carlos Azevedo†, Graça Casal††, and Jaime Montes§

* Department of Cell Biology, Institute of Biomedical Sciences, University of Porto (ICBAS/UP), Lg. Abel Salazar no. 2, P-4099-003 Porto, Portugal. e-mail: azevedoc@icbas.up.pt

ABSTRACT: The sequential developmental cycle of Haplosporidium montforti, a recently described species from farmed abalone Haliotis tuberculata (Gastropoda), was studied. Ornamented and operculated mature spores were electron dense. The nucleus of the uninucleated free cell divided successively, giving rise to multinucleate plasmodia, containing up to 100–120 nuclei. Later, the plasmodia developed into sporonts inside sporocysts with irregular contours. Each of their nuclei gave rise to uninucleate sporoblasts. At the next phase of development, a very irregular membranous group of cisternae began to differentiate in the cytoplasm of each sporoblast, surrounding each nucleus and the adjacent cytoplasm. Each sporoblast differentiated into a spore. This process was characterized by the appearance of dense blisters of amorphous material at the periphery that gradually formed the prespore wall and pre-operculum. Simultaneously, in the endosporoplasm, the spherulosome and several haplosporosomes were formed. During the final phase of the maturation process, the spores became gradually denser, and the endosporoplasmic structures were barely visible.

The Haplosporidia Caullery & Mesnil, 1899, are specific and obligate pathogens or hyperparasites that occur in different tissues and organs of invertebrate hosts. These parasites occur in several geographic areas and have been seen parasitizing gills, foot, mantle, intestine diverticula, and other organs of several molluscan species (Haskin et al., 1960; Barrow, 1961; Marchand and Sprague, 1979; Ornieriès, 1980; Azevedo, 1984; La Haye et al., 1984; Hillman et al., 1990; Perkins, 1991, 2000; Azevedo et al., 2003, 2006).

Currently, there are 31 recognized haplosporidian species (Burreson and Ford, 2004) corresponding to the 3 largest genera (Haplosporidium, Nacininia, and Urosporidium). To these, H. montforti has recently been added as a new taxon (Azevedo et al., 2006). Moreover, as a result of recent molecular phylogenetic analyses, 4 new species of Bonamia have been included in the Haplosporidia: B. ostreae (Pichot et al., 1979; Carnegie et al., 2000), B. existosaa (Hine et al., 2001; Berthe and Hine, 2003), and B. persporea (Carnegie et al., 2006), and Mirkycytos roughleyi (Farley et al., 1988), later transferred to Bonamia as B. roughleyi (Coheenac-Laurca et al., 2003).

Historically, the Haplosporidia has been an order in the class Sporozoa, but it was elevated to phylum rank in 1990 (Perkins, 1990). Early DNA sequence data showed that the phylum was historically, the Haplosporidia has been an order in the class Sporozoa, but it was elevated to phylum rank in 1990 (Perkins, 1990). Early DNA sequence data showed that the phylum was located within the Alveolata clade (Siddall et al., 1995; Flores et al., 1996), but more recent molecular data place the phylum as sister taxon to the Cercozoa (Richards et al., 2004).

The ultrastructural studies of the different life-cycle stages of several haplosporidian species have yielded insufficient information, with the exception of a few species, which have been detailed over all life-cycle stages (Perkins, 1968, 1975a; Marchand and Sprague, 1979; Desportes and Nashed, 1983; Azevedo et al., 1985; Haskin and Andrews, 1988; Azevedo and Corral, 1989; Hine and Thorne, 2002).

The present study includes detailed ultrastructural descriptions of what we believe to be the sequential developmental stages of Haplosporidium montforti (Azevedo et al., 2006) that occur within the same host tissues.

MATERIALS AND METHODS

Juveniles of Haliotis tuberculata (Mollusca, Gastropoda) from Ireland were grown in barrels suspended from rafts in Galicia (NW Spain), where mortality was observed due to the presence of Haplosporidium montforti (Azevedo et al., 2006).

Smears of fresh tissues (gills, digestive gland, and mantle) of H. tuberculata from animals with signs of disease were prepared for observation by light microscopy (LM) using differential interference contrast microscopy (Nomarski-DIC).

For transmission electron microscopy (TEM), small fragments of the infected tissues were fixed in 3% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.2) at 4 C for 10 hr, washed overnight in the same buffer at 4 C, and post-fixed in 2% osmium tetroxide with the same buffer at 4 C, and post-fixed at 10 C for 1 hr. After dehydration in an ascending ethanol series, followed by propylene oxide, the tissue fragments were embedded in Epon. Semithin sections were stained with methylene blue-Azure II for LM. Ultrathin sections were double contrasted with uranyl acetate and lead citrate and observed in a JEOL 100CXII TEM operated at 60 kV.

RESULTS

Spore structure

LM observations revealed that the developmental cycle of the parasite occurred in sporocysts located side by side in the host tissues (Fig. 1). Using TEM, some free mature spores observed among host cells showed a very dense endosporoplasm with the typical haplosporidian cytoplasmic structures, i.e., spore wall with operculum and attached filaments, spherulosomes, haplosporosomes, dense bodies, mitochondria, and nuclei. However, in fully mature spores, some of these structures were barely visible (Fig. 2).

Among the different developmental stages for some uninu-
clete free cells (amoebulae) were observed (Figs. 3, 4). Before the nuclear division, the amoebulae showed the same internal organization as the spore, except for the spore wall (Fig. 5).

**Plasmodium development**

When the uninucleate amoebula began its nuclear multiplication, no apparent mitotic figures were observed. The circular nucleus of each amoebula developed with irregular contours, and some appeared striated, suggesting nuclear bipartition. During this division, the nuclear envelope did not disappear, and cytokinesis did not occur. After division, the uninucleate amoebula transformed into a binucleate plasmoid, and, by successive nucleokinesis without cytokinesis, the binucleated plasmoid gave rise to 4 nuclei (Fig. 6), becoming a multinucleate plasmoid (Fig. 7). Using ultrathin sections, it was possible to count up to 100 to 120 nuclei per plasmoid. Diplokaryotic stages were never observed in the successive nucleokinesis.

**Sporoblastogenesis**

During this phase, the cytoplasm of multinucleate plasmodia became more vacuolated. The cytoplasmic vacuoles and vesicles fused, forming continuous cisternae surrounding the isolated nuclei and some portion of the surrounding cytoplasm. This membranous system gave rise to the plasmalemma of the newly formed uninucleated cells, which subsequently formed sporoblasts (Fig. 8). The numerous sporoblasts, surrounded by an irregular wall from the previous plasmoid, become a sporocyst (Fig. 8). Again, from serial sections, it was possible to count up to 100–120 sporoblasts in each sporocyst.

**Sporulation**

In each sporoblast, the sporulation began with the differentiation of continuous cisternae within the cytoplasm of the sporoblasts, separating 2 distinct regions, i.e., the external endosporoplasm, without any contact with nuclear information, and the internal endosporoplasm, which contained the nucleus (Fig. 8). At the beginning of spore wall genesis, the spore wall primordium was characterized by the appearance of several blisters in close contact with the external portion of the membrane that surrounded the sporoplasm (Fig. 9). These blisters became gradually denser, giving rise to the spore wall (Figs. 10, 11). While the spore wall was forming, the operculum and the external filaments simultaneously differentiated (Figs. 9, 10). In the final phase of spore maturation, the nucleus generally occupied the basal portion of the spore, while the endosporoplasm became denser (Fig. 2). The numerous vesicular and membranous structures with dense material organized to form the spherosome. The spherosome migrated to the apical zone of the spore, beneath the operculum (Fig. 11). The dense vesicles of Golgi origin fused to form the haplosporosomes (Fig. 11). The spore became very electron dense during the final process of maturation (Fig. 2). The transmission of the parasite from one host to another was not observed in the present study.

**DISCUSSION**

The first proposed sequences of the development of haplosporidians found in the literature were diagrammatic drawings based on light microscope observations (Sprague, 1963; Couch et al., 1966). Later, some isolated stages of the development of some haplosporidian species were presented. However, none of these studies described the complete development (Haskin et al., 1960; Sprague, 1963; Couch et al., 1966). More recently, some life-cycle stages of the 2 most representative genera, i.e., *Haplosporidium* and *Minchinia*, were described based on ultrastructural data (Perkins, 1968, 1975a, 1975b; van Banning, 1977; Marchand and Sprague, 1979; Desportes and Nashed, 1983; La Haye et al., 1984; Azevedo and Corral, 1989; Hillman et al., 1990).

The successive karyokinesis by some haplosporidian species without cytokinesis, producing multinucleate plasmodia, seems to be a common process, at least in species of *Haplosporidium* and *Minchinia* (Perkins, 1968; Marchand and Sprague, 1979; Desportes and Nashed, 1983; La Haye et al., 1984; Azevedo et al., 1985; Burreson, 1994; Hine and Thorne, 2002). These results suggest that this parasite may have only a single host, since all stages of the development were simultaneously observed amongst the host cells, corresponding to autoinfection (Azevedo and Corral, 1989). Recent molecular phylogenetic analyses suggest evidence of the presence of an unidentified haplosporidian in polychaetes (Siddall and Aguado, 2006), possibly serving as intermediate host (Burreson and Ford, 2004). Presently, the ultrastructural characteristics of a complete life cycle of haplosporidian species have not been reported, since a complete life cycle is unknown.

The developmental cycle described in the present work shows ultrastructural differences between species of *Haplosporidium* and *Minchinia*. In several species, there is a mitotic apparatus, previously described as “Kernstab” by Jirovec (1936). This apparatus was observed during interphase and in mitotic nuclei; it consisted of 2 spindle polar bodies, a free nuclear envelope, and a bundle of numerous longitudinal microtubules between them (Perkins, 1968, 1975a, 1975b; Marchand and Sprague, 1979; Desportes and Nashed, 1983; Azevedo et al., 1985; Azevedo and Corral, 1989). This structural organization was not observed in *H. montforti*. The sequential developmental cycle stages of typical haplo-
sporidian species (Haplosporidium, Minchinia, and Urosporidium) have been well documented in several ultrastructural studies (Couch et al., 1966; Desportes and Nashed, 1983; Haskin and Andrews, 1988; Azevedo and Corral, 1989; Burreson, 1994; Azevedo, 2001). However, the life cycle descriptions of the different Bonamia species are insufficient, and the formation of true haplosporidian spores has rarely been described in most species. Only in the life cycle of B. perspora (a new species recently described) was the formation of true spores observed (Carnegie et al., 2006). The presence of these spores in B. perspora obviously suggests that other Bonamia spp. may also produce spores (Carnegie et al., 2006). However, in Bonamia sp., only unincellate stages have been observed and considered as infecting forms that penetrate into the hemocytes (Montes et al., 1994).

ACKNOWLEDGMENTS

This work was partially supported by Eng° A. Almeida Foundation, Porto, Portugal. We would like to thank João Carvalheiro for technical assistance, and the associate editor and anonymous reviewers for their most helpful comments and suggestions.

LITERATURE CITED


FIGURES 7–11. Some ultrastructural aspects of the different life-cycle stages of the parasite Haplosporidium montforti (scale bars = μm). (7) Multinucleate plasmodium (*) with irregular contour (arrows), containing mitochondria (M) and some nuclei (N), and amongst them, several cisternae (arrowheads) begin the differentiation to form the sporoblast membranes. (8) Detail of part of a sporocyst showing the initial process of the sporoblast formations, each showing a nucleus (N), mitochondria (M), and peripheral sporoblast membrane (arrowheads). (9) Detail of the initial process of spore wall formation showing several blisters of dense material (arrowheads), the pre-operculum system (Op), the nucleus (N), and some vesicular structures, possibly the primordial structures of the sporeplasmosa (arrows). (10) Several immature spores observed in spore wall process, in which the wall (Wa) forms a continuous dense structure in close association with the operculum (Op). The endosporeplasmas of each immature spore contains a nucleus (N), a pre-spherulosoma (arrowhead), and the exosporoplasm with 1–2 large vacuoles (V). (11) An immature spore showing the spore wall (Wa) and the endosporeplasma (*) with the operculum (Op), the apical sporeplasmosa (S), mitochondria (M), and basal nucleus (N).


EXAMINATION OF NATURALLY EXPOSED BOTTLENOSE DOLPHINS 
(TURSIOPS TRUNCATUS) FOR MICROSPORIDIA, CRYPTOSPORIDIA, AND GIARDIA

R. Fayer, P. A. Fair*, G. D. Bossart†, and M. Santín
Environmental Microbial Safety Laboratory, Animal and Natural Resources Institute, Beltsville Agricultural Research Center, United States Department of Agriculture, Beltsville, Maryland 20705-2350. e-mail: ronald.fayer@ars.usda.gov

Received 14 March 2007; revised 7 June 2007; accepted 3 August 2007.
* National Oceanic and Atmospheric Administration, NOS Center for Coastal Environmental Health and Bimolecular Research, Charleston, South Carolina 29412-9110.
† Division of Marine Mammal Research and Conservation, Harbor Branch Oceanographic Institution, Fort Pierce, Florida 34946.

ABSTRACT: Bottlenose dolphins (Tursiops truncatus) captured in the estuarine waters off the shores of South Carolina and Florida were examined for the presence of Microsporidia, Cryptosporidium sp., and Giardia sp. DNA extracted from feces or rectal swabs was amplified by polymerase chain reaction using parasite-specific small subunit ribosomal RNA gene primers. All positive specimens were subjected to gene sequence analysis. Of 83 dolphins, 17 were positive for Microsporidia. None was positive for Cryptosporidium or Giardia. Gene sequence data for each of the positive specimens were compared with data in GenBank. Fourteen specimens were found similar to, but not identical to, the microsporidian species Kabatana takedai, Tetramicro brevicilium, and Microgemma tinca, reported from fish, and possibly represent parasites of fish eaten by dolphins. Gene sequence data from 3 other specimens had ~87% similarity to Enterocyrtospora bovis, a species known primarily to infect humans and a variety of terrestrial mammals, including livestock, companion animals, and wildlife. It is not clear if these specimens represent a species from a terrestrial source or a closely related species unique to dolphins. There were neither clinical signs nor age- or gender-related patterns apparent with the presence of these organisms.

Reports of marine mammals infected with protist parasites were recently summarized (Dubey et al., 2003; Fayer et al., 2004). Giardia sp. cysts were detected in feces from a California sea lion; ringed seals in the Arctic; harp, grey, and harbor seals in the Gulf of St. Lawrence; and ringed seals and right and bowhead whales from the North Atlantic (Olson et al., 1997; Measures and Olson, 1999; Deng et al., 2000; Hughes-Hanks et al., 2005), but there are no reports of Giardia sp. detected in dolphins. Likewise, Cryptosporidium spp. oocysts were detected in an Australian dugong, California sea lions, seals in the Arctic, and ringed seals, as well as right and bowhead whales from the North Atlantic (Hill et al., 1997; Deng et al., 2000; Hughes-Hanks et al., 2005; Santín et al., 2005), but there are no reports of Cryptosporidium sp. detected in dolphins. Toxoplasma gondii was identified histologically in seals, dolphins, sea lions, a manatee, and a Beluga whale from sites worldwide (Dubey et al., 2003) and antibodies have been detected by serology from a variety of marine mammals worldwide (Fayer et al., 2004). In bottlenose dolphins captured in estuarine waters near Charleston, South Carolina (CHS) and in the Indian River Lagoon, Florida (IRL), a high percentage of dolphins had antibodies to T. gondii (Dubey et al., 2005). In contrast to the aforementioned protists, no reports were found for Microsporidia infections in any species of marine mammals.

The Dolphin Health and Risk Assessment Project was established as a scientific study to evaluate the health of dolphins in CHS and the IRL. Fecal specimens were collected for the present study in addition to a comprehensive suite of nonlethal morphologic and clinicopathologic parameters. These specimens were subjected to DNA extraction, polymerase chain reaction (PCR) amplification of the small subunit (SSU) ribosomal (r)RNA gene, and gene sequence analysis to determine the prevalence and species of Microsporidia, Cryptosporidium, and Giardia.

MATERIALS AND METHODS

Study sites

The IRL is a shallow water ecosystem, 250 km long, comprising 40% of the central east coast of Florida, including the estuaries of the Indian River, Banana River, and the Mosquito Lagoon. Because there is limited exchange of waters between the IRL and the Atlantic Ocean, the IRL is vulnerable to pollutants from land drainage brought primarily by fresh- and storm-water discharges (Scott et al., 2003). All of the sugarcane crop, approximately 38% of the citrus crop, and 42% of the vegetable crops grown in Florida drain into the IRL (Miles and Pfeuffer, 1997). Wastewater treatment plants also discharge into the IRL. Dolphins in the IRL experienced a high mortality event (Marine Mammal Commission, 2002) and many were diagnosed with skin lesions from protozoans, fungi, dolphin pox, and an unknown vesicular agent (Bossart et al., 2003).

The Charleston Harbor estuary has the second largest container port on the Atlantic coast, is surrounded by urban development, and receives discharge from 2 sewage treatment plants. This 1.920 km² estuary formed by the Cooper, Ashley, and Wando rivers drains over 26,000 ha of marsh and tidal habitat on the South Carolina coast (Tiner, 1977). The average depth is 12 m at low tide, with mean semidiurnal tides of 1.6 m near the ocean (NOS, 1988). Sites with high levels of heavy metals and organic compounds have been identified (Long et al., 1998). Sediments from Charleston Harbor and the Ashley and Cooper rivers contain high concentrations of trace metals, polychlorinated biphenyls, and pesticides (Long et al., 1998). The study area extends to the adjacent Stono River estuary, a C-type salt marsh estuary with little freshwater inflow (Day et al., 1989), which is influenced by residential development.

Naturally exposed dolphins and specimen collection

The present study was part of a multidisciplinary study to evaluate the health of bottlenose dolphins (Tursiops truncatus) in the IRL and CHS. In 2004 and 2005 (June and July in the IRL and August in CHS), 83 bottlenose dolphins were captured, sampled, and released as described in Fair et al. (2006). A standardized evaluation included physical and ultrasound examination, morphometric measurements, and collection of blood, urine, feces, blubber, and skin tissue for hematology, serum chemistry, microbiology, immunology, contaminants, and a suite of biomarkers. A tooth was extracted and age was estimated by examination of postnatal dentine layers (Hohn et al., 1989). Fecal specimens were obtained via enema and rectal swabs. The capture location, age, and gender for dolphins in this study are found in Table I.

Fecal processing, DNA extraction, and molecular characterization

Sterile wrapped cotton-tipped swabs with hardwood handles were used to swab the rectum of each captured dolphin. The swabs were then inserted into 15-ml centrifuge tubes containing 360 µl of ATL buffer (Qiagen, Valencia, California). Tubes were capped and placed on ice.
for the day, all centrifuge tubes and fecal cups were transferred to a 5°C refrigerator. Liquid feces from each cup were transferred to a 15-ml capped, labeled, and also placed on ice.

The remaining protocol followed manufacturer’s instructions with 1 exception. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 µl of AE buffer. For species of Microsporidia, generic primers that amplify a fragment (~292 base pairs [bp]) were used (Fedorko et al., 1995; Fayer, Santin, and Trout, 2003). For *Giardia* sp. and *Cryptosporidium* sp., a fragment of the SSU rRNA (~292 bp and 800 bp, respectively) genes were amplified by nested PCR as previously described (Hopkins et al. 1997; Xiao et al., 1999). Negative and positive controls were included in all PCR sets. The negative control from the first PCR was amplified in the second reaction to check for low-level contamination. Positive controls consisted of DNA from *Cryptosporidium parvum*, *Giardia duodenalis* assemblage C, and *Encephalitozoon cuniculi*.

The PCR products were subjected to electrophoresis in a 1% agarose gel and were visualized by staining the gel with ethidium bromide. All PCR-positive samples were directly sequenced. PCR products were purified using EXO-SAP enzyme (USB, Cleveland, Ohio). Purified products were sequenced with the same PCR primers used for the original amplification in 10-µl reactions, Big Dye® chemistries, and an ABI3100 sequencer analyser (Applied Biosystems, Foster City, California). Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Madison, Wisconsin). Sequences obtained were compared with sequences in the GenBank database by BLAST analysis. Nucleotide sequences were deposited in GenBank under accession numbers EF672508 to EF672514.

**RESULTS**

PCR results in the present study detected Microsporidia in feces from 17 of 83 dolphins, including 10 males and 7 females ranging from 3.5 to 23 yr of age (Table II). All PCR attempts to find species of *Cryptosporidium* and *Giardia* were negative.

**2004 samples**

Seven of the 32 dolphins captured in the IRL and 3 of the 19 captured in the CHS were positive for Microsporidia (Table II). Four distinct genetic groupings of Microsporidia were identified (1B, 2, 3A, and 3B). Within each group were isolates with identical gene sequences. Each group in turn was similar or identical to Microsporidia previously reported from fish (Table III). For example, group 2, found in 1 male and 1 female, exhibited 100% similarity to a small-bp gene sequence of *Kabatana takedai* (GenBank AF356222). Group 3A, found in 4 males and 1 female, and group 3B, found in 1 female, exhibited 97.4 and 98.9% similarity, respectively, with *Microgemma tinaeae* (GenBank AY651319). Group 1B, found in 2 males, exhibited 94.1% similarity with *Tetramicra brevifilum* (GenBank AF364303).

**2005 samples**

Of 14 dolphins captured in the IRL and 18 captured in the CHS, 3 and 4, respectively, were found positive for Microsporidia (Table II). Two groups of Microsporidia were identified that were similar to species reported from fish (1A and 2).
This finding suggests the likelihood that dolphins had eaten fish, as fish were recovered from the dolphin gastrointestinal tract. Five genetic groups were found with similarity to Mi-

crosporidia previously found only in extraintestinal tissues of fish. Although too few dolphins were found to be excreting Microsporidia previously found only in extraintestinal tissues of fish. Two other groups, slightly different from each other, 4A and 4B, were similar to Enterocytozoon bieneusi, a species with many genotypes reported from humans and terrestrial animals. Group 4A, found in 2 females, and group 4B, found in 1 male, exhibited 87.6 and 86.5% similarity, respectively, with E. bieneusi (GenBank AF023245 and AF024657). These 2 groups exhibited 85.3 and 85.7% similarity, respectively, with Nucleospora salmonis (GenBank AF185996).

**Age, gender, and location of PCR-positive dolphins**

Age and gender data are summarized by year and location in Table I. Ages were obtained for 49 of 51 dolphins captured in 2004 and 32 of 32 dolphins captured in 2005. Overall, there were more specimens from males than females (52 vs. 31) and more dolphins examined from the IRL than from the CHS (46 vs. 37). Over the 2-yr study, a similar range in the ages of captured males and females was found at both sites (Table I). Although too few dolphins were found to be excreting Microsporidia of groups 1A, 1B, 3B, 4A, and 4B, for comparison it was apparent that all 5 dolphins excreting group 2 were from CHS and 4 of 5 dolphins excreting group 3A were from IRL (Table II). There did not appear to be gender- or age-related patterns related to the detection of Microsporidia.

**DISCUSSION**

There are no earlier reports of Microsporidia infecting dolphins or other sea mammals. Bootstrap analysis of the SSU rRNA gene from 125 microsporidian isolates placed these isolates into 5 clades (Vossbrinck and Debrunner-Vossbrinck, 2005). The groups 1A, 1B, 2, 3A, and 3B identified in the present study fall within clade III designated as Class Marinosporidia. Groups 4A and 4B fall within clade 4, designated as Class Terresporidia of terrestrial origin.

In the present study, dolphin fecal specimens yielded PCR products with gene sequences similar to 3 species of Microsporidia previously found only in extraintestinal tissues of fish. This finding suggests the likelihood that dolphins had eaten fish infected with Microsporidia and undigestable spores from those fish were recovered from the dolphin gastrointestinal tract. If so, it is unlikely that the spores represent species that infect dolphins. Five genetic groups were found with similarity to Microsporidia reported from fish whose known geographic ranges are distant from the sites where the dolphins were captured. Perhaps other species of fish located along the Atlantic coast of North America harbor the same Microsporidia that heretofore have not been identified as parasites of these fish. Microgemma tineae is a xenoid-inducing microsporidian that infects the liver of the peacock wrasse Symphodus tinca (Mansour et al., 2005). These fish range from Spain to Morocco, including the Mediterranean Sea and Black Sea. Estimates of the prevalence of M. tineae in fish caught along the Tunisian coast have been as high as 43% (Mansour et al. 2005). Kabatana takedai is an important pathogen affecting the heart and trunk muscles of wild and cultured salmonid fish and has been reported only from the Pacific waters near Japan and Russia (Fujiyama et al., 2002). At different times, this organism has been assigned to Pleistophora, Glugea, and Microsporidium, but phylogenetic analysis has now shown with high confidence that it is most closely related to the Microsporidia Spraguea lophii and Microgemma sp. (Lom et al., 2001). Tetramicra brevifilum, an important pathogen in turbot (Scophthalmus maximus) fish farms along the Atlantic coast of northwest Spain, has been found in 10% of wild turbot off the northern coast of Cornwall, and in black anglerfish (Lophius budogassaga) along the Mediterranean coast of Barcelona, Spain (Maillo et al., 1998). Spores were detected by PCR at highest density in turbot muscles beneath the dorsal fin (Leiro et al., 1998) and xenomas have been found in turbot liver parenchyma (Lom and Dykova, 2005).

The presence of spores of E. bieneusi or N. salmonis or closely related species in dolphin feces raises the question as to whether dolphins were actually infected with these organisms or whether they were simply passing through the gastrointestinal tract. A variety of terrestrial mammals and 2 avian species (chickens and pigeons) are the only known hosts that excrete spores of E. bieneusi (Rietz et al., 2002; Haro et al., 2005). Infected animals or persons can excrete millions of E. bieneusi spores that could be washed into coastal waters via runoff or discharged from wastewater treatment plants or leaky septic tanks. Spores of E. bieneusi have been detected in surface waters (Sparfel et al., 1997). Sera from all of the captured dolphins in the present study were found to have antibody against Toxoplasma gondii, suggesting that runoff or wastewater was a likely source of contamination of coastal waters, with oocysts from cat feces at both sites (Dubey et al., 2005). If data from the present study indicating the presence of E. bieneusi-like organisms in dolphins are confirmed by finding the same genotypes in terrestrial animals, environmental contamination with feces

---

**Table III. Comparison of Microsporidia genotypes detected in dolphins with named species previously reported in fish, birds, and mammals.**

<table>
<thead>
<tr>
<th>Microsporidia group (GenBank accession no.)</th>
<th>No. positives</th>
<th>No. of base pairs (bp)</th>
<th>bp/bp (%) similarity</th>
<th>Species name (GenBank accession no.)</th>
<th>Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (EF672508)</td>
<td>0</td>
<td>251</td>
<td>86.5</td>
<td>Tetramicra brevifilum (AF364303)</td>
<td>Turbot</td>
</tr>
<tr>
<td>1B (EF672509)</td>
<td>2</td>
<td>287</td>
<td>96.1</td>
<td>Tetramicra brevifilum (AF364303)</td>
<td>Turbot</td>
</tr>
<tr>
<td>2 (EF672510)</td>
<td>2</td>
<td>295</td>
<td>95.6</td>
<td>Kabatana takedai (AF356222)</td>
<td>Salmonid</td>
</tr>
<tr>
<td>3A (EF672511)</td>
<td>5</td>
<td>292</td>
<td>141/149 (94.6)</td>
<td>Microgemma tineae (AY651319)</td>
<td>Labridae</td>
</tr>
<tr>
<td>3B (EF672512)</td>
<td>1</td>
<td>267</td>
<td>95.1</td>
<td>Microgemma tineae (AY651319)</td>
<td>Labridae</td>
</tr>
<tr>
<td>4A (EF672513)</td>
<td>0</td>
<td>251</td>
<td>86.5</td>
<td>Enterocytozoon bieneusi (AF023245/AF024657)</td>
<td>Bird/mammal</td>
</tr>
<tr>
<td>4B (EF672514)</td>
<td>0</td>
<td>251</td>
<td>86.5</td>
<td>Enterocytozoon bieneusi (AF023245/AF024657)</td>
<td>Bird/mammal</td>
</tr>
</tbody>
</table>
from land would appear to be a logical source of the organisms in dolphins. If no identical or closely related genotype is found in terrestrial animals, the organisms detected in the present study might represent a new genotype or closely related species unique to dolphins. Atlantic salmon (Salmo salar) are the only reported hosts for N. salmonis (Genbank AF185996). Neither Atlantic salmon nor other salmonids are known to inhabit the waters in or near CHS or IRL. Other species of fish might serve as hosts to other species of Nucleospora or the Microsporidia of groups 4A and 4B.

In the present study primers previously used successfully to detect Cryptosporidium spp. and Giardia spp. by PCR yielded negative results. Although both genera have been detected in other aquatic mammals (Fayer et al., 2004), neither has been reported in any species of dolphins. If there is an age-related preference of these genera for young dolphins, as shown for some species of Cryptosporidium in cattle (Fayer et al., 2007), their presence would have been missed because all dolphins in the present study were over 2.5 yr of age.

The 2 dolphin capture sites were geographically distant and reflected different ecological conditions. Although dolphins at both sites were exposed to a variety of chemical contaminants, there were no reports that dolphins from South Carolina had been diagnosed with skin lesions from infectious agents as had those from the IRL (Bossart et al., 2003). It was interesting to observe in the present study that 5 of 6 dolphins excreting Microsporidia of groups 4A and 4B were from IRL; the closest reported species of Microsporidia to those of groups 4A and 4B were found in Labridae (Table III), and Labridae are apparently common along the Atlantic shore near the south IRL. Seven species of Labridae were found in Coral Cove and Carlin Park areas, north and south of the inlet to the south IRL (Lindeman and Snyder, 1999). All 5 dolphins excreting Microsporidia of group 2 were from CHS and the closest reported species of Microsporidia to those of group 2 were reported in salmonid fishes (Table III). However salmonids have not been reported in the CHS region. It is not known if other fish species in the CHS region might also serve as hosts for Kabatana sp.-like Microsporidia.

ACKNOWLEDGMENTS

The dolphin health assessment is a collaborative project between the National Ocean Service Center of Coastal Environmental Health and Biomolecular Research and the Harbor Branch Oceanographic Institution. Samples were collected under National Marine Fisheries Permit No. 998-1978-00, issued to G.D.B. We thank Wayne McFee for age analysis of teeth dentine and Jeff Adams for the spatial distribution graphical data. We also thank Barbara Lyon of NOAA and Brooke Reich and Kristen Cameron of the EML for technical support. This research was supported in part by the NOAA Fisheries Marine Mammal Health and Stranding Response Program, NOAA National Ocean Service, and the Florida Protect Wild Dolphins License Plate Fund.

LITERATURE CITED


Technical Memorandum NOS ORCA 128, Silver Spring, Maryland, 289 p.


FIRST RECORD OF TRYPANOSOMA CHATTONI IN BRAZIL AND OCCURRENCE OF OTHER TRYPANOSOMA SPECIES IN BRAZILIAN FROGS (ANURA, LEPTODACTYLIDAE)

M. Lemos, D. H. Morais*, V. T. Carvalho†, and M. D’Agosto‡
Departamento de Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Campus Universitário, Martelos, 36.036-330, Juiz de Fora, Minas Gerais, Brazil. E-mail: marta.dagosto@ufjf.edu.br

ABSTRACT: The present study provides the first record of Trypanosoma chattoni Mathis and Leger, 1911, in a new host, Leptodactylus fuscus Schneider, 1799 (Anura, Leptodactylidae), and the occurrence of Trypanosoma rotatorium–like species in Leptodactylus chaquensis Cei, 1950. The anurans were captured in the State of Mato Grosso, Brazil. Blood samples were obtained by cardiac puncture, and blood smears were examined for the presence of hemoparasites. The Trypanosoma rotatorium–like species in this study refers to a short-bodied trypanomastigote that has a conspicuous undulating membrane but lacks a free flagellum; T. chattoni refers to a monomorphic parasite that has a rounded body, a kinetoplast adjacent to the nucleus, and a short flagellum.

Anuran trypanosomes have a wide geographical distribution and have been described from many countries (Billet, 1904; Bouet, 1909; Feng and Chung, 1940; Mackerras and Mackerras, 1961; Bardsley and Harmsen, 1973; Guerrero and Ayala, 1977; Barta et al., 1989; Martin and Desser, 1990; Misra and Chandra, 1995). According to Desser (2001), there are more than 70 species of Trypanosoma that parasitize anurans, although the validity of several species is questioned because some may be pleomorphic. To date, most trypanosomes of amphibians have been found in species of Rana (Anura, Ranidae) and Bufo (Anura, Bufonidae) in Europe, the Americas, Africa, and Asia (Brumpt, 1928; Fantham et al., 1942; Ruiz and Alfaro, 1958; Diamond, 1965; Bardsley and Harmsen, 1973; Werner and Walowski, 1976; Miyata, 1978; Jones and Woo, 1986; Werner, 1993; Desser, 2001; Žickus, 2002). In Brazil, trypanosomes have been recorded by Marchoux and Salimbeni (1904), and by Carini (1907), Machado (1911), Gonçalves da Costa Silva (1969), Pessoa (1969), Pereira et al. (1973), and Lemos et al. (2004) in leptodactylids (Anura, Leptodactylidae).

Trypanosoma rotatorium (Mayer, 1843) is a polymorphic species with a wide geographical distribution, while T. chattoni Mathis and Leger, 1911, is a monomorphic species that is found in Asia, Europe, and the Americas (Diamond, 1965; Bardsley and Harmsen, 1973; Jones and Woo, 1986; Martin et al., 2002). The purpose of the present study is to morphologically and morphometrically characterize the species of Trypanosoma found in Leptodactylus chaquensis Cei, 1950, and Leptodactylus fuscus Schneider, 1799, in the State of Mato Grosso, Brazil. It is also an opportunity to record the occurrence of T. chattoni in a new host, Leptodactylus fuscus Schneider, 1799 (Leptodactylidae). The study was conducted as part of a fauna survey and rescue operation, accomplished in the vicinity of the Guaporé Hydroelectric Power Station, in the State of Mato Grosso, Brazil.

MATERIALS AND METHODS
During the fauna rescue activities at the Guaporé Hydroelectric Power Station (15°07’32”S, 58°57’16”W) at the boundary between the towns of Vale de São Domingos, Pontes, and Lacerda, Mato Grosso, Brazil, leptodactylid anurans were captured under permits nos. 011/02, 030/02, and 001/UHE Guaporé from the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA). Blood samples were obtained by cardiac puncture. The trypanosomes found were photographed using a ×100 objective and ocular ×10 magnification in Olympus BX40. Data were analyzed statistically with the Mann–Whitney U-tests and the Spearman correlation. P values were considered to be significant when equal to, or less than, 0.05.

RESULTS

Trypanosoma rotatorium–like species

Description

Diagnosis: Various morphometric parameters are given in Table I. The body is short and round and lacks a free flagellum. The nucleus is oval and elongate; it is circled by a lightly stained area, situated in posterior end of body (IN > 1); and it is peripheral and close to undulating membrane (Fig. 1). A round kinetoplast is pink in color, located near nucleus, and encircled by lighter stained area. The cytoplasm is granular, with vacuoles along cytoplasmic extension. Some individuals have densely pigmented cytoplasm and light canals inside cellular cytoplasm (Fig. 1). An undulating membrane is conspicuous, with 3 or 4 undulations (Figs. 2, 3). Statistical analysis, i.e., Spearman correlation tests, of morphometric data from the T. rotatorium–like species showed that body length and width increase with increasing nucleus length and width, (r = 0.67 and P < 0.003) and (r = 0.69 and P < 0.003), respectively.

Taxonomic summary

Host: Leptodactylus chaquensis Cei, 1950, family Leptodactylidae.

Type locality: Vicinity of São Domingos, Pontes, and Lacerda, Mato Grosso, Brazil (15°07’32”S, 58°57’16”W), Prevalence in type locality: 50% of analyzed hosts.

Date of collection: November 2005.

Specimens deposited: Trypanosoma rotatorium–like species.

The specimens were deposited in the Protozoological Collection of the Oswaldo Cruz Institute (PCIOC), Manguinhos, Rio de Janeiro, Brazil, slide number series B 2007-050.

Habitat: Bloodstream of L. chaquensis.

Remarks

The Trypanosoma rotatorium–like species observed in this study (Figs. 1–3) possesses a round shape, lacks a free flagell-
lum, and has a conspicuous undulating membrane similar to that reported by Machado (1911); it is morphologically similar to those reported by Jacoño (1938), Vucetich and Giacobbe (1949), Miyata (1978), and Werner (1993). According to Machado (1911), the round shapes precede *T. rotatorium*’s division phase. In this pre-reproductive period, the kinetoplast migrates toward the nucleus, and the cytoplasm and the undulating membrane become short (Machado, 1911). The position of the undulating membrane varied among the analyzed individuals. In some specimens, the membrane was found in a midbody position, marking the cytoplasm with clear canals (Figs. 2, 3); in others, it was observed at the periphery (Fig. 1). Vucetich and Giacobbe (1949) in Argentina and Miyata (1978) in Japan verified this variation in *T. rotatorium*. The membrane position was attributed to the parasite’s position at the moment the smear was prepared.

**DESCRIPTION**

*Trypanosoma chattoni*

**Diagnosis:** Various morphometric characters of *T. chattoni* are presented in Table II. This species has a round to oval body, and the cytoplasm is densely stained, vacuolated (Fig. 4), and granular. Is has a spherical nucleus (Fig. 5), with little condensed chromatin, in the midbody region. Kinetoplast is juxtaposed with the nucleus (Fig. 6); in 3 specimens, kinetoplast is superimposed on the nucleus (Fig. 5). The flagellum is small, marks cytoplasm with a clear canal, and does not exceed half the body length (Figs. 4–6). Statistical analysis of morphometric data from *T. chattoni*, according to the Spearman correlation test, showed that nucleus length and width increase with increasing body length and width (*r* = 0.67 and *P* < 0.008) and (*r* = 0.72 and *P* = 0.01), respectively. A Mann–Whitney U-test showed that morphometric data for width (*P* < 0.0003), nucleus length (*P* < 0.008), and nucleus width (*P* < 0.002) differed significantly between *T. rotatorium*-like species and *T. chattoni*.

**Taxonomic summary**

**Host:** *Leptodactylus fuscus* (Schneider, 1799). Family Leptodactylidae.

**Type locality:** Vicinity of São Domingos, Pontes, and Lacerda, Mato Grosso, Brazil (15°07’32”S, 58°57’16”W).

**Prevalence in type locality:** 100% of analyzed hosts.

**Date of collection:** November 2005.

**Specimens deposited:** *Trypanosoma chattoni*. The specimens were deposited in the Protozoological Collection of the Oswaldo Cruz Institute (PCIOC), Manguinhos, Rio de Janeiro, Brazil, slide number series B 2007-51.

**Habitat:** Bloodstream of *L. fuscus*.

**Remarks**

*Trypanosoma chattoni* reported in the present study is morphologically similar to the shapes found by Diamond (1965), Miyata (1978),

---

**Table I.** Morphometric data of the *Trypanosoma rotatorium* found in the Americas and Asia. Data are presented in µm, disposed by mean ± standard deviation and amplitude (lower and higher values).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Trypanosoma rotatorium–like</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>42.25 ± 3.57 (39–48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>23 ± 4.1 (18–29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WN</td>
<td>3 ± 1 (2–4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td>9.66 ± 0.57 (9–10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>21.33 ± 1.15 (20–22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN</td>
<td>23.33 ± 2.88 (20–25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KN</td>
<td>1.33 ± 0.57 (1–2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>28.28 ± 2.43 (23–30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK</td>
<td>12.71 ± 1.68 (8–19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* BL = body length, BW = body width, WN = width of nucleus, LN = length of nucleus, AK = distance from anterior end to kinetoplast, PK = distance from posterior end to kinetoplast.

**Table II.** Morphometric data of the *Trypanosoma chattoni* found in the Americas and Asia. Data are presented in µm, disposed by mean ± standard deviation and amplitude (lower and higher values).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Leptodactylus fuscus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>48.95 ± 4.6 (39–57)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>36.05 ± 6.2 (20–43)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WN</td>
<td>4.37 ± 2.3 (2–9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td>5.82 ± 2.1 (2–9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>1.21 ± 0.22 (0.93–1.56)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>8.09 ± 2.38 (2–12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Rana clamitans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>52 ± 7.27 (37–64)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>39.93 ± 7.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN</td>
<td>5.43*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KN</td>
<td>9.1 ± 0.24 (7–11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>9.47 ± 2.89</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Rana pipiens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>40.72 ± 7.63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>39.93 ± 7.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN</td>
<td>3.8 ± 0.51*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KN</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>7.2 ± 2.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* BL = body length, BW = body width, WN = width of nucleus, LN = length of nucleus, AK = distance from anterior end to center of nucleus, PK = distance from posterior end to center of nucleus.

* Distance from kinetoplast to posterior end to nucleus.

1 Refers to diameter.

Figures 1–6. Photomicrographs of Trypanosoma rotatorium–like species from Leptodactylus chaquensis and Trypanosoma chattoni from Leptodactylus fuscus; scale bar = 10 μm. Trypanosoma rotatorium–like species: (1) rounded form with a conspicuous undulating membrane; the arrow shows the kinetoplast (Kp); (2) undulating membrane marking the cytoplasm with light canals (arrow) and rounded kinetoplast (arrow); (3) undulating membrane located in midbody position (arrow). Trypanosoma chattoni: (4) parasite with a spherical nucleus (arrow) and a short flagellum, F (arrow); (5) parasite with kinetoplast superimposed onto the nucleus (arrow) and flagellum marking the cytoplasm with light canal; (6) a short flagellum emerging near the kinetoplast adjacent to the nucleus (arrows).

Jones and Woo (1986), and Werner (1993). However, these forms were smaller and more slender than those described by Mathis and Leger (1911), larger and more slender than those found in the United States by Diamond (1965) and Canada by Jones and Woo (1986), and smaller than those found in China by Werner (1993) (Table II).

Discussion

The morphometric analysis of the T. rotatorium–like species found in this study is similar to the one made by Fantham et al. (1942) and Werner (1993) (Table I). However, the report provided by Werner (1993) indicated that the kinetoplast was situated next to the posterior end of the body. In the present study, the kinetoplast is next to the nucleus. We did not observe any cellular division of the parasites. However, it is possible that the analyzed trypanosomes were in the pre-reproductive period, since, according to Machado (1911), it is in this phase that the kinetoplast migrates toward the nucleus.

The morphometric variation observed in T. chattoni in the present study and that reported in China by Werner (1993) and the Americas by Diamond (1965) and Jones and Woo (1986), could be associated with the low host specificity of this species.

Diamond (1965) and Jones and Woo (1986) found T. chattoni in the visceral circulation, mainly in the kidneys, of Rana p nipiens. In the present study, these parasites were detected in the peripheral blood. Mathis and Leger (1911) reported in their description of T. chattoni that the kinetoplast was intranuclear. Vucetich and Giacobbe (1949) found a parasite similar to T. chattoni and mentioned an intranuclear kinetoplast in their description. However, Diamond (1965) questioned their report, suggesting that the kinetoplast was probably displaced during the preparation of the blood smear. This condition was observed in 0.13% of the specimens observed by Jones and Woo (1986) and 0.2% in the present study (Fig. 5).

Several authors have recorded the polymorphism of T. rotatorium (Machado, 1911; Kudo, 1922; Fantham et al., 1942; Vucetich and Giacobbe, 1949; Ruiz and Alfaro, 1958; Diamond, 1965; Creemers and Jadin, 1966; Gonçalves da Costa and Silva, 1969; Bardsley and Harmsen, 1973; Miyata, 1978; Barta et al., 1989; Desser, 2001). Vucetich and Giacobbe (1949) and Creemers and Jadin (1966) established morphotypes for variations of T. rotatorium. Vucetich and Giacobbe (1949) and Scorza and Dagert (1958) proposed the occurrence of a single species of trypanosome in anurans in the Americas, recognizing the others as variations of T. rotatorium. However, according to Werner and Walewski (1976), T. chattoni is synonymous with T. rotatorium. Dutton et al. (1907) and Werner and Walewski (1976) both suggested that latent bodies are formed by rounding the cellular body via contraction of the cytoplasm, retraction of the undulating membrane, and loss of the free flagellum.

Nevertheless, the two species of Trypanosoma found in the present study were morphologically and statistically distinct.

Acknowledgments

The authors thank the Comissão de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) and Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) for financial support and Dr. Juliane Lopes for a critical review of the manuscript.
LITERATURE CITED


EFFECT OF AGE OF THE INTERMEDIATE HOST TRIBOLIUM CONFUSUM (COLEOPTERA) ON INFECTION BY HYMENOLEPIS DIMINUTA (CESTODA)

Allen W. Shostak
Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 1W4, Canada. e-mail: al.shostak@ualberta.ca

ABSTRACT: A cross-sectional study of 27 cohorts of Tribolium confusum aged 2–78 wk was done to examine effects of host age on exposure to eggs of Hymenolepis diminuta under standardized conditions. Pre-exposure, fasting, and postexposure mortality were low, sex ratio was equal, and fecundity of hosts was high during the first 30 wk, followed by increasing mortality and male bias of the sex ratio, and declining fecundity, in older beetles. These changes in the host were not associated with pronounced changes in infection results. Prevalence of infection was higher in females than males, but was unaffected by age in both sexes. Intensity of infection was similar between sexes in beetles up to 30 wk old, and thereafter declined in females, but not in males. Age-related changes in hosts were gradual, but unexpected levels of short-term variation in infection results suggest that some undetermined proximate factors may override general host age effects on the infection process.

Host age affects infections of insect hosts where age correlates with body size, such as in larval insects infected with parasitoids (Colinet et al., 2005) or where defensive capabilities of invertebrate hosts change with age (Blaser and Schmid-Hempel, 2005). Beetles (Tenebrionidae) are commonly used as experimental hosts for protozoan and helminth parasites, particularly the cestode Hymenolepis diminuta. Control of host age is common in these experimental studies, but only 3 studies have specifically examined the effect of varied host age on parasite establishment following a controlled exposure of flour beetles Tribolium confusum to eggs of the cestode H. diminuta. Kelly et al. (1967) infected virgin beetles 4–51 wk old at the time of infection. Dunkley and Mettrick (1971) infected beetles that were 4–12 days old. Keymer (1982) infected beetles over the range 2–14 wk posteclosion. All studies reported age effects. Other studies (Soltice et al., 1971; Mankau, 1977; Yan and Norman, 1995; Robb and Reid, 1996) have used a single age class of beetle, virgin or mated, but only up to 6 wk old. Collectively, these studies fall short of encompassing the normal life span of T. confusum, which can live 77 wk or more (Pearl et al., 1941), particularly for mated hosts, which are more likely to reflect the status of older individuals. Moreover, comparisons among previous studies are hampered by variations in methods used to infect beetles.

This article reports a cross-sectional study on cohorts of mixed-sex, adult T. confusum, aged 1–78 wk at the time of exposure to eggs of H. diminuta. Patterns of host survival and fecundity were used to identify different phases of adult life, and prevalence and intensity of infection and parasite growth were compared among these phases.

MATERIALS AND METHODS

Animals

History and general maintenance conditions of hosts and parasites are described elsewhere (Shostak et al., 2006). Twenty-seven cohorts of known-age beetles were established between August 2004 and August 2006 by removing up to 200 pupae of T. confusum at a time from stock cultures, and storing them at 26°C in the dark in a 10-cm-diameter glass dish with 15 g of flour/brewer’s yeast medium. The time of pupa collection was termed age 0. The actual time of emergence of adults was not recorded, but typically would occur 1–7 days after collection of pupae. Each 4 wk or less the contents of each dish were passed through a sieve. Adults (live and dead) were censused to determine pre-exposure survival, and live adults were transferred to 15 g of fresh medium; any eggs, larvae, and pupae were discarded. To minimize injury of beetles during the censuses, beetles were poured onto an 18.5-cm-diameter filter paper, which they generally gripped well. Inversion of the paper allowed eggs and debris to fall off, and the adults were counted as they were brushed gently off the paper. Any live adults that fell off the paper were transferred by scooping them gently onto a small piece of filter paper. Eggs of H. diminuta for this specific study were obtained from the same 2 rats, each infected with 10 cysticercoids 8 mo prior to the first exposure. Fresh fecal pellets were collected on each day of exposure, washed through sieves, cleaned with the use of sucrose gradient centrifugation, and diluted to the required concentration in distilled water.

Experimental design

Five of the oldest cohorts died out before any infections were attempted, but these provided data on host mortality rates. Experimental infections were initiated after heavy mortality in the oldest of the remaining 22 cohorts (February 2005) indicated that those beetles were also nearing the end of their life span. Two of 22 cohorts contained a large number of surviving beetles and were subdivided and exposed on separate occasions, so 24 exposures in all were done. Exposures were done over a period of 3 mo for logistic reasons. The order in which cohorts were exposed was selected to minimize gaps in host age in the final data set.

The medium in a dish selected for infection was passed through a sieve. Up to 35 beetles were removed for infection and placed in a clean dish without food (26°C, dark) for 4 days. If ≤35 live beetles remained, they were placed on fresh medium to age further and be infected at a later date. Following the 4-day fast, beetles were censused to determine fasting survival and up to 32 were selected at random for infection. Beetles were placed individually in infection arenas comprising a 44-mm inside diameter acrylic ring placed on a 55-mm-diameter Whatman no. 1 filter paper, on which was placed 400 μl distilled water estimated to contain 1,500 eggs from a suspension mixed with the use of a magnetic stirrer. For logistic reasons, a quick initial estimate of egg density was required and was done with the use of counts on 8 20-μl samples from the stirred suspension, followed by concentration or dilution of the egg suspension to the required density. Some beetles landed on their back when added to the arena. The time required for the beetle to right itself was noted and the midpoint of the range for all beetles in an exposure was recorded as a representative righting time. The arena was covered with a glass dish to prevent beetle escape and to retard drying of eggs and left for 6 hr (room temperature and lighting), then beetles were removed and stored individually in 25-mm glass vials containing 1 g fresh medium for 2 wk (26°C, dark). After beetles had been added to arenas, a more time-consuming total count of eggs in 3 separate, 400-μl samples of the egg suspension was done to confirm the actual exposure dose. At 2 wk, hosts were again censused to determine “post-exposure survival”. The remaining hosts were killed, sexed, and dissected to count and photograph cysticercoids for size determination according to published procedures (Shostak et al., 2006). Beetle egg counts were done after passing the medium from each vial through a 250-μm sieve.
The maintenance procedure appeared to maintain each cohort of uninfected Tribolium confusum. Each line represents a different cohort and each point represents a census day.

**Analysis**

Pre-exposure survival was assessed by 2 methods. Census records for some cohorts were complete from the time of adult emergence and these enabled expression of survival over time relative to the original number of adults. Census records for these as well as the remaining cohorts also provided initial ($N_i$) and final ($N_f$) numbers of live hosts for each cohort and census interval of $t$ wk from which a weekly mortality rate, $\lambda = \log(N_f/N_i)/t$ (Shell et al., 1995) was calculated. Statistical analysis was done with the use of SAS 9.1 (SAS Institute Inc., Cary, North Carolina) PROC CORR, PROC GLM, and PROC FREQ, with the exception of comparisons of prevalence (percent of hosts with 1 or more parasites) and intensity of infection (number of parasites per infected host), which were done with the use of Quantitative Parasitology 3.0 (Rozsa et al., 2000). Data are reported as mean ± SD unless indicated otherwise. Statistical significance was determined with the use of $\alpha = 0.05$.

**RESULTS**

The 5 aspects of host biology that were monitored varied in prevalence (percent of hosts with 1 or more parasites) and intensity of infection (number of parasites per infected host), which was initially even, but became gradually more uniform near 50%. Spearman correlations were used to assess whether other experimental variables contributed to this pattern. The order in which the exposures were done was not correlated with prevalence ($R_{s22} = 0.05, P = 0.83$) or intensity ($R_{s22} = -0.17, P = 0.42$). Similarly, the date on which each cohort was removed from the source culture was not correlated with prevalence ($R_{s22} = -0.01, P = 0.99$) or intensity ($R_{s22} = -0.04, P = 0.85$). The number of eggs per arena within an exposure was fairly uniform, with the coefficient of variation (CV = mean/SD) averaging 5% in the 24 exposures. There was variation in mean number of eggs/arena used in different exposures (CV = 8.7%) with the actual number being 1,440 ± 126 in the 24 exposures. However, the actual number of eggs per exposure of each cohort was not correlated with resulting prevalence ($R_{s22} = 0.35, P = 0.09$) or intensity ($R_{s22} = 0.13, P = 0.55$).

The 5 aspects of host biology that were monitored varied in a complex manner over time (Fig. 2A–E). Although infections of each cohort started with 6–32 hosts (mean = 26), mortality during pre-exposure fasting and following exposure reduced the number of hosts that were necropsied to 1–32 per cohort (mean = 22), with only 1–22 infected hosts per cohort (mean = 7). Therefore, data were pooled into 6 age classes to increase sample sizes for statistical analysis of infections and to smooth age-related patterns. The boundaries of each class were chosen to achieve a compromise between a consistent age range of each class and number of cohorts included, and the possession of a unique set of properties by each class (Fig. 2F–J). Class 1 (0–5 wk) were hosts with low pre-exposure mortality rates (λ), high fasting and postexposure survival, an even sex ratio, and moderate fecundity. Class 2 (5–15 wk) were hosts with low pre-exposure mortality rates, high fasting and postexposure survival, an even sex ratio and higher fecundity. Class 3 (15–30 wk) were hosts with the lowest pre-exposure mortality rates, high fasting, and postexposure survival, a slightly male-biased sex ratio but the highest fecundity. Class 4 (30–45 wk) were hosts with moderate pre-exposure mortality, reduced fasting, and postexposure survival, a slightly male-biased sex ratio and low fecundity. Class 5 (45–60 wk) were hosts with higher pre-exposure mortality rates, reduced fasting, and postexposure survival, a strongly male-biased sex ratio, and low fecundity. Class 6 (>60 wk) were hosts with the highest (although variable) pre-exposure mortality rates, the lowest fasting and postexposure survival, and with females absent.

Age-related trends in host biology (Fig. 2F–J) were not always unidirectional with age. Weekly mortality rates were initially low, dropped to negligible levels, then increased markedly (Fig. 2F). The proportion of beetles that survived the pre-exposure fast (Fig. 2G) increased initially, then decreased ($\chi^2 = 113.3, P < 0.001$). The proportion of beetles that survived the 2-wk period from exposure through to necropsy (Fig. 2H) was initially high and then declined ($\chi^2 = 148.0, P < 0.001$). The sex ratio (Fig. 2I) was initially even, but became gradually more male biased until the oldest beetles were male only ($\chi^2 = 62.2, P < 0.001$). Egg production by females was low initially, increased for a period, then declined sharply; the oldest females produced few eggs (Fig. 2J).
Figure 2. Changes in host properties over time determined for individual cohorts of *Tribolium confusum* (A–E) or following grouping of cohorts into age classes (F–J). (A, F) Weekly pre-exposure mortality rate (\( \lambda \)). (B, G) Percentage survival during a 4-day pre-exposure fast. (C, H) Percentage survival from the day of exposure to 2 wk postexposure. (D, I) Percentage of female beetles alive at 2 wk postexposure. (E, J) Mean weekly egg production by female hosts that did not acquire an infection. All error bars are 95% CL. Vertical dotted lines and numbers above (A–E) indicate the boundaries of the 6 age classes used in (F–J).

Hosts were pooled into 6 age classes as before to determine if prevalence and intensity were affected by the different combinations of host properties represented by that classification. Prevalence (Fig. 3C) did not differ among age classes in female hosts \( (\chi^2 = 2.38, P = 0.665) \), but it did differ among male hosts \( (\chi^2 = 17.02, P = 0.004) \). Examination of confidence limits (Fig. 3C) suggests that low prevalence in age class 3 contributed to the difference in males. Prevalence in males was consistently
lower in males than females (Fig. 3C), but statistical testing (Fisher exact test) supported this only for age classes 1 ($P = 0.024$), 3 ($P < 0.001$), and 5 ($P = 0.008$). Overall prevalences, ignoring host age, were 44.5% in 220 females and 21.6% in 333 males (Fisher's exact test, $P < 0.001$). Differences of intensity among age classes (Fig. 3D) were marginally significant in female hosts (Mood's median test, $P = 0.080$), tending to decline with age, but were not different in male hosts ($P = 0.956$). Intensity was similar between sexes except in age class 5, where intensity in males was significantly higher ($P = 0.003$). Overall mean intensities, ignoring host age, were 2.47 in females and 2.12 in males (bootstrap $t$-test, $P = 0.254$).

The analysis of prevalence and intensity based on 6 age classes of hosts produced few significant results with respect to age. This may be due to a lack of effect, or that the magnitude of effects was too low to be detected given the distribution of sample sizes. Hosts were further pooled into just 2 classes: young and old. Young hosts (age classes 1–3, age 0–30 wk) had generally high survival and fecundity and old hosts (age classes 4–6, age 30 wk or older) had generally poor survival and low fecundity (Fig. 2). Prevalence (Fig. 3E) did not vary between young and old female beetles or young and old male beetles, but male beetles in both age classes had a lower prevalence than in females. Intensity in young male and female beetles (Fig. 3F) was similar. Intensity did not decline in old males, but old females had lower intensity than young females and also than old males (Fig. 3F).

Mean volume of cysticercoids from single-parasite infections was $0.0195 \pm 0.0034 \text{ mm}^3$ (n = 73). Effects of host age and sex were tested by factorial ANOVA following log$_{10}$ transformation of volumes. There was no effect of host sex ($F_{1,72} = 1.56$, $P = 0.216$) or age ($F_{4,72} = 1.08$, $P = 0.137$) and there was no host sex/age interaction ($F_{4,72} = 1.18$, $P = 0.329$).

**DISCUSSION**

This is the first study to evaluate infections of *H. diminuta* over the entire life span of *T. confusum*. With dramatic changes noted in host mortality and fecundity as the hosts aged, it was surprising that their parasite infections seemed relatively insensitive to these changes.

The basic survivorship curves for *T. confusum* in this study were similar in shape and duration to those reported previously (Pearl et al., 1941), with periods of initial and late high mortality. The only major difference noted was that the intervening period of relatively low mortality in this study was longer than observed by Pearl et al. (1941). Similarly, temporal changes in fecundity were similar to patterns reported previously (Maema, 1986), with fecundity increasing for several weeks and then declining. The results in the present study would appear to en-
compass and reflect the normal lifetime of a typical population of *T. confusum* under culture conditions.

Previous studies exposed *T. confusum* of different ages to eggs of *H. diminuta*. Dunkley and Mettrick (1971) reported that the number of cysticercoids recovered per beetle increased with beetle age over the narrow range from 5–6 to 15–16 days (corresponding to class 1), which is corroborated by the continuous increase in prevalence and intensity during the first 5 wk of the present study. Keymer (1982) reported between 2 and 14 wk posteclosion (corresponding to classes 1 and 2) an exponential decline from about 16 to 2 mean parasites per host. Kelly et al. (1967) examined young, middle-aged, and old (corresponding to classes 1, 3, and 5) virgin *T. confusum* and reported that both prevalence and intensity were high in young and middle-aged females and declined in the old females, but were highest in middle-aged, compared to young or old, males. These 2 studies (Kelly et al., 1967; Keymer, 1982) contrast each other and with results of the present study, which indicated that prevalence, although lower in males than females, did not vary with age in either sex, and that intensity declined with age in females, but not males. The methods used in the other studies were not reported with sufficient detail to assess whether a methodological explanation for the differences exists. Strains of *T. confusum* vary in susceptibility to *H. diminuta* (Yan and Norman, 1995) and perhaps the populations of *T. confusum* used by Kelly et al. (1967), Keymer (1981), and in the present study also vary in their age-related response to infection by *H. diminuta*.

This is the first study to examine the size of cysticercoids resulting from infection of such an age range of hosts, and no age-related variation was found. This corroborates results from separate experiments (Shostak et al., 2006) in which the shape of cysticercoids varied minimally, or not at all, in hosts 2–52 wk old at the time of exposure.

The considerable variation among cohorts of similar age was surprising because it was expected that age-related changes in infections would be gradual and smooth, as were the changes in host properties. The source of variation may be host-age-dependent variation in the infection process, host-age-independent variation among cohorts in some property that affects the infection process, or variation resulting from the methodology. Many aspects of *T. confusum* change markedly with age, often over relatively short time spans. Response to pheromones increases dramatically during the first 3 wk of life, then declines markedly in females but not males (Obeng-Ofori and Coaker, 1990). Egg production increases during the first 7 wk, then declines until at least 12 wk of age (Maema, 1986), although in *Tribolium castaneum* a general decline in egg production from about 1 to 17 wk is punctuated by periods of sharp increases (Mertz, 1969). The overlap of different physiological and behavioral processes as the age host should result in irregular patterns of susceptibility to infection such as those observed. Populations of *Tribolium* spp. in culture undergo fluctuations in numbers and demographics for a variety of reasons (Mertz, 1969). Some cohorts in the present study exhibited unusual survivorship curves. It may be that pupae removed from the stock cultures at different times vary in properties that affect infection probability when adult. The nature of these properties is unknown, although laboratory populations of *T. confusum* subject to selection have yielded strains with large variation in susceptibility to *H. diminuta* (Yan and Norman, 1995). The stock colony of *T. confusum*, from which pupae were chosen to populate each cohort, has been allowed to breed freely without intentional selection, but presumably harbors a similar range in genetically based susceptibility. Subtle shifts in genetic makeup as the colony goes through population cycles could result in age-independent variation among cohorts. These explanations must be regarded at present as hypotheses requiring specific testing, such as through longitudinal studies in which the same cohort is tested at different times.

Methodological explanations for variation in infection among cohorts can be addressed more directly. Beetles were exposed individually, not in groups, reducing the chance that some event during the exposure would affect the infection results for the whole group. There was small variability in number of eggs placed in different arenas, and in average number of eggs used among treatments. Large differences in egg density could affect resulting infections (Keymer, 1981), but parasite egg density was not a significant factor in this study. The logistic requirement of conducting the infections over a period of about 3 mo raises the possibility of a systematic change in infectivity of eggs of *H. diminuta* that beetles were exposed to, or in ambient conditions under which exposures were conducted. This would be evident as an effect of the order in which infections were done, but again this effect was not significant. Without doubt, some of the variation among cohorts relates simply to the sample size of hosts available. Prevalence and intensity are difficult to estimate with high confidence due to the aggregated distribution typical of helminth infections (Rozsa et al., 2000). Although the present study used about 30 hosts in each exposure, which is typical (Kelly et al., 1967; Dunkley and Mettrick, 1971), sample-size problems become more acute in attempts to evaluate infections in beetles nearing the end of their life where, by definition, few have survived.

The only major observed effect for host age on infection was a decline in infection intensity in the older (class 4–6) females. This corresponded with a decline in the proportion of females, indicative of differential mortality at some point, which can occur in the absence of infection (Pearl et al., 1941). Host mortality severely confounds the interpretation of intensity determined at the time of necropsy. Intensity-dependent mortality is reported for *T. confusum* infected with *H. diminuta* (Keymer, 1980) and would remove heavily infected individuals, resulting in lower intensity at necropsy than immediately after infection. The present study did not generate the high intensities that Keymer (1980) did using multiple exposures, and a more recent study (Hurd et al., 2001) suggests that infection with *H. diminuta* may actually increase life span in female *Tenebrio molitor*. In the present study, host sex was determined only at the time of necropsy, as insufficient beetles were available to sample sex ratios at earlier times. Although there may have been mortality of heavily-infected females, it should also be noted that there was increased mortality in older beetles even during the pre-exposure fast, suggesting that mortality in the older beetles was more likely due to a general weakening of the beetles and not by the parasites per se. Therefore, although the low-intensity infections indicate clearly that the old females would contribute relatively few cysticercoids to the transmission process, it cannot be resolved whether they failed to acquire the infection initially, or whether they acquired similar or even higher num-
bers of parasites than younger beetles, followed by increased parasite-induced mortality.

There are a number of behavioral and physiological barriers in the infection process that must be overcome to produce a successful infection of *H. diminuta* in *T. confusum*, even under simplified laboratory conditions. The initial barriers are behavioral, where the beetle must first encounter a viable egg and then ingest it (although these behaviors may be influenced by underlying physiological processes). Subsequent barriers are largely physiological. The oncosphere must be released from the egg membranes and enter the hemocoel, the parasite must survive within the hemocoel long enough to develop to the cysticercoid stage, and the host must survive the infection. It is this very complexity of the infection process that may explain the relatively uniform results of infection over the life span of *T. confusum* when such dramatic changes are taking place in the host. Some barriers may become less effective as the host ages and others become more effective. Previous studies (Kelly et al., 1967; Keymer, 1982) attribute age-related differences in infections of *T. confusum* to changes in host susceptibility or resistance, although other explanations such as differences in maternal influence of exposure time and host density. Parasitology 84: 157–166.

**LITERATURE CITED**


GpMyoF, A WD40 REPEAT-CONTAINING MYOSIN ASSOCIATED WITH THE MYONEMES OF GREGARINA POLYMORPHA

Matthew B. Heintzelman and Marcus J. Mateer
Department of Biology, Program in Cell Biology and Biochemistry, Bucknell University, Lewisburg, Pennsylvania 17837.
e-mail: mheintze@bucknell.edu

ABSTRACT: This study presents the first characterization of a WD40 repeat-containing myosin identified in the apicomplexan parasite Gregarina polymorpha. This 222.7 kDa myosin, GpMyoF, contains a canonical myosin motor domain, a neck domain with 6 IQ motifs, a tail domain containing short regions of predicted coiled-coil structure, and, most notably, multiple WD40 repeats at the C-terminus. In other proteins such repeats assemble into a β-propeller structure implicated in mediating protein-protein interactions. Confocal microscopy suggests that GpMyoF is localized to the annular myonemes that gird the parasite cortex. Extraction studies indicate that this myosin shows an unusually tight association with the cytoskeletal fraction and can be solubilized only by treatment with high pH (11.5) or the anionic detergent Sarkosyl. This novel myosin and its homologs, which have been identified in several related genera, appear to be unique to the Apicomplexa and represent the only myosins known to contain the WD40 domain. The function of this myosin in *G. polymorpha* or any of the other apicomplexan parasites remains uncertain.

The cytoskeletal architecture of apicomplexan parasites has been the subject of numerous studies over the course of many years (for reviews see Tilney and Tilney, 1996; Morissette and Sibley, 2002; Fowler et al., 2004). The earliest studies were ultrastructural descriptions of the elaborate cortical architecture characteristic of these parasites, but over the past decade a renewed interest in the molecular characterization and dynamics of the cortical cytoskeleton has occurred as investigators have sought to understand the details of parasite locomotion, parasite-host cell interactions, host cell invasion and egress, as well as the cellular mechanics of parasite replication. Reflecting the morphological diversity of apicomplexan parasites, their individual use of cytoskeletal elements to establish and maintain their shape, support their locomotion, or permit morphological transformations during their life cycle will vary somewhat from one parasite species to the next. Nevertheless, all of the major cytoskeletal systems including actin filaments, microtubules, and intermediate filament-like elements may be employed by apicomplexan parasites.

One well-studied element of the cortical cytoskeleton is an array of subpellicular microtubules, so named because of its location beneath a tripartite membrane system, the pellicle, which includes the cell membrane, together with a closely apposed double membrane system known as the inner membrane complex (IMC; Vivier and Schrevel, 1964; Dubremetz and Torpier, 1978; Russell and Sinden, 1982; Nichols and Chiappino, 1987; Dyson et al., 1994; Morissette et al., 1997; Raibaud et al., 2001; Kuriyama et al., 2005). As in most eukaryotic cells, the parasite microtubules play an important role in defining cell shape and establishing functional polarity. Parasites whose microtubules have been disrupted by drug treatments are nonpolar, nonmotile, and noninvasive (Morissette and Sibley, 2002).

In species of *Toxoplasma* and *Plasmodium*, a second scaffold, termed the subpellicular network and comprised of a meshwork of 8- to 10-nm filaments associated with the cytoplasmic face of the pellicle, has also been described (Mann and Beckers, 2001; Khater et al., 2004). This membrane-cytoskeletal network plays an important role in defining cell shape and maintaining the structural integrity of the cell. Targeted disruption of this network in *Plasmodium* spp. sporozoites can lead to abnormal cell shape, a loss of structural integrity, as well as impairment in motility and host-cell invasiveness (Khater et al., 2004).

In the gregarines, some groups of which are characterized by the presence of numerous epicytic folds, ridgelike elaborations of the cell surface, and a pellicle that runs the length of the parasite, additional, but as yet uncharacterized, scaffolding elements appear to support these cell surface features. Most notably, a set of longitudinally oriented ~12-nm filaments subjacent to the pellicle membranes are in position to support the top of each epicytic fold, while a continuous dense internal lamina, morphologically reminiscent of a basal lamina, is found in the cortical cytoplasm at the base of the epicytic folds (Regier, 1967; Walsh and Callaway, 1969; Rühl, 1976; Hildebrand, 1980; Schrevel et al., 1983). These cytoskeletal networks have not been experimentally targeted, but it could be expected that their disruption would similarly compromise normal cell shape, gliding, and other cellular behavior.

Of late, it is the actin cytoskeleton of apicomplexan parasites that has received a significant amount of attention largely because of the demonstrated role for an actin-myosin system powering the gliding locomotion and host cell invasion mechanisms of these parasites (Keeley and Soldati, 2004; Sibley, 2004; Heintzelman, 2006). Parasite actin has been studied most thoroughly in *Toxoplasma gondii*, and to a lesser extent in *Plasmodium* spp., and other related parasites, but one hurdle in these studies has been the actual visualization of the actin cytoskeleton. In contrast to many eukaryotic cells that maintain comparable amounts of filamentous and globular actin, at least in *T. gondii*, greater than 97% of the actin is found in globular form (Dobrowolski, Niesman et al., 1997). More filamentous actin may be recovered in *Plasmodium* spp., but even here, the filamentous fraction appears to represent a collection of short polymers, possibly reflecting some intrinsic limitation on the ability of parasite actin to polymerize more extensively (Field et al., 1993; Schmitz et al., 2005; Schüller et al., 2005). With the use of refined morphological techniques (Schatten et al., 2003) or with the application of the F-actin stabilizing drug jasplakinolide (Shaw and Tilney, 1999), cortical actin filaments can be visualized, reinforcing immunolocalization studies that place much of the actin in association with the parasite cortex (Dobrowolski, Carruthers et al., 1997; Dobrowolski, Niesman...
et al., 1997). These short actin polymers are thought to be a highly dynamic and essential component of the cortical actin-myosin motor complex, the glideosome, which is responsible for the gliding locomotion of these parasites. In essence, gliding is powered by a population of myosin motors immobilized on the IMC. This myosin actively transports an assembly of short actin filaments, which are themselves linked to a transmembrane adhesive protein via aldolase. Rearward translocation of this actin-aldolase-substrate complex propels the parasite forward atop the immobilized substrate (Keeley and Soldati, 2004; Sibley, 2004; Heintzelman, 2006).

In the gregarines actin is present in the epicytic folds and presumably participates in a glideosome-like complex that powers the gliding motility of these cells as it does for other apicomplexan parasites (Heintzelman, 2004, 2006; Keeley and Soldati, 2004; Sibley, 2004). However, an additional and robust population of actin filaments has also been described in the cortical domain of many gregarines. These filaments form a series of annular myonemes that gird the cortex along the length of the parasite (Beams et al., 1959; Warner, 1968; Hildebrand and Vincikier, 1975; Rühl, 1976; Hildebrand, 1980; Heintzelman, 2004), an architectural feature not described in coccidian or haemosporidian parasites. In addition to a skeletal role, these annular myonemes likely serve as contractile organs that are responsible for the bending or peristaltic motions observed in some gregarines (Fowell, 1936; Beams et al., 1959; Hildebrand and Vincikier, 1975; Hildebrand, 1980).

Associated with the parasite actin cytoskeleton is a collection of myosins, mechanoenzymes, that have the ability to generate force in association with actin in an ATP-dependent fashion. Three primary functional domains characterize most myosins. These include the myosin head domain, in which resides the motor activity of the protein, a neck domain that serves as a binding site for myosin light chains, which can play a role in modulating myosin behavior, and a myosin tail domain, that portion of the myosin thought to dictate much of how and where a given myosin motor will be used in the cell (Krendel and Mooseker, 2005). The first parasite myosins discovered established a novel class within a growing myosin superfamily (Heintzelman and Schwartzman, 1997), a designation supported by phylogenetic analysis of the primary structure of myosin motor (head) domains (Cheney et al., 1993; Hodge and Cope, 2000; Foth et al., 2006). These class XIV parasite myosins play important roles in gliding motility and host cell invasion (Meissner et al., 2002; Keeley and Soldati, 2004) and may also be involved in aspects of parasite replication (Delbac et al., 2001).

A second novel class of parasite myosins, recently designated class XXII, includes representatives from species of Plasmodium, Toxoplasma, Eimeria, Cryptosporidium, Theileria, and Babesia (Foth et al., 2006). The most distinctive feature of this class is the presence of WD40 repeats in the myosin tail domain. In other proteins, WD40 repeats are known to assemble into a β-propeller structure that can serve as a scaffold for the assembly of multimolecular complexes (van der Voorn and Ploegh, 1992; Neer et al., 1994; Smith et al., 1999). This class of WD40 repeat-containing myosins, to date a class unique to the Apicomplexa, remains uncharacterized, and its role in the biology of the parasites is unknown. To begin exploring the biology of this intriguing new myosin, we present here the cloning and initial characterization of a GpMyoF, a WD40 repeat-containing myosin from Gregarina polymorpha.

**MATERIALS AND METHODS**

**Parasites**

*Trypanosoma brucei* hosts *G. polymorpha* parasites were maintained at room temperature in buckets containing bran and oat meal, supplemented daily with potato slices. The guts were dissected from mealworms into phosphate buffered saline (PBS) and the contents removed. *Gregarina polymorpha* were manually separated from any other gregarine species that were present only rarely in our mealworm cultures. Parasites were washed several times in PBS and then processed.

**Cloning of *G. polymorpha* MyoF**

Total RNA was prepared from purified cultures of *G. polymorpha* using TRizol® reagent (Invitrogen, Carlsbad, California), following the manufacturer’s suggested protocol. For polymerase chain reaction (PCR) and 5′ and 3′ RACE (rapid amplification of cDNA ends), total RNA was used as starting material for reverse transcription. For construction of the *G. polymorpha* CDNA library, poly-A RNA was prepared from total RNA using a poly(A) Quick® mRNA isolation kit (Stratagene, La Jolla, California). For the initial identification of GpMyoF, total RNA was first reverse transcribed using random hexamers and MMLV reverse transcriptase (Invitrogen), and the resultant cDNA was used as a template in PCR employing degenerate myosin primers corresponding to the well-conserved GESGAGKT and EAF- GNKTA sequences in the myosin head domain (Bement et al., 1994). DNA sequencing of this initial PCR product permitted the design of specific oligonucleotide primers, which were then used in additional PCRs in combination with other degenerate myosin primers derived from the well-conserved LDIFGFE and PHIRCKP domains occurring closer to the carboxyl (C)-terminus of the myosin head domain. The remainder of the head domain, as well as the neck and partial tail domain, was obtained by hybridization screening of a random primed *G. polymorpha* cDNA library constructed using a Lambda Zap®-cDNA library construction kit (Stratagene). Using a 32P-labeled random-primed probe (Roche Applied Science, Indianapolis, Indiana) derived from the 3′ end of the existing head sequence, hybridization screening of the cDNA library resulted in the isolation of a clone overlapping the existing sequence, but lacking the complete tail domain. The remainder of the tail domain sequence was isolated using a 3′ RACE approach following the manufacturer’s protocol (Invitrogen). Similarly, the remainder of the sequence upstream of the original PCR product was obtained by means of a 5′ RACE strategy (Invitrogen). All DNA sequencing (accomplished at the Molecular Biology Core Facility at Dartmouth College, Hanover, New Hampshire, or the Nucleic Acid Facility at the Pennsylvania State University, State College, Pennsylvania) employed analysis of both sense and antisense strands, and the final sequence was subsequently confirmed by sequence analysis of PCR products spanning the entire sequence using cDNA derived independently of that used for the initial sequencing. Sequence analysis was accomplished through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) and employed the Basic Local-Alignment and Search Tool (BLAST; Altschul et al., 1990) and the Conserved Domain Search service (CD-Search; Marchler-Bauer and Bryant, 2004). Additional analysis of conserved protein domains employed the Pfam database (http://www.sanger.ac.uk/Software/Pfam; Finn et al., 2006). Phylogenetic tree analysis was accomplished as described in Heintzelman and Schwartzman (1997) using the default parameters of the ClustalW program (Thompson et al., 1994) and TreeView (Page, 1996).

**Polyclonal antibody production**

A PCR product encompassing the sequence corresponding to ~100 amino acids at the amino-terminus of the myosin head domain was subcloned into both pGEX (GE Healthcare, Piscataway, New Jersey) and pQE (Qiagen, Valencia, California) bacterial expression vectors. Both fusion proteins were soluble when expressed and were purified using glutathione sepharose (GE Healthcare) or nickel NTA agarose (Qiagen) according to manufacturers’ protocols. The GST-myosin head
fused protein was used as the immunogen for the production of rabbit polyclonal sera (Pocono Rabbit Farm and Laboratory, Canadensis, Pennsylvania), and the resultant immune sera were affinity purified over a column generated by coupling the His-tagged myosin-head fusion protein to cyanogen bromide-activated sepharose 4B (GE Healthcare). Antibody fractions from pre-immune sera were prepared using an anti-rabbit IgG affinity column (ICN Biomedicals, Aurora, Ohio) and served as the control antibody for subsequent experiments.

**Immunolocalization**

For immunofluorescence studies, parasites were fixed for 5 min in methanol at −20 °C, rinsed in PE buffer (1× PBS containing 50 mM K-EGTA), and then permeabilized for 10 min in PEAT buffer (PE containing 0.1% bovine serum albumin [BSA] and 0.1% Triton X-100). Parasites were then incubated in affinity purified anti-GpMyoF antibody diluted to 10 µg/ml in PEAT, or with pre-immune IgG at the same concentration, for 30–60 min at room temperature with gentle agitation. Parasites were washed several times in PEAT buffer and then incubated in Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) diluted to 2.5 µg/ml in PEAT buffer for 30–60 min at room temperature. Following additional washes in PEAT buffer, parasites were mounted in Vectashield (Vector Laboratories, Burlingame, California), and images were captured using a Nikon PCM-2000 confocal system. Control images stained with pre-immune antibodies were captured with the same settings and processed in concert with experimental samples.

**Immunoblotting and extraction studies**

Whole parasite protein homogenates were obtained by manually dousing parasites on ice in PBS and processing for SDS-PAGE using 4–16% gradient mini-gels. Gels were electrotransferred to nitrocellulose membrane and processed for immunoblot analysis. Membranes were blocked for 1 hr in 5% nonfat dry milk in PBS, incubated for 2 hr at room temperature in primary antibody at 1 µg/ml, washed several times in PBS, and then incubated for 1 hr in a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Promega, Madison, Wisconsin) at a 1:7,500 dilution in PBS. Following additional washing in PBS, the immunoblots were developed using NBT and BCIP (Sigma, St. Louis, Missouri) in alkaline phosphatase buffer.

For protein extractions, parasites were dounced in PBS with cell lysis monitored by phase contrast microscopy. The homogenate was centrifuged at 16,000 g for 15 min at 4 °C after which the supernatant was collected and its proteins precipitated for 30 min on ice with the addition of trichloroacetic acid to 10%. The proteins were recovered by centrifugation as above and processed for SDS-PAGE. The original pellet of parasite ghosts was resuspended and extracted for 30 min on ice in 1 of the following solutions: PBS; PBS containing 0.6 M NaCl, 10 mM ATP, and 1% Triton X-100; Na2CO3, pH 11.5; or 0.25% sarkosyl in 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA. Following extraction, samples were centrifuged as above, supernatants collected, pellets resuspended in the extraction buffer to equal volume, and then all samples were processed for SDS-PAGE and immunoblotting as described above.

**RESULTS**

**Identification of WD40 repeat-containing myosin in G. polymorpha**

Using a combination of PCR-based strategies together with hybridization screening of a G. polymorpha cDNA library, the full-length sequence for a novel myosin expressed by G. polymorpha was obtained (Fig. 1). Sequence analysis predicted a protein of 222,722 daltons, which contained the well-defined head, neck, and tail domains of a canonical myosin as revealed by the BLAST and CD-Search tools (Altschul et al., 1990; Marchler-Bauer and Bryant, 2004). Defining the neck domain were 6 IQ motifs (Fig. 1, shaded residues), most of which adhered well to the consensus sequence of IQXXXXRGXXXR as defined by Cheney and Moosoker (1992) with ‘X’ representing any amino acid residue. Following the neck domain, the initial portion of the tail domain showed regions of predicted coiled-coil structure (Fig. 1, wavy underline), and the terminal portion of the tail contained multiple WD40 repeats (Fig. 1, boxed residues). Both CD-Search as well as a search of the Pfam database (Finn et al., 2006) detected 2 to 4 WD40 repeats located near the carboxy (C)-terminus of the protein. Two of these repeats were more easily recognized as fitting the consensus WD40 repeat motif (Smith et al., 1999; BioMolecular Engineering Research Center website: http://bmerc-www.bu.edu/projects/wdrepeat), while the other 2 were more degenerate. Further visual inspection of the tail domain sequence between, or flanking, the more well-defined WD40 repeats revealed other possible, albeit degenerate, WD40 repeats. In total, 7 putative WD40 repeats were found in the tail domain of this G. polymorpha myosin (Fig. 2), but as discussed below, identification of such repeats is complicated by the severe degeneracy of the WD40 consensus sequence itself (Smith et al., 1999).

Results of the BLAST search demonstrated greatest similarity of the G. polymorpha myosin to homologs expressed in T. gondii (myosin F), and species of Plasmodium (myosin C, recently renamed myosin F; Foth et al., 2006), Theileria (myosin F), and Cryptosporidium (myosin F). These myosins shared ~35–45% amino acid identity over the entire protein with somewhat greater conservation seen in the head compared to the tail domain of the myosin. To confirm the association of this G. polymorpha myosin with this group of myosins, and to place the G. polymorpha myosin within the context of the established classes of the myosin superfamily, phylogenetic analysis using myosin head domain sequences representative of the recognized myosin classes was performed. The phylogenetic tree confirmed the association of the G. polymorpha myosin with the class XXII myosins (Foth et al., 2006) expressed in several apicomplexan parasites (Fig. 3). In keeping with established naming conventions, the G. polymorpha myosin has been designated GpMyoF.

**GpMyoF expression**

To facilitate studies on the expression of GpMyoF; polyclonal antibodies were generated against a fusion protein encompassing the N-terminal 100 amino acid residues of the myosin head domain, a region that shows no homology to the other characterized myosins of G. polymorpha (Fig. 1, underlined residues). Immunoblots of whole parasite homogenates using affinity purified antibody revealed a single band at the expected molecular mass, while pre-immune IgG showed only weak, diffuse reactivity with the parasite sample (Fig. 4). To assess the association state of GpMyoF within the parasite, cells were homogenized and the parasite ‘ghosts’ extracted with buffer alone or with the addition of the nonionic detergent Triton X-100, salt (0.6 M NaCl), and ATP. Neither of these extraction conditions resulted in the solubilization of any detectable GpMyoF; all of which instead remained associated with the high-speed cytoskeletal pellet as assessed by immunoblot of the cell fractions (Fig. 5). The extraction of parasite ghosts with sodium carbonate pH 11.5 resulted in the release of a fraction (roughly half) of the myosin, and complete solubilization of the protein was possible with extraction in the presence of the ionic detergent sarkosyl (Fig. 5). Unfortunately the solubilized myosin fractions
Figure 1. Deduced amino acid sequence of the 222.7 kDa Gregarina polymorpha myosin F. GenBank EF626805. The 6 IQ motifs defining the myosin neck domain are indicated by shading. Regions of predicted coiled coil are indicated by the wavy underline. The cassette of repeats spanning the C-terminus of the myosin tail domain are boxed. The amino acids underlined at the beginning of the myosin head domain were inactive at least as evidenced by the inability of the protein to bind with F-actin, a behavior diagnostic of all myosins. For this reason additional biochemical analysis of the myosin was precluded.

The GpMyoF-specific antibody was also used to immunolocalize the myosin in methanol-fixed parasites using confocal microscopy. At low magnification, and at a focal plane passing through the middle of the parasite, GpMyoF was localized to the parasite cortex in both protomerite and deutomerite segments of the trophozoite as well as to the septum dividing these 2 cellular regions (Fig. 6b). Upon closer inspection, it was apparent that the cortical staining was not continuous, but rather punctate, in distribution (Fig. 6c). Pre-immune IgG used as a control showed only the weak cortical fluorescence typical of the reactivity of most nonspecific antibodies with G. polymorpha (Fig. 6d). With the focal plane passing through the parasite cortex, GpMyoF was seen to be distributed as a series of closely packed myonemes or riblike structures oriented perpendicular to the long axis of the cell in both protomerite and deutomerite cell segments (Fig. 7). No staining of the epicytic folds or of any other cellular structures was observed.

**DISCUSSION**

The description of GpMyoF presented here brings to 3 the number of myosins from a gregarine that have received some attention (Heintzelman, 2004), although a fourth gregarine myosin, a ~90kDa class XIV myosin, has also been identified in G. polymorpha, but remains uncharacterized (GenBank EF608157) (Table I). It is common for most eukaryotic cells to...
express several myosins representing multiple classes of the myosin superfamily (Bement et al., 1994; Gavin, 2001; Bezanilla et al., 2003), and this holds true for the Apicomplexa as well. The parasites of this phylum express multiple class XIV myosins (Heintzelman and Schwartzman, 1997, 2001; Hettman et al., 2000; Matuschewski et al., 2001; Lew et al., 2002; Chapparo-Olaya et al., 2005), as well as a WD40 repeat-containing myosin (Hettman et al., 2000; Foth et al., 2006) designated class XXII by Foth et al. (2006). The repertoire of myosins expressed by a given eukaryotic cell may serve a variety of functions within that cell ranging from contraction and cytokinesis to endocytosis and vesicle transport to signal transduction and cell locomotion (Mermall et al., 1998; Gavin, 2001; Krendel and Mooseker, 2005). In the Apicomplexa most all of the attention has focused on the class XIV myosins and, particularly the myosins A from Toxoplasma, Plasmodium, and other parasite species, because of the demonstrated role for this myosin in powering the gliding motility and host-cell invasion process essential for the obligate intracellular lifecycle of these important pathogens (Meissner et al., 2002; Keeley and Soldati, 2004; Sibley, 2004; Heintzelman, 2006). Other apicomplexan myosins may participate in parasite division (Delbac et al., 2001), but the function of most of the apicomplexan myosins remains unresolved, although several do show variable expression patterns at different stages of the parasite life cycles (Chapparo-Olaya et al., 2005; Herr-Götz et al., 2006; Siden-Kiamos et al., 2006).

The behavior of a given myosin is dictated by its 3 functional

---

**Figure 2.** Alignment of WD40 repeats from GpMyoF with the consensus WD40 repeat. The most common amino acid substitutions for the highly degenerate WD40 consensus are given, in order of frequency, below the consensus sequence. The consensus contains 2 sites (*1, *2), where inserts of variable length may be found. The starting amino acid number for each GpMyoF repeat is given to the left of the sequence. The consensus or one of its common substitutions are shaded. Only strands a, b, and c of the 4-stranded WD40 repeat are indicated, the fourth d strand was omitted because no clear consensus sequence exists for this portion of the repeat.

<table>
<thead>
<tr>
<th>Consensus</th>
<th>strand a</th>
<th>strand b</th>
<th>strand c</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH x x x V x x x F x</td>
<td>G H N Y A T L</td>
<td>T D G A</td>
<td>S D G A</td>
</tr>
</tbody>
</table>

**Figure 3.** An unrooted phylogenetic tree showing the association of GpMyoF (G. polymorpha) with other class XXII, WD40 repeat myosins from T. gondii (TgMyoF, ABA01554), Plasmodium sp. (XP728361), and Cryptosporidium sp. (CAD98272). One representative from each of the other classes of the myosin superfamily is included as follows: for myosins I–XVII, accession numbers are those employed by Heintzelman (2004), with the additional myosins; XIV: T. gondii myosin A (A59283); XVIII: Mus musculus myosin XVIIIa (CA244242); XIX: Canis familiaris myosin (XP_548248); XX: D. melanogaster myosin isoform A (AAF52683); XXI: Typosoma brucei myosin (EAN80576); XXIII: T. gondii MyoG (DQ131540); XXIV: C. parvum Myol (CAD98475).

**Figure 4.** Characterization of anti-GpMyoF polyclonal antibody. Lane (a) Coomassie-stained SDS-PAGE sample of whole G. polymorpha parasite homogenate with molecular weights (in kDa) indicated on left; (b) parasite homogenate probed with anti-GpMyoF antibody showing specific reactivity with a single band at ~220 kDa; (c) parasite homogenate probed with pre-immune IgG fraction.
domains, the head or motor domain, the light chain-binding neck domain, and the tail domain, the structure and function of which vary most among the different types of myosins. The myosin head domain contains the actin-binding and ATP-binding sites that are essential for the mechanochemical function of the myosin, in essence, its motor properties. The myosin head is the most well-conserved domain among different myosin classes, but sufficient divergence in this domain does permit the various myosins to be assigned to different classes using phylogenetic analyses (Cheney et al., 1993; Hodge and Cope, 2000; Korn, 2000; Foth et al., 2006). In addition to the binding sites necessary for motor function, some myosins have head domains that contain N-terminal extensions in which reside additional functional domains such as kinases (myosins III), ankyrin repeats (myosins VI), or PDZ domains (myosins XVIII; Thompson and Langford, 2002; the myosin home page at http://www.mrc-lmb.cam.ac.uk/myosin/myosin.html). The head domain of GpMyoF shares the canonical features of all myosin motor domains, but appears to lack any accessory domains. Whether or not the head domain of GpMyoF exhibits the diagnostic properties of a myosin motor, such as ATP-sensitive actin binding, an actin-dependent Mg2+-ATPase activity, and an ability to translocate actin filaments has not yet been determined because this myosin can be extracted from G. polymorpha only under conditions that likely result in some degree of protein denaturation sufficient to impair the normal function of the enzyme. It will be important to develop protocols for either re-­
aturation of the isolated protein, isolation of the protein in its native form, or in vitro expression of the protein or its motor domain so as to permit additional biochemical characterization of this unique myosin.

Of interest is the observation that although other apicomplexan class XXII myosins are predicted to contain an SH3-like domain as an N-terminal extension of the myosin head, this motif was not found in GpMyoF using the same battery of search algorithms that reveal this domain in the other apicomplexan myosins. The N-terminal SH3-like domain, common to many different classes of myosin, could permit interaction of the myosin head with proteins containing SH3-binding domains (Mayer, 2001) or, as has been recently shown, may mediate interactions within the myosin motor domain itself (Fujitabecker et al., 2006). That such a domain is found in other apicomplexan class XXII myosins, but is not apparent in GpMyoF, may reflect the early phylogenetic position of the gregarines in the Apicomplexa (Carreno et al., 1999; Leander et al., 2003) and the obvious structural differences in terms of size and cortical architecture between the gregarines and the coccidian, haemosporidian, and piroplasmid parasites. That is, given the lack of cortical myonemes in the nongregarine parasites, it may be that the N-terminal SH3 domain in the GpMyoF homologs mediates novel associations appropriate to the partic-

Table I. Myosins of Gregarina polymorpha.

<table>
<thead>
<tr>
<th>Class</th>
<th>Mr (kDa)</th>
<th>Neck domain (canonical IQ motifs)</th>
<th>Features of tail domain</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>GpMyoA</td>
<td>XIV</td>
<td>93</td>
<td>Short, highly basic</td>
<td>Epicytic folds, myonemes</td>
</tr>
<tr>
<td>GpMyoB</td>
<td>XIV</td>
<td>96</td>
<td>Short, highly basic</td>
<td>Epicytic folds</td>
</tr>
<tr>
<td>GpMyoF</td>
<td>XXII</td>
<td>222</td>
<td>Coiled-coil segments, WD40 repeats</td>
<td>Myonemes</td>
</tr>
<tr>
<td>Unnamed</td>
<td>XIV</td>
<td>90</td>
<td>Short, highly basic</td>
<td>No data</td>
</tr>
</tbody>
</table>
Immunolocalization of GpMyoF in _C. polymorpha_ by confocal microscopy with the focal plane set in the parasite cortex beneath the epicytic folds. A pattern of cortical striations can be seen in both protomerite (p) and deutomerite (d) cell segments at low magnification (a). Intermediate (b) and high (c) magnifications of the deutomerite cortex demonstrate the association of this myosin with the annular myonemes that gird the parasite cortex. Bar in (a): 25 μm, (b, c): 10 μm.
or fewer blades may occur (Madrona and Wilson, 2004). Proteins that contain WD40 repeats may participate in a broad spectrum of intracellular activities ranging from signal transduction to gene regulation and RNA processing to the regulation of cytoskeletal dynamics (Neer et al., 1994). The unifying feature of these proteins is their ability to participate in multimolecular complexes through interactions that may be mediated, in part, by the β-propeller scaffold (Smith et al., 1999; Madrona and Wilson, 2004; Wilson et al., 2005).

When searching the GpMyoF sequence for conserved protein domains using the CD-Search and Pfam tools, 4 WD40 repeats were found near the end of the myosin tail domain (Fig. 2, repeats indicated in bold). Given the spacing among these repeats and the precedents for 7-bladed β-propellers, it seemed prudent to visually examine the surrounding tail sequence for additional hidden WD40 repeats not recognized by the available protein domain search algorithms. Two additional WD40 repeats were easily defined and fit cleanly into the spaces between the original repeats. A third, but more anomalous, repeat (the repeats indicated in bold). Given the spacing among these repeats were easily defined and fit cleanly into the spaces between the original repeats. A third, but more anomalous, repeat (the repeats indicated in bold).

In addition to WD40 repeats, the proximal portion of the GpMyoF tail contained some regions of predicted coiled-coil structure marked by multiple heptad repeats (Fig. 1). As another common structural motif found in several classes of myosin, coiled-coil domains are most typically associated with the ability of a myosin to dimerize (Krendel and Mooseker, 2005), though such a region could potentially mediate heteromeric interactions with other binding partners as well. There is as yet no evidence for or against the dimerization of GpMyoF in vivo.

The immunolocalization studies presented here indicate that GpMyoF is associated with the annular myonemes of the parasite cortex, the pattern of its distribution being similar to that of myoneme-associated proteins described previously (Heintzelman, 2004). Early ultrastructural studies have described the filamentous nature of the myonemes, including the suggestion that they may consist of actin microfilaments (Beans et al., 1959; Warner, 1968; Hildebrand and Vinckier, 1975; Rühl, 1976; Hildebrand, 1980), and more recent studies have indeed confirmed these structures to be actin-rich (Heintzelman, 2004) and thus an appropriate environment for a myosin. In addition, Ghazali et al. (1989) have identified a putative spectrin-like protein, which, in gregarines that also have annular myonemes, localizes to these structures in a pattern similar to that seen for actin and GpMyoF: As an actin-binding protein, spectrin could potentially mediate a flexible connection between the myoneme system and the cortical membrane system thus allowing myoneme contraction to more directly influence cell shape. What other proteins may contribute to the myoneme scaffold has yet to be determined, though Heintzelman (2004) has already demonstrated the presence of at least 1 additional myosin associated with the myonemes, the class XIV GpMyoA. This myosin, together with a second class XIV myosin, GpMyoB, are also present in the epicytic folds, another actin-rich domain of the parasite cortex, but one in which GpMyoF is not found.

What role the myonemes play, and what contributions, unique or redundant, the 2 myoneme-associated parasite myosins make to the structure or function of the myonemes remains speculative. It has been suggested that the myonemes may play a structural role in parasite architecture as well as a role in some of the movements exhibited by the parasites (Fowell, 1936; Beams et al., 1959; Hildebrand and Vinckier, 1975; Hildebrand, 1980). As gliding motility is most likely associated with the glososomes-associate class XIV myosins present in the epicytic folds in a motility complex akin to what has been thoroughly dissected in T. gondii (Keey and Soldati, 2004; Sibley, 2004; Heintzelman, 2006), the myonemes are more likely responsible for the more macroscopic cellular movements seen in some gregarines.

Various gregarines may engage in a range of different movement patterns, including rolling or pendular movement in the case of Selenidium, peristaltic-like movement in the case of Urospora and Didymophyes, or a more simple ‘bending’ of the cell body as can be seen in G. polymorpha as it changes direction during gliding (Fowell, 1936; Hildebrand and Vinckier, 1975; Hildebrand, 1980; Schrével and Philippe, 1993). This diverse repertoire of movements, no doubt, reflects the equally diverse morphology and coincident cytoskeletal architecture underpinning the surface features of different gregarines. For in-
stance, the surface of Selenidium sp. exhibits a small number of broad epicytic ridges in contrast to the surface of D. gigantea or Gregarina spp., which is replete with epicytic folds supported by an elaborate cytoskeletal network. While the cortex of Selenidium is distinguished by an elaborate array of longitudinally disposed microtubules, it appears to lack the actin-rich annular myonemes that are a distinct feature in G. polymorpha, D. gigantea, and some other gregarines (Beams et al., 1959; Vivier and Schrével, 1964; Hildebrand and Vinckier, 1975; Hildebrand, 1980; Heintzelman, 2004). It might be expected, then, that those gregarines with annular myonemes may engage in additional, or distinct, movement patterns not seen in parasites lacking this structural feature.

Apart from influencing the physical movements of the motile gregarine trophozoites, it can be easily envisioned that the contractile actin-myosin myoneme network could also contribute to the morphogenetic remodeling of the parasites as they progress in their life cycle from syzygy between elongate gamonts into the formation of spherical gametocytes (Schrével and Philippe, 1993). The enlistment of other cytoskeletal systems, e.g., microtubules, is also likely to be important in such a process (Kuriyama et al., 2005). Further studies examining the dynamics of cytoskeletal proteins such as actin, the myosins, spectrin, and others among these dramatic morphological events should shed additional light on the mechanisms involved.

Certainly one question of interest that remains is the role of the WD40 repeat myosins in other apicomplexan parasites such as Plasmodium spp. (PlMyoF) and T. gondii (TgMyoF). Given the very discrete localization of GpMyoF to the myonemes in G. polymorpha, and the fact that comparable myoneme-like structures have not been described in these other apicomplexan parasites, the role played by the myosin F in these cells remains a mystery. Does this myosin contribute in some way to the architecture of the known cytoskeletal scaffolds such as the subpellicular network, or the array of subpellicular microtubules (Nichols and Chiappino, 1987; Mann and Beckers, 2001; Khatzer et al., 2004; Patrón et al., 2005), or is this myosin employed in a novel fashion in these parasites? Clearly there is substantial work to be done in characterizing this unique myosin and its contribution to the elaborate cytoskeletal architecture of apicomplexan parasites.

ACKNOWLEDGMENTS

The authors wish to acknowledge Scott Sperling for his tireless enthusiasm during hours of parasite isolation procedures as well as Dr. Joe Moore for his assistance with the confocal microscopy.

LITERATURE CITED


Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.

Characterization of the subpellicular network, a filamentous membrane skeletal component in the parasite Toxoplasma gondii. Molecular and Biochemical Parasitology 115: 257–268.


KINETICS OF ENCEPHALITOZOO SPP. INFECTION OF HUMAN MACROPHAGES

Jeffrey Fischer, Diana Tran, Richard Juneau, and Hollie Hale-Donze*

Department of Biological Sciences, Louisiana State University, 202 Life Sciences Building, Baton Rouge, Louisiana 70803.
e-mail: hhaled1@lsu.edu

ABSTRACT: Microsporida are obligate intracellular, eukaryotic parasites that are known to infect a variety of invertebrate and vertebrate species and have been reported to include a broad range of host specificities for various cell types. Although it is clear that some species of microsporida have the ability to disseminate, causing multiorgan infections, it is not understood how dissemination occurs. One hypothesis suggests that mononuclear phagocytes engulf the pathogen and migrate to various organs while the parasite persists and proliferates. This implies that microsporida have developed methods by which to escape intracellular degradation and can, instead, use the host as a source of nourishment and a vehicle for dissemination. In our study, we investigated the infection kinetics of 2 Encephalitozoon spp. known to cause disseminated disease in humans. Using fluorescence and scanning electron microscopy, it was determined that spore adherence to the host was rapid (3–6 hr), as was the uptake and organization of internal parasitophorous vacuoles (24 hr). Furthermore, replication was shown to occur within macrophages at 72 hr, as measured by the bromodeoxyuridine proliferation assay, and the production of mature spores occurred in host cells at 120 hr. Parasite replication could be reduced by pretreatment of macrophages with interferon-gamma and bacterial lipopolysaccharide.

For many years, microsporidians have been recognized as pathogens of invertebrate and vertebrate species, including silk-worms, honeybees, mosquitoes, mice, rabbits, foxes, pigs, and cows (Weiss, 2001; Didier et al., 2004), but have only recently gained greater notoriety for their ability to cause chronic and fatal disseminated infections in humans diagnosed with HIV/AIDS or in patients on immunosuppressive therapies (Schottelius and da Costa, 2000; Orenstein, 2003; Carlson et al., 2004; Didier et al., 2004). Of the more than 1,200 microsporidian species, only 14 have been reported to infect humans (Didier et al., 2004). When left untreated, immunocompromised patients suffer from chronic diarrhea, leading to dehydration and malnutrition. Often, in these patients, disseminated disease has been reported to cause keratoconjunctivitis, sinusitis, tracheobronchitis, encephalitis, interstitial nephritis, hepatitis, cholecystitis, osteomyelitis, and myositis (Schottelius and da Costa, 2000; Orenstein, 2003; Didier, 2005).

Microsporidians are obligate intracellular, eukaryotic parasites that were once classified as protozoans, but are now suggested to be more closely related to fungi, based on phylogenetic analysis (Gill and Fast, 2006). Microsporidians are found worldwide and are shed in the feces and urine of infected animals, thereafter residing as environmentally resistant spores. Microsporidian infections are believed to occur when the spore is ingested from contaminated water or food sources (Didier et al., 2004). Infections of epithelial and endothelial cells and macrophages are common (Khan et al., 2001). Classically, cellular invasion occurs when the spore encounters a host cell and injects a polar tube, thereby penetrating the cell membrane and injecting the sporoplasm. Alternatively, spores can be internalized through phagocytosis or endocytosis. Further proliferation and spore production occur through merogony and sporogony within a parasitophorous vacuole (PV), followed by lysis of the host cell and release of mature spores (Franzen, 2004).

The microsporidians Encephalitozoon spp. have been described in several case reports in which the cases culminated in disseminated infections (Gunnarsson et al., 1995; Soule et al., 1997; Carlson et al., 1997; 2004) and these microsporidians have been reported as having a promiscuous tropism for a variety of mammalian tissues and cell lines (Visvesvara, 2002). Some reports have suggested that dissemination is the result of migrating macrophages that act as reservoirs and allow for the vehicular dispersal of the parasite, all the while providing host metabolites for their replication (Soule et al., 1997; Orenstein, 2003; Fischer et al., 2007). To elucidate the role of macrophages in microsporidiosis including dissemination events, it is critical to establish the timing of parasitic entry, replication, and lysis in primary human macrophages. The infection kinetics and production of mature spores have been well established in several nonhuman cell lines and animal models (Cox et al., 1979; Gannon, 1980; Didier et al., 2001; Salat et al., 2001; Wasson and Barry, 2003), whereas most studies on infection kinetics in human cell lines and primary cells are limited (Couzin et al., 2000; Fasshauer et al., 2005; Franzen, Hartmann, and Salzberger, 2005; Franzen, Hosl et al., 2005; Franzen, Muller et al., 2005; Leitch et al., 2005). Although these studies suggest that a variety of cell types can act as permissive hosts for microsporidian replication, the role of primary human macrophages in these infections is not well defined.

Studies on infections of murine models with Encephalitozoon spp. have described methods of parasitic evasion in macrophages (Weidner, 1975) and the consequent increase of mature spores (Didier and Shadduck, 1994) which suggests that parasite replication is possible in these cells. Furthermore, it was established that host resistance to the parasite could be induced by supplementing the cells with interferon-gamma (IFN-γ) or tumor necrosis factor-alpha (TNF-α) (Didier and Shadduck, 1994; Khan and Moretto, 1999), which lead to the subsequent release of reactive nitrogen intermediates that contributed to the success of parasite demise (Didier, 1995).

Recently, studies conducted on Encephalitozoon spp. infections of resting human macrophages reported elevated levels of various cytokines needed for pathogenic clearance, i.e., IFN-γ and TNF-α; however, these studies failed to detect increases in nitric oxide that may lead to microbial killing (Franzen, Hartmann, and Salzberger, 2005). Further investigations determining the kinetics of infection in primary human macrophages showed that these infections did not result in the production of mature spores when followed for 12 days (Franzen, Muller et al., 2005). However, in the discussion and subsequent review, it was proposed that parasites could escape from the phagoly-
sosome yielding a productive infection (Franzen, 2005; Franzen, Muller et al., 2005). Taken together, these studies suggest that microsporidians activate resting macrophages to become microbicidal and do not allow for the pathogen to gain an intracellular stronghold. Both human and murine studies of infection have found that pathogenic clearance is mediated through the induction of cytokines, such as IFN-γ and TNF-α; however, the same studies disagree about the likelihood of Encephalitozoon spp. directly activating resting macrophages to invoke the microbicidal response.

Using 3 different methods to identify the adherence and uptake of the spore, the active replicating stages of the vegetative meronts, and the formation of PV, the present study uniquely defines the life cycle for Encephalitozoon spp. in primary human macrophages. Additionally, we confirm the role for IFN-γ and bacterial lipopolysaccharide (LPS) treatment of resting human macrophages in reducing parasite replication.

MATERIALS AND METHODS

Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats of blood samples from healthy donors (Our Lady of the Lake Regional Medical Center, Baton Rouge, Louisiana) by gradient centrifugation on lymphocyte separation media (Cambrex, Walkersville, Maryland). Monocyte-derived macrophages (MDM) were obtained by adherence assays, as previously reported (Fischer et al., 2007). Briefly, monocytes were plated onto 12-well culture plates (1 × 10⁶ monocytes/well) containing cover glasses (Greiner Bio-One, Cellstar, Monroe, North Carolina) and cultured for 3 hr in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 μg/ml gentamicin at 37°C in 5% CO₂. Cells were stringently washed with PBS to remove nonadherent PBMC and then allowed to differentiate for 7 days in complete media with 10% fetal calf serum (FCS) (Cambrex) at 37°C in 5% CO₂. Cells were viewed using a Leica DMI 2000 inverted fluorescence microscope, and captured and analyzed with a Leica DFX300 FX CCD camera and Image Pro Plus v5.1 software (MediaCybernetics, Silver Springs, Maryland).

Parasites

Encephalitozoon cuniculi III and Encephalitozoon intestinalis were grown in a rabbit kidney cell line (ATCC CCL-37) and harvested from tissue culture supernatants. Spores were washed once in PBS containing 0.2% Tween-20, resuspended in supplemented DMEM, and counted with a hemacytometer (Didier et al., 1991). Some spores were inactivated by treatment with 10% bleach solution for 30 min and washed once with water; others were heat-inactivated in a 95°C water bath for 30 min. Spores were used at a 5:1 spore-to-MDM ratio (Fischer et al., 2007), and infected cultures were washed free of unadhered spores at 6 hr and maintained at 37°C in 5% CO₂.

Scanning electron microscopy (SEM)

SEM was used to monitor immediate adherence of spores to MDM. Cells grown on culture plates were challenged with spores of E. intestinalis for 5 min and fixed with 2% glutaraldehyde and 1% formaldehyde in 0.75% sucrose and 0.1 M sodium cacodylate buffer for 1 hr, washed 4 times with 1.5% sucrose and 0.02 M glycine in 0.1 M sodium cacodylate buffer, postfixed with 2% osmium tetroxide for 1 hr, and rinsed with distilled water. Cells were dehydrated with a graded series of ethanol and air dried. Well bottoms were cut from culture dishes and mounted on aluminum specimen holders with conductive tape, coated with gold/palladium (60/40) in an Edwards S-150 sputter coater, and imaged with a Cambridge S-260 SEM.

Adherence, uptake, and proliferation assays

Kinetics of adherence and uptake were measured by enumerating macrophages that were positive for spores and contained a perinuclear parasitophorous vacuole (PV) of spores, respectively. In adherence assays, macrophages were challenged with spores for 1, 3, or 6 hr, washed with PBS, and fixed in methanol for 10 min. Cells were stained with a 0.2% solution of SCRI Renaissance Stain 2200 chitin label (Renaissance Chemicals Ltd., North Yorkshire, U.K.) for 20 min at room temperature to allow for visualization of the spores. Cover glasses were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, California). MDM with fluorescent blue spores attached were counted.

In uptake assays, spores were prelabeled with PKH26 Fluorescent Cell Linker per manufacturer’s instructions (Sigma, St. Louis, Missouri). MDM were challenged for 3, 6, 12, 24, 48, or 72 hr. MDM challenged for 3 hr were washed of free spores and fixed. All other cultures were washed with PBS at 6 hr postchallenge to remove free spores. Cells were fixed in 10% formalin for 30 min and cover glasses were mounted in Prolong Gold antifade reagent with DAPI to detect nuclear staining of MDM. MDM with perinuclear organized vacuoles of red spores were counted.

Microsporidia proliferation and infection were monitored using the bromodeoxyuridine (BrDU) assay system (Invitrogen). MDM were challenged with spores for 24, 48, 72, 120, 168, or 240 hr and fixed in 4% paraformaldehyde. Free spores were washed from cultures with PBS at 6 hr postinfection (PI) and the medium was replaced. Twenty-four hours prior to fixation, 100 μM of BrDU was added to each well. Cells were washed with 1% Triton X-100 and treated with 1 N HCl for 10 min on ice, 2 N HCl for 10 min at room temperature, and 2 N HCl for 20 min at 37°C. Cells were neutralized in a 0.1 M borate buffer and washed with 1% Triton X-100. MDM were incubated on ice with a 1:50 dilution of Alexa Fluor 488 anti-BrDU (Invitrogen) in 0.5% bovine serum albumin and 1% Triton X-100 for 90 min. Cells were washed with 1% Triton X-100 and mounted in Prolong Gold antifade reagent. In some cultures, MDM were stimulated with various concentrations of human recombinant IFN-γ (eBioscience, San Diego, California) or LPS (Sigma) 24 hr prior to spore challenge. MDM containing green fluorescent vacuoles of replicating parasite were counted.

The kinetics of spore production was monitored with Differential Interference Contrast (DIC) microscopy by counting MDM that contained mature PV of spores.

Ten fields of view (FOV) with an average number of 115 MDM per experiment were counted with a ×630 magnification in all assays, unless otherwise stated. Cells were viewed using a Leica DMI 6000 B inverted fluorescence microscope, and captured and analyzed with a Leica DFX300 FX CCD camera and Image Pro Plus v5.1 software (MediaCybernetics, Silver Springs, Maryland).

Statistical analysis

Three experiments were performed at each time point and for each parasite condition, unless otherwise stated. Each experiment consisted of a single donor and was measured in duplicate. Statistical significance was determined by the unpaired, 2-tailed t-test, and values of P < 0.05 were considered significant. All error bars shown in this paper are SEM. Analyses were performed using InStat software (GraphPad 3.0, San Diego, California).

RESULTS

Encephalitozoon spp. rapidly adhere to macrophages and are internalized by a majority of the cells within the first 24–48 hr

Spore adherence to macrophages is a rapid process as seen by SEM. MDM exposed to E. intestinalis spores for 5 min prior to fixation and mounting demonstrate that minimal time was needed for some of the cells to acquire several spores on their surface (Fig. 1A). Typically, aggregates of spores were seen bound on MDM, which suggests that cells may cluster specific surface molecules needed for microsporidia adherence (Fig. 1B). Host membranes were observed intimately associated with the spores.

Using fluorescence microscopy, we established that MDM exposure to spores for 6 hr is required to generate greater than 75% of MDM with adhered spores. Cells cultured with E. ca-
niculi or E. intestinalis for 1, 3, or 6 hr were washed free of spores, fixed, and stained with a fluorescent chitin label to visualize the spore wall (Fig. 2A). Nearly 50% of MDM had at least 1 spore attached after only 1 hr of challenge with either species (Fig. 2B). The percentage of MDM with attached spores and the number of spores per macrophage (Fig. 2C) continued to increase over time up to 6 hr. At 6 hr, approximately 79% of MDM showed adherence of E. cuniculi, whereas 85% of cells were positive for E. intestinalis. The average number of spores per macrophage observed at 6 hr was approximately 11 and 17 for E. cuniculi and E. intestinalis, respectively. However, some MDM did not have any spores attached. This suggests that a population of MDM may be present that does not express a surface molecule needed for immediate attachment. These initial results determined that greater than 75% of MDM would have attached spores after 6 hr of exposure. Therefore, in all performed experiments, nonadhered spores were washed out at this time point, unless otherwise stated.

To monitor the kinetics of microsporidian uptake, MDM were challenged with spores prelabeled with an amphipathic lipid fluorescent dye that binds plasma membranes and allows one to follow the parasitic membranes during intracellular development. In previous reports that studied kinetics (Didier and Shadduck, 1994; Couzin et al., 2000; Franzen, Muller et al., 2005), parasites have been detected with fluorescent chemicals or antibodies directed against the spore coat, which is subsequently removed or lost during parasite replication. MDM were monitored for perinuclear vacuoles containing red-labeled spores at various times (Fig. 3A). By 6 hr PI (Fig. 3B), approximately 27% of MDM contained vacuoles and demonstrated minor increases in uptake at 12 hr and 24 hr. Comparison of uptake data at time points consecutive to each other revealed significant increases in spore uptake occurring at both 48 hr and 72 hr, with 56% and 69% of MDM observed to contain labeled vacuoles, respectively.

**Pronounced replication of E. cuniculi in MDM begins at 72 hr PI**

MDM infected with E. cuniculi spores at various times were observed for microsporidia replication using the BrdU assay. The nucleotide analog (BrdU) was added to infected cultures 24 hr prior to fixation and stained with an anti-BrdU antibody conjugated to a fluorescent dye to visualize vacuoles of replicating parasites (Fig. 4A). MDM are terminally differentiated cells and therefore, do not incorporate BrdU into the DNA, whereas the actively replicating meront stage of the parasite will utilize this nucleotide. Little replication was observed at 24 hr and 48 hr, whereas approximately 32% and 34% of MDM were positive for vacuoles with BrdU labeling at 72 hr and 120 hr, respectively (Fig. 4B). By 168 hr and 240 hr, a majority of the cells contained replicating vacuoles of microsporidians as detected by BrdU labeling.

The production of mature spores is analogous with the formation of mature PV containing easily identifiable spores of light microscopy. Using DIC microscopy, MDM that contained PV with mature spores were counted at various times (Figs. 4C–D). By 120 hr, 15% of MDM contained vacuoles of spores, increasing to 27% and 22% by 168 hr and 240 hr, respectively. It was first noted that a modest amount of MDM appeared to have ruptured and were releasing spores by 168 hr; this was also observed at 240 hr. The release of spores and destruction of the MDM could account for an unexpected lower percentage of cells containing mature PV. This also signifies the end of 1 round of the life cycle.

In control studies, some MDM were infected with chlorine-treated or heat-inactivated E. cuniculi spores in which approximatively 1% of MDM stained positive for chlorine-treated spores and 1.6% for heat-inactivated spores at 72 hr. These positive results from the chlorine-treated and heat-inactivated studies could be attributed to minor emissions of cellular autofluorescence. The production of mature PV was not observed in either treatment.

**Replication kinetics of Encephalitozoon spp. are similar in MDM**

To determine if both species of microsporidians are able to replicate in MDM and have similar kinetics of infection, MDM were infected with E. intestinalis and observed for proliferation using the BrdU assay. As with E. cuniculi, E. intestinalis was
able to replicate and complete sporogony as evaluated by PV formation in human MDM. Approximately 26% of the MDM were positive for replicating parasites at 72 hr and this number increased to 47% of cells by 120 hr. The BrdU incorporation for E. intestinalis was statistically significant from that reported for E. cuniculi at 120 hr; however, the percentage of cells with E. intestinalis PV did not differ at the same time point (Table I).

**DISCUSSION**

Macrophages have been described as essential players for parasitic clearance in many model systems and for various pathogens (James, 1995). Resting macrophages require signals from Th1 cells, such as IFN-γ and CD40L, which result in their activation and increased intracellular microbicidal effects (Jeneway et al., 2005). Cases of microsporidiosis in HIV/AIDS patients and organ recipients receiving immunosuppressive therapies, who have significantly lower numbers of T cells, report...
that full clearance of the pathogen is difficult, and in some cases impossible, leading to dissemination and death (Soule et al., 1997; Schottelius and da Costa, 2000; Salat et al., 2001). In our system, Encephalitozoon spp. could successfully exploit the host surface molecules and intracellular environment of human macrophages in the absence of Th1 activation signals and achieve a productive infection resulting in the accumulation of mature spores.

To address critical questions about infection dynamics within a larger population and to establish a more defined time between spore attachment and PV formation, this study has used different approaches to better define the infectious process in primary human macrophages. Prior methods to detect the spore coat with UV brighteners or antibodies are problematic in that the sporoplasm will eventually dissociate from the spore coat to undergo merogony. Because the meront stage has only previously been shown using TEM, limited information about the total population dynamics could be addressed. The present study, using a variety of methods, specifically addresses the need to determine adherence, uptake, replication, and spore formation in primary human macrophage, because no clear kinetics or methods have been established for following the infectious process, which is critical for looking at immune parameters related to entry (vs.) replication.

Our results indicate that spore adherence to the host cell is a rapid process; adherence could be detected by SEM within 5 min after exposure of MDM to spores (Fig. 1) and three-quarters or more of MDM were adorned with numerous microsporidia by 6 hr postchallenge (Fig. 2). This work is in agreement with several other research groups (Couzinier et al., 2000; Franzen, Hosl et al., 2005; Leitch et al., 2005) that have reported that spore uptake is a rapid, actin-dependent process. Additionally, we observed a subset of macrophages that did not contain any spores on their surface, which may represent a population

<table>
<thead>
<tr>
<th>Table I. Comparison of replication kinetics between Encephalitozoon cuniculi and Encephalitozoon intestinalis. SEM = ±.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Encephalitozoon cuniculi</td>
</tr>
<tr>
<td>Encephalitozoon intestinalis</td>
</tr>
</tbody>
</table>

* P < 0.05 in comparison to E. cuniculi at 120 hr.
of cells that do not express a surface molecule required for spore adherence. Recently, Hayman et al. (2005), has shown that spore adherence can be mediated by the expression of sulfated glycosamoinoglycans, specifically, heparan sulfate, which has been established as an abundant component of the activated human MDM cell surface (Clasper et al., 1999). Based on these reports, the disparity in number of spores we observed in association with MDM may reflect the activation status of the cells.

In experiments with prelabeled spores, we looked to define the amount of MDM present that contained organized vacuoles of microsporidia and were perinuclear in the host cell at various times (Fig. 3). Similar to the findings of Franzen, Muller et al. (2005), greater than 30% of MDM were observed to contain these vacuoles at 24 hr PI. In contrast to the results described by Franzen, Muller et al. (2005), which show that the number of MDM-internalized spores decreases over the next 48 hr and that new developing parasites were not observed for the remainder of a 12-day infection, we report that 50% of MDM contain vacuoles of the parasite between 24-48 hr and nearly 70% at 72 hr. This discrepancy could be because of our differences in staining techniques used to follow the parasite's uptake and intracellular fate. Our staining technique utilizes an amphiphatic dye that incorporates into cell membranes, thereby allowing us to follow the sporoplasm of the parasite, not the spore coat, which must be removed prior to replication.

Furthermore, proliferation in MDM was confirmed by BrdU incorporation as early as 72 hr PI, whereas ruptured and broken cells were observed at 168 hr PI (Fig. 4). This indicates that these microsporidians are capable of completing their life cycle within MDM as early as 5 days PI and commit to successive rounds of reinvasion. Differences in kinetics between Encephalitozoon spp. were not detected and, therefore, demonstrate that both species have similar infection kinetics (Table I).

Knowledge of the kinetics of replication can be used to identify differences in host cell gene expression between initial, preproliferative events and those that occur during replication, as well as for studies of meront genomics. This information could lead to a better understanding of which genes and proteins are being expressed or suppressed to produce a permissive environment and foster parasite development.

Treatment of macrophages with extracellular signals, such as IFN-γ and LPS, has been shown to prevent various intracellular pathogens from proliferating and to promote parasitic clearance. These signals sensitize and activate the cells to release reactive oxygen and nitrogen species into membrane-bound vesicles containing the parasite and promote fusion of degradative lysosomes (Janeway et al., 2005). Our studies suggest that an extracellular signal must be present in order to reduce parasitic replication (Fig. 5), because IFN-γ production was not detected upon spore exposure to resting MDM (data not shown). Cultures pretreated with IFN-γ or LPS showed moderate decreases in parasitic replication, whereas cultures treated with a combination of the 2 produced a significant decrease in MDM with replicating parasites; approximately a 44% decrease was observed. Although a decrease in parasitic burden was observed, none of the treatments completely inhibited replication. Similar results were reported by Didier and Shadduck (1994), when activating a murine macrophage cell line with either IFN-γ or LPS decreased the number of intracellular E. cuniculi spores over time in a dose-dependent response. Additionally, a combination of the 2 signals had an even greater decrease in the number of spores observed, but did not result in complete clearance of the spores (Didier and Shadduck, 1994). In another study, SCID mice infected with E. cuniculi were reported to have 90% of their macrophages infected with spores by day 14, whereas spores were not observed in macrophages of wild type mice (Salat et al., 2001). Taken together, these reports indicate a necessity for a diverse immune response, possibly including CD8+ and γδ T cells (Khan et al., 1999; Moretto et al., 2001), for a full recovery.

The intracellular fate of microsporidia has been the topic of debate in the literature (Weidner, 1975; Franzen, 2004). Our studies clearly indicate that Encephalitozoon spp. can avoid destruction by naïve macrophages and replicate within these permissive cells, resulting in the formation of mature spores. We have recently reported that infected macrophages can induce a functional chemokine gradient leading to the recruitment of additional macrophages (Fischer et al., 2007), which correlate to the onset of parasitic replication. Taken together, these studies suggest that microsporidia have a means of utilizing phagocytes responding to sites of infection as reservoirs for proliferation and as a medium for the continuous growth and dissemination of the infection.

ACKNOWLEDGMENTS

This study was supported by NIH RR020159-01 and partially by the Louisiana Board of Regents LEQSF (2004-7)-RD-A-10. We thank Dr. Elizabeth Didier and Lisa Bowers for donating E. cuniculi III strain, Cindy Henk with the Socolofsky Microscopy Center at LSU for her technical assistance with SEM, and the staff at Our Lady of the Lake Blood Center for providing the buffy coats. We also thank Dr. James Moroney for his critical review of the manuscript, and Jeffery West and undergraduates for their technical assistance.
LITERATURE CITED


———, J. D. Schwartzman, L. H. Kasper, and M. Moretto. 1999. CD8+ CTLs are essential for protective immunity against Encephalitozoon cuniculi infection. Journal of Immunology 162: 6086–6091.


A NEW SPECIES OF *ALLOCREADIDUM* (TREMATODA: *ALLOCREAIDIIDAE*) FROM FRESHWATER FISHES IN THE DANJIANGKOU RESERVOIR IN CHINA

Dian Gao, Gui Tang Wang*, Bing Wen Xi, Wei Jian Yao, and Pin Nie

State Key Laboratory of Freshwater Ecology and Biotechnology, and Fish Immunology and Parasitology Group of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, Hubei Province, P.R. China. e-mail: stnwang@ihb.ac.cn

**ABSTRACT:** A new species of *Allocreadium*, *Allocreadium danjiangensis* n. sp., is described from the intestine of several species of freshwater fish, including *Abbottina rivularis* (Basilewsky, 1855), *Sarcocheilichthys nigripinnis nigripinnis* (Günther, 1873), *Gnathopogon argentatus* (Sauvage et Dabry 1874), *Opsariichthys unicirrhis bidentis* (Günter, 1873), and *Erythroculter mongolicus mongolicus* (Basilewsky, 1855) (Cyprinidae) from the Danjiangkou Reservoir in central China. The main morphological characters of the new species are as follows: vitelline follicles numerous, extending from the level of acetabulum to posterior extremity, distributed over both sides around the ceca; cirrus sac relatively large, developed, lying obliquely anterior to the acetabulum, extending from the level of the intestinal bifurcation to the central level of acetabulum, and overlapping left or right cecal; and ovary much smaller than testes, generally close to or even overlapping the anterior border of anterior testis. Observation by scanning electron microscopy shows only 2 kinds of tegumental formations, i.e., papillae and tubercles, instead of 3 types of tegumental formations, i.e., papillae, bosses, and minute sensor receptors observed on other species of the Allocreaidiidae. The tegumental striations of the present species vary on the different parts of the body. In addition, a new structure, identified as the "groove" with a tonguelike tubercle, was observed on the inner wall of acetabulum.

In Asia, species of *Allocreadium* Loose, 1902 are among the most common and widely distributed freshwater fish parasites. So far, more than 10 species have been described in China (Wang et al., 1983; Wang, 1983, 1984; Zhang and Yang, 1994; Lu and Wu, 1996; Feng and Wang, 1997). Most of them have been recorded in the southern drainage basin, which includes the Yangtze and Minjiang rivers.

The Danjiangkou Reservoir (110°08'–110°34'E, 32°14'–32°58'N), as the headwater of the middle route of the south-to-north Water Transfer Project of China, is located on the Hanjiang River, which is the largest branch of the Yangtze. It possesses rich fishery resources, but only a few data on the helminth parasites of fishes in this reservoir have been presented to this point (Wu et al., 2006). From February 2004 to December 2006, we examined more than 50 species of fish and identified more than 20 species of digenean trematodes. Among them was a new species of *Allocreadium* (Trematoda: Allocreaidiidae).

**MATERIALS AND METHODS**

The fish in the present study were captured by local fishermen in the Danjiangkou Reservoir or the Hanjiang River below the reservoir dam. The fork length of each fish was measured. Fish were then necropsied according to Gao et al. (1997). Some specimens were washed by 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C, postfixed in 1% osmium tetroxide for 2 hr at 4°C, dehydrated through an ethanol series and critical-point dried, then sputter-coated with gold, and examined using a scanning electron microscope according to Gao et al. (2003).

All measurements are given in micrometers. Type specimens have been deposited in 70% ethanol in the Institute of Hydrobiology, Chinese Academy of Sciences in China. Scientific names of the fishes are given according to Froese and Pauly (2005).

*Received 27 February 2007; revised 13 July 2007; accepted 21 July 2007.*

*To whom correspondence should be addressed.*

**FIGURE 1.** *Allocreadium danjiangensis* n. sp. (A) Entire worm, ventral view; (B) terminal genitalia, ventral view; (C) ovarian complex, dorsal view. Bar = 100 μm.
**DESCRIPTION**

*Allocreadium danjiangensis* n. sp.

(Figs. 1–4)

*Diagnosis:* Allocreadiidae. Description and measurements on the basis of 7 specimens from *Abbottina rivularis* (Basilewsky, 1855), 3 from *Sarcocheilichthys nigripinnis nigripinnis* (Gunther, 1873), 2 from *Gnathopogon argentatus* (Sauvage et Dabry, 1874), 1 from *Opsarichthys uncirostris bidens* (Gunther, 1873), and 2 from *Erythroculter mongolicus mongolicus* (Basilewsky, 1855). Body elongate, flattened (Figs. 1A, 2A), with maximum width at level of acetabulum just anterior to midbody (Fig. 2A), 138–274 × 380–960 in size. Tegument smooth by light microscopy, but with some papillae and occasionally with tubercle clusters on the forebody by scanning electron microscopy (SEM) (Figs. 2B, C, 4). Surface striations on anterior body different from posterior part; striations on posterior part much denser, but shallower than at anterior end of body (Fig. 3A–D). Protuberant ruga densely covering dorsal surface of forebody, whereas ventral tegument between oral sucker and acetabulum resembles a tortoise shell. Numerous papillae distributed over both ventral and dorsal surface of forebody, extending from oral sucker to anterior region of acetabulum (Fig. 2B, C). Papillae arranged somewhat symmetrically on ventral surface, but scattered irregularly on dorsal surface. All papillae relatively large, rounded, almost equal in size, and in proximity to each other. Tubercle clusters observed on dorsal surface of forebodies of a few specimens much larger than papillae (Fig. 4). Oral sucker oval to round, terminal, 144–228 × 96–208 in size, with muscular grids at its edge and tegumental papillae around it. Prepharynx absent. Pharynx muscular, rounded, 64–104 × 56–96 in size. Esophagus short, bifurcating into ceca between pharynx and acetabulum. Ceca broad, blind, long, reaching almost to posterior end. Acetabular region caelate; acetabulum oval to round, submedian, 144–324 × 144–320 in size, with muscular grids at its edge similar to those of

**Table 1. Infection of Allocreadium danjiangensis n. sp. in various fishes in the region of the Danjiangkou Reservoir, China.**

<table>
<thead>
<tr>
<th>Fish species</th>
<th>No. of fish examined</th>
<th>Fish fork length (mean ± SD) (cm)</th>
<th>Prevalence (%)</th>
<th>Abundance (mean ± SD)</th>
<th>Intensity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abbottina rivularis</em></td>
<td>182</td>
<td>8.44 ± 1.4</td>
<td>19.78</td>
<td>0.71 ± 4.22</td>
<td>3.61 ± 9.01</td>
</tr>
<tr>
<td><em>Sarcocheilichthys nigripinnis nigripinnis</em></td>
<td>124</td>
<td>8.24 ± 1.09</td>
<td>4.03</td>
<td>0.08 ± 0.43</td>
<td>2.00 ± 1.00</td>
</tr>
<tr>
<td><em>Gnathopogon argentatus</em></td>
<td>196</td>
<td>10.30 ± 1.17</td>
<td>14.80</td>
<td>0.45 ± 1.75</td>
<td>3.03 ± 3.64</td>
</tr>
<tr>
<td><em>Opsarichthys uncirostris bidens</em></td>
<td>328</td>
<td>13.66 ± 2.44</td>
<td>4.88</td>
<td>0.08 ± 0.45</td>
<td>1.56 ± 1.41</td>
</tr>
<tr>
<td><em>Erythroculter mongolicus mongolicus</em></td>
<td>244</td>
<td>26.43 ± 4.75</td>
<td>1.64</td>
<td>0.05 ± 0.47</td>
<td>2.75 ± 2.87</td>
</tr>
</tbody>
</table>
FIGURE 3. Muscular striations on body surface. (A) Dorsal view of anterior body; (B) dorsal view of posterior body; (C) ventral view between oral sucker and acetabulum; (D) ventral view of posterior body.

oral sucker, but no tegumental papilla observed in it. Several grooves arranged regularly on inner wall of acetabulum, each groove with a tonguelike tubercle (Fig. 2F). Ratio of oral sucker to acetabulum 1:0.97 to 1.48. Two testes globular or oval, entire, tandem, near middle of hindbody; anterior and posterior testes 112–344 × 96–360 and 96–224 × 160–376 in size, respectively. Cirrus sac relatively large, well developed, clavate, with anterior end slightly curved ventrally, lying obliquely anterior to acetabulum, extending from level of intestinal bifurcation to central level of acetabulum, overlapping left or right cecum, 144–304 × 144–320 in size (Fig. 1A, B). Cotied seminal vesicle and long, rectilinear pars prostatica inside cirrus sac. Genital pore submedian in forebody, generally at level of intestinal bifurcation. Ovary rounded, submedian, much smaller than testes, generally close to, or overlapping, anterior border of anterior testis, 64–200 × 64–144 in size (Fig. 1A). Mehlis’ gland situated between ovary and anterior testis, Laurer’s canal long, opening dorsally at level of posterior border of ovary (Fig. 1C). Seminal receptacle very small, pyriform, situated between ovary and posterior testis, often invisible. Vitelline gland developed, with numerous follicles, 56–112 × 24–56 in size, extending from level of acetabulum to posterior extremity, distributed over both sides of ceca, partly overlapping testes and ovary. Uterus between intercecal, extending windingly from acetabulum to posterior testis. Eggs oval, large, 56–112 × 24–56 in size. Excretory vesicle tubular, reaching anterior border of anterior testis. Excretory pore opening at posterior end of body (Fig. 2E).

Taxonomic summary

Type host: Abbottina rivularis (Basilewsky, 1855).
Other hosts: Sarcocheilichthys nigripinnis nigripinnis (Günther, 1873), G. argentatus (Sauvage et Dabry, 1874), O. uncirostris bidens (Gunther, 1873), and E. mongolicus mongolicus (Basilewsky, 1855).
Site of infection: Intestine.
Locality: The Danjiangkou Reservoir (110°08’–110°34’E, 32°14’–32°58’N), Hubei Province, P.R. China.
Specimens deposited: Holotype and paratypes deposited in the Mu-
Epidemiologic data: The prevalence, mean abundance, and intensity of the digenean, and the number and fork length of its hosts are listed in detail in Table I.

Remarks
Yamaguti (1971) divided Allocreadium into 3 subgenera, i.e., Allocreadium, Allocreadioides, and Neoallocreadium. The morphological characters of the present new species are consistent with those of Allocreadioides. Caira and Bogga (2005) considered Allocreadioides and Neoallocreadium as junior synonyms of Allocreadium, and parasitologists should avoid discussion of subgenera of Allocreadium until a thorough examination of species assigned to Allocreadium has been conducted. Therefore, discussion on the new species with similar species is restricted to all species in Allocreadium.

Yamaguti’s (1971) scheme of Aquatic Organism in the Institute of Hydrobiology, Chinese Academy of Sciences, in Wuhán, Hubei Province.

Etymology: The specific name relates to the locality where it was found.

Discussion
So far, there have been many species of Allocreadium reported from many locations in the world. In several neighboring countries of China, digeneans in this genus have also been found. For example, there have been about 15 species of the genus described in Japan; 3 of them, i.e., Allocreadium gotoi (Hasegawa and Ozaki, 1926), Allocreadium tosai Shimazu, 1988, and Allocreadium aburahaya Shimazu, 1988, resemble this new spe-
cies. In India, only 1 species, *Allocreadium singhi* Rai, 1962 (Rai, 1962), is similar to the present species. In the former U.S.S.R., there are 4 species, i.e., *Allocreadium dogieli* Koval, 1951, *Allocreadium markevitschii* Koval, 1949, *Allocreadium maculate* Akhmerov, 1960, and *Allocreadium hemibarbi* Roitman, 1963, considered to be similar to the new species (Yamaguti, 1971). Thomas (1957) regarded the following characters as important to differentiate between the various species of *Allocreadium*: (1) relative size of the oral and ventral suckers; (2) extent of the vitelline glands; (3) position of the ovary; (4) extent of the intertesticular space; (5) length of the esophagus; (6) size of pharynx; and (7) position of the genital pore. According to these characters, the new species can be differentiated from the above similar species on the basis of some of the following criteria: *A. tosai* and *A. aburahaya* have much larger ovaries than their testes; *A. gotoi* has a much more developed seminal receptacle (Shimazu, 1988, 2003). The others distinguish themselves from the present new species in the position of the ovary very close to the acetabulum and far from the anterior testis.

To date, no observation by SEM has been reported for any species of *Allocreadium*. The present SEM observations reveal just 2 kinds of tegumental formations on the surface of the new species, namely papillae and tubercles. This differs from other allocreadiid species such as *Crepidostomum farionis* (Muller, 1784) and *Crepidostomum metoeus* (Braun, 1900), which have 3 types of tegumental movements, i.e., papillae, bosses, and minute sensor receptors (Moravec, 2002). Unlike present new species, the papillae of *C. metoeus* were found to have 5 types of presumed ciliate sensory receptors by transmission electron microscopy (Zd’arska and Nebesárová, 2004). However, the tegumental structures of the new species are different in number and arrangement compared with species of other genera in the family. The papillae of the present species are not only distributed over the ventrolateral fields between the head end and the acetabulum, but are also scattered on the dorsal surface of the forebody, and have a higher density. Moreover, the tegumental striations of the forebody are much more striking than those on the hindbody. In addition, we observed “grooves” in the inner wall of the acetabulum, which have not been mentioned previously for species of Allocreadiidae, although their function remains unclear.

ACKNOWLEDGMENTS

The research was financially supported by the National Natural Science Foundation of China through the projects 30371102 and 30571413. The authors thank many colleagues for their assistance in the collection of the parasites.

LITERATURE CITED


NEW AND ALREADY KNOWN ACANTHOCEPHALANS FROM AMPHIBIANS AND REPTILES IN VIETNAM, WITH KEYS TO SPECIES OF PSEUDOACANTHOCEPHALUS PETROCHENKO, 1956 (ECHINORHYNCHIDAE) AND SPHAERECHINORHYNCHUS JOHNSTON AND DELAND, 1929 (PLAGIORHYNCHIDAE)

Omar M. Amin, Nguyyen Van Ha*, and Richard A. Heckmann†
Institute of Parasitic Diseases, P.O. Box 28372, Tempe, Arizona 85285. e-mail: omaramin@aol.com

ABSTRACT: Adults of 2 new species in 2 orders of acanthocephalans obtained from the intestines of terrestrial amphibians and reptiles collected between 1998 and 2004 in Vietnam are described here. Pseudoacanthocephalus nguyenthileae n. sp. (Palaeacanthocephala: Echinorhynchidae) was collected from 5 species of terrestrial amphibians: (1) the common Sunda toad Bufo melanostictus Schneider (Bufoidea); (2) Paœ verucospinosa (Bourret); (3) Gunther’s Amoy frog Rana guntheri Boulenger; (4) Taipei frog R. taipehensis Denburgh (Ranidae), and (5) the Burmese whipping frog Polypedates mutus (Smith) (Racophoridae); as well as from the Chinese cobra Naja atra Cantor (Reptilia: Elapidae) and house gecko Hemidactylus frenatus Dumeril and Bibron (Reptilia: Gekkonidae). Sphaerechinorhynchus maximesossipinus n. sp. (Plagiorhynchidae: Sphaerechinorhynchinae) was isolated from a king cobra Ophiophagus hannah (captor) (Reptilia: Elapidae), Cystacanths of Porrorchis houdemeri (Joyeux and Baer, 1935) Schmidt and Kuntz, 1967 (Plagiorhynchidae: Porrorchiniidae) obtained from the mesenteries of banded krait Bungarus fasciatus (Schneider) (Reptilia: Elapidae), a paratenic host, are reported for the first time. Keys to the species of Pseudoacanthocephala and Sphaerechinorhynchus are included. Characteristic features distinguishing the new species from related taxa include: 
P. nguyenthileae has 15–19 (usually 16–18) proboscis hook rows, each with 5–6 hooks that progressively increase in length and size posteriorly. The largest, intermediate, and smallest proboscis hooks of S. maximesossipinus are the middle, anterior, and posterior hooks, respectively; the proboscis and neck are enclosed in a membrane. Morphometric characteristics of P. nguyenthileae show host-related variability.

The material reported in this work has been collected since 1998 as part of a larger faunistic research program from a wide variety of terrestrial vertebrates conducted by the Institute of Ecology and Biological Sciences (IEBS), Hanoi, Vietnam. Free-ranging hosts were examined for parasites. Acanthocephalans were made available by N.V.H., who was instrumental in the conduct of the IEBS program. The number and uniqueness of the new taxa attest to the extraordinary status of these unusual and new acanthocephalan hosts. The present report adds new and interesting knowledge and understanding of the parasite fauna of Vietnam, which is just now opening for international collaborative research in parasitology sponsored by the IEBS. Few acanthocephalans have been recently reported from Vietnam, e.g., Amin et al. (2000, 2004). A total of only 28 species of Acanthocephala (17 in Echinorhynchida, 7 in Gyracanthocephala, and 4 in Neoechinorhynchida) have been reported from Vietnamese fishes to date (Arthur and Te, 2006). None has been identified from amphibians or reptiles.

MATERIALS AND METHODS

Collection data included host name and size, number examined, prevalence, parasite names, mean and range of parasites per examined host, as well as collection localities and number of specimens available for study (Table 1). The amphibian and reptilian hosts were purchased from farmers in various localities and subsequently examined for parasites. Freshly collected specimens were relaxed for a few hours or overnight in 0.9% NaCl and were then transferred to 80% ethanol. Worms were then punctured with a fine needle and subsequently stained in Mayer’s pineol, then

of Acanthocephala (17 in Echinorhynchida, 7 in Gyracanthocephala, and 4 in Neoechinorhynchida) have been reported from Vietnamese fishes to date (Arthur and Te, 2006). None has been identified from amphibians or reptiles.

Finally whole mounted in Canada balsam. Some thick specimens were sliced before mounting to reveal internal structures.

Measurements are reported in micrometers unless otherwise stated. The range is followed by the mean (in parentheses). Length measurements are given before the width; the latter refers to maximum width. Trunk length does not include the neck, proboscis, or bursa. Eggs refer only to fully developed mature eggs removed from the body cavity. Specimens were deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland.

RESULTS

The taxa described in the present study include 2 new and 1 already known species of acanthocephalans belonging in 2 orders and 2 families of Palaeacanthocephala Meyer, 1931 collected from 5 species of terrestrial amphibians and 4 species of reptiles between 1998 and 2004. This collection is organized according to the taxonomy scheme of Amin (1985).

DESCRIPTION

Pseudoacanthocephalus nguyenthileae n. sp
(Figs. 1–10)

General: Echinorhynchidae, with characters of the genus. All shared characters markedly larger in females than in males. Trunk cylindrical, aspinose widest near middle, but more anteriorly in females and gradually tapering toward both ends (Figs. 1, 2, 4). Body wall thick, with markedly pronounced reticular lacunar system. Proboscis cylindrical, rounded anteriorly, with 15–19 longitudinal and regularly alternating hook rows (usually 16 in males and 17–18 in females), each with 5 or 6 hooks (5 in males and 5 or 6 in females) (Fig. 10). Hooks increase in length and size posteriorly and all are rooted; roots simple and directed posteriorly; no manubria (Fig. 6). Neck of moderate size, broader at base, with clusters of 50–60, mostly lateral, nucleated sensory pits. Proboscis receptacle simple, double-walled, with brain at its posterior end and with cellular elements associated with retractor muscles just exterior to its posterior tip (Fig. 10). Lemnisci congested, with many small nuclei similar to, but often larger than, those of brain; equal, shorter, or longer than receptacle, in anterior end of trunk (Fig. 10).

Male (based on 11 mature adults with sperm): Trunk 4.12–8.62 (6.98) mm long, 0.67–1.55 (1.18) mm wide. Proboscis 364–541 (469) long, 208–375 (288) wide, with 15–19 (16.18) hook rows, each with 5
Table I. Collections of acanthocephalans from the intestine of amphibians and reptiles from Vietnam during 1998–2004.

<table>
<thead>
<tr>
<th>Hosts (Amphibia)</th>
<th>Common name</th>
<th>No. examined</th>
<th>Locality</th>
<th>Length SVL (cm) mean ± SD (range)</th>
<th>Prevalence</th>
<th>Intensity# mean (range)</th>
<th>Study specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amphibia</strong> (B. melanostrictus Schneider**)</td>
<td>Common Sunda toad</td>
<td>24</td>
<td>Tam Dao*</td>
<td>10.8 ± 0.2 (10.5–11.5)</td>
<td>58%</td>
<td>PN: 7.8 (1–15)</td>
<td>11</td>
</tr>
<tr>
<td><strong>Amphibia</strong> (Rana guentheri) **</td>
<td></td>
<td>30</td>
<td>Xuan Son†</td>
<td>7.2 ± 1.7 (5.5–10.2)</td>
<td>60%</td>
<td>PN: 7.3 (1–15)</td>
<td>23</td>
</tr>
<tr>
<td><strong>Amphibia</strong> (Polypedates mutus (Smith)**)</td>
<td>Burmese whipping frog</td>
<td>5</td>
<td>Xuan Son</td>
<td>4.8 ± 0.2 (4.4–5.3)</td>
<td>100%</td>
<td>PN: 3.5 (2–4)</td>
<td>4</td>
</tr>
<tr>
<td><strong>Reptilia</strong> (Banded krait) **</td>
<td>Banded krait</td>
<td>149</td>
<td>Red River Delta;</td>
<td>150.1 ± 75.5 (56.0–217.5)</td>
<td>4%</td>
<td>PH: 5.6 (1–27)</td>
<td>6</td>
</tr>
<tr>
<td><strong>Naja atra</strong> (Schneider)</td>
<td>Chinese cobra</td>
<td>157</td>
<td>Red River Delta</td>
<td>135.7 ± 60.4 (70.2–165.0)</td>
<td>2%</td>
<td>PN: 0.6 (1–2)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Ophiophagus hannah</strong> (Cantor)</td>
<td>King cobra</td>
<td>2</td>
<td>Vinh Phuc§</td>
<td>200</td>
<td>100%</td>
<td>SMM: 4.0 (4)</td>
<td>7</td>
</tr>
<tr>
<td><strong>Reptilia</strong> (Hemidactylus frenatus Dumeril &amp; Bibron)</td>
<td>House gecko</td>
<td>25</td>
<td>Babe Park¶</td>
<td>4.7 ± 1.2 (4.0–6.0)</td>
<td>4%</td>
<td>PN: 2.0</td>
<td>2</td>
</tr>
</tbody>
</table>

† Xuan Son National Park Museum, Thanh Son District, Phu Tho Province (21°12’N, 104°50’W).
‡ Red River Delta in Hai Hau District, Nam Dinh Province (20°13’N, 106°18’W); Dong Hung District, Thai Binh Province (20°30’N, 106°22’W); Kim Bang District, Ha Nam Province (20°34’N, 105°53’W); Duy Tien District, Ha Nam Province (20°37’N, 105°55’W); Kien Thuy District, Hai Phong Province (20°45’N, 106°40’W); Chi Linh District, Hai Duong Province (21°7’N, 106°23’W).
§ Vinh Phuc Province (21°13’N, 105°31’W).
¶ Babe Park: Babe District, Bac Kan Province (105°37’N, 22°25’W).
# PN: Pseudoacanthocephalus nguyenthileae n. sp.; PH: Porrorchis houdemeri; SMM: Sphaerechinorhynchus maximesospinus n. sp.

hooks; average of 81 hooks on proboscis. Proboscis hook length from anterior: 42–83 (66), 62–94 (80), 62–94 (83), 62–94 (86), 62–104 (91). Neck 52–117 (102) long, 239–354 (309) wide. Proboscis receptacle 499–884 (696) long, 208–343 (255) width. Lemnisci 624–1,250 (932) long, 150–312 (235) wide. Whole reproductive system post equatorial (Figs. 1, 2). Testes equal, oblong to avoid, contiguous but occasionally overlapping. Anterior testis 489–1,150 (758) long, 300–750 (467) wide; posterior testis 530–1,000 (744) long, 343–700 (461) wide. Cement glands 8, multiloculated, in 2 overlapping anterior and posterior tiers, 364–1,175 (947) long, 100–200 (146) wide, with 2 posterior sets of long cement ducts surrounding and joining at the base of Saefftigen’s pouch, 624–936 (785) long, 208–374 (287) wide (Fig. 5). Bursa with many uninucleated sensory pits similar to those of the neck, 350–825 (585) long, 416–875 (633) wide (Fig. 9). Gonopore terminal.

Female (based on 27 gravid specimens): Trunk 8.00–28.00 (16.60) mm long, 0.95–2.40 (1.49) mm wide. Neck 83–208 (123) long, 302–426 (352) wide. Proboscis 354–700 (526) long, 302–425 (363) wide; with 15–19 (17.4) hook rows, each with 5 or 6 hooks; average of 94 hooks on proboscis. Prostate hooks relatively larger in 6-hook rows than in 5-hook rows; length from anterior of 5-hook rows: 62–88 (74), 83–99 (87), 88–104 (91), 88–104 (95), 88–104 (96), and of 6-hook rows: 67–94 (80), 73–114 (93), 73–114 (96), 73–114 (97), 84–118 (97). Proboscis receptacle 749–1,300 (991) long, 300–475 (355) wide. Lemnisci 624–1,825 (1,386) long, 104–400 (275) wide. Reproductive system (Figs. 7, 8) 1.22–1.65 (1.38) mm long, 6.1–10.3 (8.3)% of trunk length. Mature eggs fusiform elongate without polar prolongation of middle membrane (Fig. 3), outer shell 70–80 (75) long, 20–26 (24) wide. Gonopore subterminal.

Figs. 1–10. Pseudoacanthocephalus nguyenthileae n. sp. (1) Robust paratype male from Rana guentheri; note lemnisci longer than proboscis receptacle. (2) Typical smaller male from Paa verrucospinosa, with smaller lemnisci drawn to same scale as male in Figure 1. (3) Egg teased out of the body cavity of a paratype female. (4) A paratype female from R. guentheri. (5) Detail of the reproductive system of the male in Figure 2. (6) One row of proboscis hooks and roots of a paratype female from P. verrucospinosa. (7, 8) Dorsal and lateral views of female reproductive system of paratype specimens collected from R. guentheri and drawn to same scale. (9) The bursa of a paratype male from Polypedates mutus, showing uninucleated sensory pits (lower left) and penis. (10) Anterior end of specimen in Figure 2 showing the relationship between the proboscis, receptacle, and lemnisci in worms typically found in P. verrucospinosa. Note the vaculated structure of the lemnisci, the packed nucleated cephalic ganglion, and the cell-like appendage at the posterior tip of the receptacle. Scale bars: 1, 2, 4, 5, 2.0 mm; 3, 30 μm; 6, 200 μm; 7, 8, 1.0 mm; 9, 10, 300 μm.
Table II: Measurements of *Pseudoacanthocephalus nguyenthileae* males from the intestines of 3 species of terrestrial amphibian hosts and of Chinese cobra *Naja atra* in Vietnam.

<table>
<thead>
<tr>
<th>Character</th>
<th><em>Rana guentheri</em></th>
<th><em>Paa verrucospinosa</em></th>
<th><em>Rana taipehensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 2)</td>
<td>(n = 1)</td>
</tr>
<tr>
<td>Trunk L × W (mean) (mm)</td>
<td>5.00–8.62 (7.53) × 0.80–1.55 (1.30)</td>
<td>4.12–5.37 (4.75) × 0.67–0.090 (0.79)</td>
<td>7.00 × 1.00</td>
</tr>
<tr>
<td>Proboscis L × W (mean)</td>
<td>447–541 (484) × 250–374 (299)</td>
<td>364–458 (411) × 208–260 (234)</td>
<td>468 × 312</td>
</tr>
<tr>
<td>Hooks/row × rows (mean)</td>
<td>5 × 15–19 (16.4)</td>
<td>5 × 15–16 (15.5)</td>
<td>5 × 16</td>
</tr>
<tr>
<td>Hook L (mean)</td>
<td>#1</td>
<td>57–83 (72)</td>
<td>42–52 (45)</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>73–94 (83)</td>
<td>62–73 (66)</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>83–94 (86)</td>
<td>62–73 (69)</td>
</tr>
<tr>
<td></td>
<td>#4</td>
<td>83–94 (90)</td>
<td>62–78 (71)</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>88–104 (95)</td>
<td>62–83 (76)</td>
</tr>
<tr>
<td>Hook rows measured</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neck L × W (mean)</td>
<td>52–135 (95) × 260–354 (326)</td>
<td>52–177 (114) × 239–260 (250)</td>
<td>135 × 312</td>
</tr>
<tr>
<td>Receptacle L × W (mean)</td>
<td>499–884 (682) × 208–343 (260)</td>
<td>728–800 (764) × 229–275 (252)</td>
<td>675 × 225</td>
</tr>
<tr>
<td>Lemnisci L × W (mean)</td>
<td>624–1,250 (979) × 187–312 (257)</td>
<td>676–700 (688) × 150–177 (163)</td>
<td>1,050 × 200</td>
</tr>
<tr>
<td>Ant. testis L × W (mean)</td>
<td>600–1,150 (828) × 300–750 (491)</td>
<td>489–600 (544) × 333–400 (366)</td>
<td>625 × 475</td>
</tr>
<tr>
<td>Post. testis L × W (mean)</td>
<td>600–1,000 (797) × 375–700 (478)</td>
<td>530–550 (540) × 343–400 (371)</td>
<td>725 × 500</td>
</tr>
<tr>
<td>Cement gl. L × W (mean)</td>
<td>625–1,375 (1,014) × 100–200 (148)</td>
<td>364–750 (557) × 114–150 (132)</td>
<td>1,000 × 150</td>
</tr>
<tr>
<td>Saffitgen's p. L × W (mean)</td>
<td>728–936 (828) × 208–375 (287)</td>
<td>624–728 (676) × 229–333 (281)</td>
<td>785 × 287</td>
</tr>
<tr>
<td>Bursa L × W (mean)</td>
<td>550–825 (675) × 500–875 (710)</td>
<td>350–374 (362) × 416–468 (442)</td>
<td></td>
</tr>
</tbody>
</table>

* One male from *N. atra* measured: Trunk 5.87 × 0.95 mm, proboscis 486 × 260 with 17 rows of 5 hooks each, hook length from anterior 57–68 (62), 62–73 (66), 63–75 (70), 68–75 (72), 73–83 (80) (n = 3), proboscis receptacle 832 × 229, lenmisci 468 × 239, anterior testis 551 × 468, posterior testis 593 × 285, cement glands 572 × 114, Saffitgen's pouch 624 × 229.

**Taxonomic summary**

*Type host:* Gunther's Amoy frog, *R. guentheri* Boulenger (Ranidae).

*Other hosts:* Four amphibians: common Sunda toad, *B. melanostictus* Schneider (Bufonidae); *Paa verrucospinosa* (Bourret), Teipei frog, *R. taipehensis* Denburgh (Ranidae); Burmese whistling frog, *P. mutus* (Smith) (Racophoridae), as well as 2 reptiles: Chinese cobra *N. atra* Cantor (Elapidae) and house gecko *H. frenatus* Dumeril and Bibron (Gekkonidae).

*Type locality:* Tam Dao National Park, Vinh Phuc Province (21°27'N, 105°40'W).

*Other localities:* Xuan Son National Park, Babe Park, Red River Delta, Vinh Phuc Province, and Hanoi Zoological Park; see footnotes (Table I) for more details.

*Type material:* USNPC no. 98345 (holotype male); no. 98346 (allo-type female); nos. 98347–98353 (paratypes).

*Etymology:* The new species is named for Professor Nguyen Thi Le in recognition of her notable contributions to Vietnamese parasitology.

**Remarks**

The new species is distinguished from the other 10 known species of the genus by its proboscis hooks that progressively increase in size and length posteriorly. It and *P. rauschi* Gupta and Fatma, 1985, have 8 instead of 6 cement glands. Some of the other features of the new species, which are not reported in other species, include the multipletuned lemmisci and the many sensory pits on the neck and bursa. These differences, among others, are included in the following key to the other species of *Pseudoacanthocephalus*. Petrochenko, 1956. It should be noted that *P. rauschi* is provisionally retained in *Pseudoacanthocephalus* until type material become available for examination, to verify the position of its cephalic ganglion (Gupta and Fatma, 1985). It should also be noted that *P. bufonis* (Shipley, 1903) is also retained in *Pseudoacanthocephalus* despite its redescription as *Acanthocephalus bufonis* (Shipley, 1903) from *B. melanostictus* Schneider in Indonesia by Kennedy (1982) who believed it to be compatible with Shipley's (1903) *Echinorhynchus bufonis*. Kennedy (1982) made no reference to *Pseudoacanthocephalus* and offered no justification for or discussion of generic assignments. See discussion of the taxonomic status of *Pseudoacanthocephalus* below.

This was the only species of acanthocephalan collected from all 5 species of terrestrial amphibians and 2 species of reptiles from the various localities surveyed (Table I). It was more common in its amphibian hosts from Xuan Son than from the Tau Dao National Park and highest in intensity in *B. melanostictus* Schneider from the Xuan Son National Park (Table I). All specimens collected included sexually mature adult males with sperm or females with eggs. There was a full range of morphological variability of the new species from all 5 amphibian host species that was represented in 3 hosts, *R. guentheri* Boulenger, *Paa verrucospinosa* (Bourret), and *R. taipehensis* Denburgh. Comparisons between the morphometric characteristics of males and females collected from each of these 3 host species are presented in Tables II and III. Measurements of specimens from the other 2 amphibian species were closest to those from *R. taipehensis* and *P. verrucospinosa*. Measurements of the 1 male from the Chinese cobra, *N. atra* Cantor, are included in Table II. Two immature adults (a male and a female) were also collected from another reptilian, the house gecko *H. frenatus* Dumeril and Bibron.

Qualitative description of the new species is based on all specimens studied from all 5 amphibian host species (Table I) to cover the full range of intraspecific variability. Measurements in the above description are, however, those of 11 males and 27 females from *R. guentheri*, *P. verrucospinosa*, and *R. taipehensis*.

**Key to valid species of *Pseudoacanthocephalus***

1. Proboscis spheroidal or cylindrical with 20–24 hook rows... 2
   - Proboscis cylindrical with 8–20 hook rows .................. 3
2. Proboscis doliform with 20–22 rows each with 10–12 hooks.
   In Lucknow, India .............. *P. rauschi* Gupta and Fatma, 1986
   - Proboscis cylindrical with 22–24 rows each with 6–7 hooks. In
     North Osetiya (Caucasus) .................... 3
     3. Proboscis with 16–20 hook rows .......................... 4
    - Proboscis with 8–16 hook rows .................... 5
4. Proboscis with 16–20 hook rows, each with 6–8 hooks; hooks
   longest anteriorly. Male with 6 cement glands. In Uzbekistan,
   Turkmenistan, Thailand, and Hong Kong ..........................
      - *P. bufonis* (Shipley, 1903) Petrochenko, 1958
      - *P. nguyenthileae* n. sp.
5. Proboscis with 8–12 hook rows. In Thailand ...... P. xenopeltidis (Shipley, 1903) Golvan, 1969
- Proboscis with 12–16 hook rows ............... 6
6. Proboscis with 12–14 hook rows, each with 4–5 hooks ...... 7
- Proboscis with 16 hook rows, each with 6–15 hooks .... 9
7. Proboscis with 14 hook rows, each with 3 or 4 hooks not equal in length; roots simple or with anterior lateral process .... 8
- Proboscis with 12–14 hook rows, each with 4–5 hooks. Hooks equal in length, 70–90 long; roots simple. In Australia ........ P. perthenis Edmonds, 1971
8. Hooks from anterior 50, 80, 100, 80 long; roots with anterior lateral process. In Madagascar .................. .
- Three hooks in males from anterior 22–53 (42), 56–66 (60), 53–66 (59) long; 3–4 hooks in females 40–63 (49), 69–79 (75), 50–82 (69), 60–75 (78) long; roots simple. In Tanzania P. rhampoleontos Smales, 2005
- Proboscis not long, with rows of 6–7 hooks each .... 10
10. Posterior 2 hooks smallest and rootless; other proboscis hooks about equal, with fourth and fifth hooks longest, reaching 107. In Central Asia, Tajikistan, and Eastern Europe ........ P. bufonicola (Kostylew, 1941) Petrochenko, 1958
- First, fourth, and seventh hooks 50, 90, and 70 long; all hooks with simple roots. In Madagascar ........ P. betisileol Golvan, Houin and Brygoo, 1969

The intraspecific variability within P. nguyenthileae is not limited to morphometric differences between the sexes (above), but appears to also be related to host species. The full range of variability in worms infecting all 5 amphibian hosts is expressed in R. guentheri, P. verrucinos, and R. taipehensis. The size of the trunk, proboscis, hooks, and reproductive structures, as well as the number of proboscis hook rows, were largest in males from R. guentheri than in males from the other 2 host species (Table II). In females, the size of the trunk, proboscis hooks, and lemnisci were also greatest in individuals from R. guentheri (Table III). These variations are not considered to be of sufficient magnitude to suggest different specific status. The small, yet sexually mature, male collected from the intestine of a Chinese cobra, N. atra (Table II) probably represents an occasional infection in a post-cyclic host that normally feeds on toads, frogs, rodents, and other snakes.

**DISCUSSION**

Petrochenko (1956) erected Pseudoacanthocephalus to accommodate P. bufonis (type species), P. bufonicola, P. caucasicus, and P. elongates based on having holoechinate acanthors normally associated with the terrestrial ecology of their amphibian hosts, and their cement glands lying in a compact cluster. He placed his new genus in Pseudoacanthocephalidae Petrochenko, 1956, which he assigned to Gigantorhynchidea Southwell and MacFie, 1925. Yamaguti (1963), Golvan (1969, 1994) and Amin (1985) accepted Petrochenko’s new genus as valid, but assigned it to Echinorhynchidae Cobbold, 1879, as emended by Golvan (1969) and Amin (1985). It should be noted that the holoechinate acanthor is not exclusive to acanthocephalans of terrestrial hosts (Golvan, 1969) and that intraspecific variability in cement gland pattern does occur in Pseudoacanthocephalus. For example, Petrochenko (1958), Southwell and MacFie (1925), and Kennedy (1982) observed cement glands of P. bufonis in a compact cluster or in a more elongate or elongate-filiform pattern, respectively. However, the clustered cement glands and terrestrial host’s ecology appear to be the rule and thus are considered primary characters distinguishing Pseudoacanthocephalus from Acanthocephalus. We continue to accept the validity of Pseudoacanthocephalus in Echinorhynchidea.
The cement gland pattern remains of crucial significance for generic and higher taxa diagnoses in the Acanthocephala (Amin and Redlin, 1980; see also Lühe (1911), Meyer (1938), and Van Cleave (1949). However, its extreme variability was noted by Amin and Redlin (1980) and Southwell and Macfie (1925), Thapar (1927), and Baylis (1944) who considered them to be "variable," "unreliable," and "artificial." Petrochenko's (1956) description of Pseudoacanthocephalus and Pseudoacanthocephalidae did not specify the number of cement glands. That was, however, later indicated as 6 by subsequent observers, e.g., Yamaguti (1963) and Golvan (1969). Eight cement glands were observed in P. nguyenthiiae and P. rausi. The number of cement glands in P. bufonicola was reported to be 6, but remains questionable because of their presence in a very tight compact cluster, unknown in P. elongatus (described from 1 female) and in P. xenopeltidis (no internal structures were described). Intra- and interspecific variability in cement gland numbers and patterns in a number of genera, including the closely related Acanthocephalus Koelreuter, 1771 and Echinorhynchus Müller, 1784 (with 6 cement glands) as well as other genera, e.g., Moniliformis Travassos, 1915 with 6 or 8 cement glands (Meyer, 1933) and Plagiorhynchus (Lühe, 1911) with 3–6 cement glands (Schmidt and Kuntz, 1966) have been reported. Four cement glands were described in A. lutzi (Hamann, 1891) Meyer, 1932 (nee Von Linstow, 1896), 1 compact cement gland in each of E. gomesi Machado Filho, 1948, E. paranensis Machado Filho, 1959, and E. salobrensis (Machado Filho, 1948), and 1 compact cluster of 6 or 8 cement glands in E. debenhami Leiper and Atkinson, 1914. Thirty-seven percent of 211 males and 10% of 1,801 males of A. dirus (Van Cleave, 1931) from New England and from Wisconsin had other than 6 cement glands (4–5, 7–11, and 0–5, 7–12, respectively) (Amin, 1975). Echinorhynchus was split by Petrochenko (1956) into 3 genera based on the cement gland pattern that were later reduced to subgenera by Golvan (1960), and then subsequently eliminated by Amin and Redlin (1980). These taxonomic questions regarding Pseudoacanthocephalus, with a special reference to cement gland patterns (see Figs. 1, 2 for P. nguyenthiiae), need to be resolved. Smales (2005) has already pointed to the inconsistencies in the interpretation of the characters used in assigning species to this genus. Smales' uncertainties were conceptually repeated regarding South American species assigned to Acanthocephalus (Smales, 2007). Such questions cannot, however, be resolved until a comprehensive revision of Acanthocephalus is made, which is beyond the scope of the present work.

The prevalence and mean intensity of P. nguyenthiiae varied in different host species collected from the Tam Dao and the Xuan So National Parks. These infection parameters were relatively higher in the latter than in the former localities (Table 1). The differences could be attributed to the distribution of the infected intermediate host(s) in the diet of the respective host species. Habitat preference within each of the 2 parks appears to play a relevant role, e.g., R. guentheri is most often found in swamps, paddy farms, and ponds of low altitude and P. verucospinosa is most often found in, and around, streams in hill and lower montane (evergreen) forests. When in their optimum habitats, host's population density increases proportional to their parasitic load. Dense populations of the green toad Bufo viridis Pallas was shown to harbor 11–14 helminth species, including P. bufonis and P. bufonicola, compared with only 5–6 helminth species when the toad was present in low population densities in Uzbekistan (Vashketov and Siddikov, 1999). The single infection in the intestine of the Chinese cobra, N. atra, is attributed to its diet, which includes mostly frogs and toads, thus making it a post-cyclic host of an acanthocephalan predominantly infecting terrestrial amphibians.

**DESCRIPTION**

*Sphaerechinorhynchus maximesospinus* n. sp.

(Figs. 11–18)

**General:** Plagiorynchidae: Sphaerechinorhynchinae, with characters of the genus. Shared characters larger in females than in males. Trunk long, cylindrical, slightly wider anteriorly, then tapers toward both ends (Fig. 11–12). Body wall relatively thick and lined with sporadic multineucleated pouches (Fig. 16). Proboscis spherical, wider than long (Fig. 18), with 2 types of hooks in 13–16 rows; 3 anterior hooks large with simple posteriorly directed roots and anterior manubria and 4–6 (usually 5 or 6) small posterior spiniform rootless hooks (spines) with anchoring discoid bases (Fig. 15). Middle hook largest, anterior hook next largest, posterior hook smallest. Neck short. Proboscs and neck enveloped in a double-layered membrane (Figs. 15, 18). Proboscis receptacle with strong double walls and brain at its middle. Lemnisci subequal very long, ribbon-shaped, broader anteriorly, branched, multineucleated, appear vacuolated (Fig. 18).

Male (based on 3 specimens with sperm): Trunk 34.00–35.50 (34.75) mm long, 2.5–2.62 (2.56) mm wide. Proboscis 750–875 (825) long, 825–950 (875) wide. Proboscis hooks from anterior (n = 12 each): 114–156 (131), 156–198 (157), 94–156 (125). Proboscis hook roots from anterior (n = 12 each): 83–146 (118), 125–187 (157), 62–135 (110). Proboscis spines from anterior (n = 7 each): 73–94 (84), 73–88 (77), 62–83 (74), 62–83 (73), 62–78 (66), and 62–73 (66). Proboscis receptacle 1,900–2,575 (2,242) long, 650–800 (717) wide. Lemnisci 1,162–1,250 (1,206) long, 375–450 (400) wide. Testes oblong, contiguous, pre-equatorial (Fig. 11). Anterior testsis 1,925–2,175 (2,050) long, 650–925 (787) wide; posterior testsis slightly larger 2,100–2,250 (2,175) long, 775–925 (850) wide. Four long tubular cement glands beginning at or slightly anterior to posterior tip of posterior testsis 15,000–16,750 (15,875) long, 175–260 (228) wide. Cement ducts surround and join at posterior end of Safftigen's pouch, 1,800–2,375 (2,183) long, 700–875 (775) wide anteriorly (Fig. 11). Lobulated diverticula of genital sphincter at posterior end of reproductive system just anterior to terminal gonopore (Fig. 17).

Female (based on 4 gravid specimens): Trunk 32.50–46.25 (38.19) mm long, 2.37–3.25 (2.74) mm wide. Proboscis 728–950 (839) long, 832–950 (878) wide. Proboscis hooks from anterior (n = 5 each): 146–177 (154), 198–218 (204), 114–135 (125). Proboscis hook roots from anterior (n = 5 each): 125–156 (142), 166–198 (184), 114–125 (119).

**FIGURES 11–18.** *Sphaerechinorhynchus maximesospinus* n. sp. from *Ophiogagus hannah*: (11) Holotype male. (12) Allotype female. (13) Egg teased from the body cavity of allotype female. (14) Reproductive systems of allotype female. (15) Two adjacent proboscis rows of hooks, roots, and spines of holotype male. Note the membrane surrounding the proboscis. (16) Sliced section of the body wall of allotype female near reproductive system (Fig. 14) and at same magnification showing large nucleated cells lining the body cavity (center) and part of the lacunar canal system (right). (17) Posterior tip of holotype male (Fig. 11) showing the diverticula of the genital sphincter. (18) Anterior end of the holotype male (Fig. 11) enlarged. Note the membrane surrounding the proboscis and neck. Scale bars: 11, 12, 10.0 mm; 13, 50 μm; 14, 16, 18, 1.0 mm; 15, 17, 400 μm.
Proboscis spines from anterior (n = 6 each): 62–94 (81), 57–94 (77), 57–88 (76), 57–83 (72), 62–73 (65). Proboscis receptacle 2,250–2,800 (2,475) long, 750–775 (767) wide. Lemnisci 14,375–17,000 (16,083) long, 375–475 (425) wide. Reproductive system (Fig. 14) 2.50–3.00 (2.80) mm long, 5.4–9.1 (7.5%) of trunk length; uterus relatively long. Mature eggs elliptoid without polar prolongation of fertilization membrane 73–88 (82) long, 26–36 (31) wide (Fig. 13). Posterior end broadly flattened with terminal gonopore at its center.

**Taxonomic summary**

**Type host:** King cobra, *O. hannah* (Cantor) (Reptilia: Elapidae).

**Type locality:** Vinh Phuc Province (21°13′N, 105°31′W), Vietnam. Both king cobras *O. hannah* (Cantor) examined from the Vinh Phuc Province were infected with total of 7 sexually mature adults of this new species (3 males, 4 females) (Table 1).

**Site of infection:** Intestine.

**Type material:** USNPC no. 98354 (holotype male), no. 98355 (allo-type female), no. 98356 (paratypes).

**Etymology:** The new species is named for its middle proboscis hook being the largest.

**Remarks**

Two major characteristics distinguish *S. maximesospinus* from the other 4 species in the genus. Its proboscis and neck are ensheathed in a thin double-layered membrane through which the hooks partially penetrate. This membrane was observed to be a well-defined structure consistently evident in all specimens and definitely not an artifact of processing or mounting. The proboscis and neck are free in the other species. The middle proboscis hook is the largest in *S. maximesospinus* and the anterior hook is the next largest. The middle hook is also the largest in *S. ophiograndis* Bolette, 1997 from the same host, *O. hannah*, but its posterior hook is the next largest (see Amin et al. [1998] for additional differences). The following key distinguishes the new species from the others, using the most significant characters.

**Key to species of the genus Sphaerechinorhynchus**

1. Proboscis with 12–17 hook rows each with 2 anterior rooted hooks and 5–6 posterior rootless spines. Posterior female trunk simple. In *Naja naja* and *O. hannah* from Borneo (Malaysia) and Thailand . . . . . . . . . . *S. serpenticola* Schmidt and Kuntz, 1966
   2. Proboscis with 13–18 hook rows, each with 3 anterior rooted hooks and 3–7 posterior rootless spines. Posterior female trunk simple or bifid. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
directed roots and 6 posterior spine-like hooks with reduced roots with anterior manubria, except the last spine. Our specimens closely match that description but show a wider range of variability. Our corresponding counts are 23–27, 11–12, 5–6 (usually 6), and 5–7 (usually 6), in the same order.

The finding of these cystacanth in kraits represents a new paratenic host record. Coucals are known to feed on a wide range of insects, caterpillars, and small vertebrates, which in this case may have included young krait. To the best of our knowledge, no other paratenic hosts have been reported for that lesser known species of Paracaenetus. To the best of our knowledge, no other paratenic hosts have been reported for that lesser known species of Paracaenetus. Coucals are known to feed on a wide range of insects, caterpillars, and small vertebrates, which in this case may have included young krait. To the best of our knowledge, no other paratenic hosts have been reported for that lesser known species of Paracaenetus. Our specimens closely match those of other species of the genus as per the revised description of Golvan and Brygoo (1965), among others. The designation of this species as “tentative” by Schmidt and Kuntz (1967) is inadmissible.

LITERATURE CITED


———. P. V. LUC, AND P. N. DOANH. 2000. Revision of the Genus Palissinsis (Acanthocephala: Quadrirgidrae) with the erection of three new subgenera, the description of Palissinsis (Brevitirostri­pus) vietnamensis subgen. et sp. n., a key to species of Palissinsis, and the erection of a new quadrirgid genus, Pararaosentis gen. n. Comparative Parasitology 67: 40–50.


SMALES, L. R. 2005. Acanthocephalans from some frogs and toads (An­ura) and chameleons (Squamata) from Tanzania with the description of new species. Journal of Parasitology 91: 1450–1464.


ERRATUM...

It has been brought to my attention that several numbers in Table II (Merkel et al. 93: 495–503) are incorrect. The correct version is printed below. The measurement bar in Figure 2 of the same paper is also in error and should be 80 μm.

Table II. Measurements (μm) of microfilariae from peripheral blood of flightless cormorants (*Phalacrocorax harrisi*; n = 3 birds) and Galápagos penguins (*Spheniscus mendiculus*; n = 3 birds).

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Flightless cormorants (n = 30) (mean and range)</th>
<th>Galápagos penguins (n = 21) (mean and range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>164 (128–184)</td>
<td>164 (136–200)</td>
</tr>
<tr>
<td>Maximum width</td>
<td>5.3 (4.0–6.8)</td>
<td>5.3 (4.8–6.0)</td>
</tr>
<tr>
<td>Cephalic space</td>
<td>6.4 (4.0–8.8)</td>
<td>5.2 (4.0–8.0)</td>
</tr>
<tr>
<td>Nerve ring (%)</td>
<td>25 (18–31)</td>
<td>24 (20–31)</td>
</tr>
<tr>
<td>Excretory pore (%)</td>
<td>38 (32–47)</td>
<td>37.6 (32–46)</td>
</tr>
<tr>
<td>Inner body (%)</td>
<td>64 (54–73)</td>
<td>63 (52–72)</td>
</tr>
<tr>
<td>G 1 cell (%)</td>
<td>74 (63–90)</td>
<td>75 (62–84)</td>
</tr>
<tr>
<td>Tail</td>
<td>18.1 (12.0–22.4)</td>
<td>19.7 (12.8–29.6)</td>
</tr>
</tbody>
</table>

* Proportion of distance from anterior end of microfilariae.
NEW SPECIES OF PARATHELANDROS (NEMATODA: PHARYNGODONIDAE) IN NYCTIMYSES TRACHYDERMIS (ANURA: HYLIIDAE) FROM PAPUA NEW GUINEA

Charles R. Bursey, Stephen R. Goldberg*, and Fred Kraus†
Department of Biology, Pennsylvania State University, Shenango Campus, Sharon, Pennsylvania 16146. e-mail: cxb13@psu.edu

ABSTRACT: Parathelandros allisoni n. sp. from the intestines of the frog Nyctimytes trachydermisis (Hylidae) from Central Province, Papua New Guinea is described and illustrated. It is the 12th species assigned to the genus and the ninth species of Parathelandros to be reported from the Australo-Papuan region. Parathelandros allisoni n. sp. is most similar to Parathelandros australiensis in that the vulva occurs near the esophageal bulb, but differs in that males lack the spherical postcloacal lobe characteristic of P. australiensis. The description of P. andersoni is amended to include operculate eggs and rosette papillae on the tail of the male.

In an ongoing helminthological survey of frogs of Papua New Guinea, 4 of 18 Morobe big-eyed tree frogs, Nyctimytes trachydermisis Zweifel, 1983 (Hylidae), were found to harbor 58 (2, 2, 18, 36) nematodes of an undescribed species of Parathelandros and 4 of 4 southern Owen Stanley alpine frogs, Oxydactyla crassa (Zweifel, 1956) (Microhylidae), were found to harbor 19 (3, 4, 5, 7) nematodes fitting the description of Parathelandros allisoni Moravec, 1990. Nyctimytes trachydermisis ranges throughout the Owen Stanley Mountains of Papua New Guinea, from Wau in the north to Mt. Simpson in the south (Zweifel, 1983; Kraus and Allison, 2004). Oxydactyla crassa is known only from Mt. Dayman and Mt. Simpson in the southern Owen Stanley range (2,000–2,900 m elevation) of northern Milne Bay Province, Papua New Guinea (Zweifel, 2000; Kraus and Allison, 2004). To our knowledge, there are no reports of helminths from N. trachydermisis or O. crassa. The purpose of this paper is to describe a new species of Parathelandros and to amend the description of P. andersoni.

MATERIALS AND METHODS

Eighteen N. trachydermisis (4 females, 14 males, mean snout–vent length [SVL] = 67.1 ± 2.3 mm, range 64–71 mm, Mt. Simpson, Central Province, 10.03894°S, 149.57650°E, 2,480 m elevation) and 4 O. crassa (4 males, SVL = 27.8 ± 3.6 mm, range 22–31 mm, Mt. Simpson, Milne Bay Province, 10.03642°S, 149.57488°E, 2,480 m elevation) were collected by hand in Papua New Guinea, 18–19 February 2003 by F.K. and local villagers, and fixed in 10% formalin. The body cavity was opened by a longitudinal incision and the gastrointestinal tract was removed by cutting across the esophagus and rectum. The esophagus, stomach, small intestine, and large intestine of each frog were examined separately for endoparasites. Only nematodes were found, which were placed in lactophenol, allowed to clear, and examined by light microscopy. Drawings were made with the aid of a microprojector. Measurements are in micrometers with mean ± 1 SD and range in parentheses unless otherwise stated. Frogs were deposited in the Bernice P Bishop Museum (BPBM), Honolulu, Hawaii. N. crassa as BPBM 18181–18198, O. crassa as BPBM 17064–17067. Helminths were deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland and the Bishop Museum.

DESCRIPTION

Parathelandros allisoni n. sp. (Figs. 1–9)

General: Oxyuroidea Railliet, 1916, Pharyngodonidae Travassos, 1919, Parathelandros Baylis, 1930. Small, cylindrical nematodes with transversely striated cuticle. Sexual dimorphism evident, males shorter and thinner than females. Lateral alae present extending from level of nerve ring to tail filament in both sexes. Oral opening triangular, each lip with thin, distal cuticular flange; dorsal lip with 2 papillae, each sublateral lip with 1 papilla and 1 amphid. Esophagus composed of anterior cylindrical corpus, short isthmus, and posterior bulb. Tail in both sexes forming long, flexible terminal process.

Male (on the basis of holotype and 9 paratypes): Length including tail filament 1.84 ± 0.31 mm (1.41–2.30 mm), width at excretory pore 161 ± 34 (128–218). Lateral alae approximately 25 in width. Esophageal corpus 202 ± 13 (183–226) in length, isthmus 17 ± 2 (15–18) in length, bulb 60 ± 12 (49–92) long, 58 ± 7 (46–67) wide. Nerve ring 112 ± 9 (104–128) and excretory pore 304 ± 40 (244–378) from anterior end. Genital cone present supporting 2 pairs of small digitiform papillae, 1 pair precloacal, 1 pair postcloacal. One pair sessile rosette papillae on tail filament, ventral in position and 76 ± 12 (55–92) posterior of postcloacal pair. Spicule 56 ± 2 (52–58), distal half tapering to a point. Genuberculatum not found. Conical tail tapering to form long, flexible, smooth, filiform process, 525 ± 65 (400–610) in length.

Female (on the basis of allotype and 9 paratypes): Length including tail filament 6.02 ± 0.50 mm (5.12–6.39 mm), width a level of vulva 288 ± 26 (255–332), body cylindrical, tapering sharply posterior to anus to form flexible, smooth, filiform process 1,344 ± 74 (1,280–1,470) in length. Lateral alae approximately 15 wide. Esophagus 568 ± 38 (523–638) in total length consisting of corpus 441 ± 33 (383–485) in length, isthmus 21 ± 3 (18–24) in length, bulb 107 ± 7 (92–116) long, 115 ± 5 (107–122) wide. Nerve ring 125 ± 7 (116–134), excretory pore 474 ± 43 (408–536), and vulva 550 ± 58 (459–612) from anterior end. Ratio of distance from anterior to vulva and length of esophagus, 0.97. Vulva slitlike, salient. Thick-walled vagina extending 435 ± 55 (357–536) posteriorly, opening onto egg reservoir 574 ± 91 (459–714), which in turn joins 2 uteri. Uteri extend posteriorly and when filled with eggs reach posterior end of body cavity. In non gravid individuals, ovaries lie midbody. Egg ovoidal, slightly flattened on one side, 130 ± 4 (125–134) by 42 ± 3 (40–46), thin, punctated shell. Indistinct subterminal operculum on flattened surface; eggs not larvated when released.

Taxonomic summary


Type locality: Mt. Simpson, Central Province, Papua New Guinea, 10.03894°S, 149.57650°E, 2,480 m elevation.

Site of infection: Large intestine.

Prevalence: 4/18 (22%) infected.

Intensity: 41/18 (22%) infected.

The new species is named for Allan Allison, Natural Sciences Department, Bishop Museum, Honolulu, Hawaii, in recognition of his contributions on the biology of the herpetofauna of Papua New Guinea.

Remarks

Parathelandros was established by Baylis (1930) for a nematode, Parathelandros mastigurus Baylis, 1930, taken from the intestine of...
Hyla caerulea (currently Litoria caerulea) collected in Australia, which was found to differ from Theladros Wedl, 1862 in the structure of the caudal end of the male and the position of the vulva of the female. Later, Parathelandros anolis Chitwood, 1934, Parathelandros oedurae Johnston and Mawson, 1947, and Parathelandros scelopori Caballero, 1938 were described and assigned to the genus; Read and Amrein (1953) transferred Pharyngodon apapillosum Koo, 1938 and Pharyngodon medinae Calvete, 1948 to the genus; and Skrjabin et al. (1960) referred 4 species, i.e., Pharyngodon bassii Walton, 1940, Pharyngodon nabuiensis Malan, 1939, Pharyngodon manuay Sandground, 1936, and Oxyuris megalocerca Skrjabin, 1916 to the genus. Inglis (1968) undertook a revision of the genus during his study of helminths of Australian frogs and concluded that because members of Parathelandros occur in Australian hosts and exhibit a morphological uniformity, i.e., lateral alae in both sexes, tail terminating in long spike in both sexes, cloacal region of male raised as distinct cone supporting 2 pairs of papillae, cloacal alae absent, pair of rosette papillae on tail of male, eggs with operculum, those species previously transferred to Parathelandros could not remain in the genus. With the exception of P. bassii, he reassigned these species to a new genus, Skrjabinodon Inglis, 1968. Inglis (1968) then described 3 new species, i.e., Parathelandros carinae Inglis, 1968, Parathelandros johnstoni Inglis, 1968, and Parathelandros maini Inglis, 1968, and transferred 3 species, i.e., Cosmocerca australiensis Johnston and Simpson, 1942, Pharyngodon linnodynastes Johnston and Mawson, 1942, and Cosmocerca propinqu 1a Johnston and Simpson, 1942, to the genus. Thus, Inglis (1968) considered the genus to contain 7 species, i.e. the type species P. mastigurus, plus Parathelandros australiensis, P. carinae, P. johnstoni, Parathelandros linnodynastes, P. maini, and Parathelandros propinqu 1a. Since the 1968 revision, 4 species have been described and assigned to the genus, i.e., Parathelandros hemidactyl 1a Wang, Zhao, Wang, and Zhang, 1979, Parathelandros orientalis Wang, 1980, and Parathelandros texanus Specian and Ubelaker, 1974, all parasites of lizards, and P. andersoni Moravec, 1990, a parasite from frogs of New Guinea. We believe some revision may be necessary because the description of P. hemidactyl 1a fails to mention rosette papillae in the male and operculate eggs, the description of P. orientalis places the third pair of caudal papillae of the male in a lateral position, and the description of P. texanus indicates that in the female the excretory pore and vulva are not adjacent, but we will wait until the type specimens can be examined before suggesting reassignment. Our examination of P. andersoni from O. crassa (deposited as USNPC 99782) finds both rosette papillae and operculate eggs present; thus, we amend Moravec’s (1990) description to “large doubled ventral rosette papilla on tail” and “mature eggs elongate, spindle-shaped, operculate, thin-walled with un­cleaved content.” Parathelandros allisoni n. sp. is the 12th species assigned to the genus and the ninth from Australo-Papuan frog hosts.

Inglis (1968) grouped the Australian species of Parathelandros by position of the vulva: (1) anterior to esophageal bulb, P. maini, P. mas­figurus, P. propinqu 1a; (2) slightly posterior to the esophageal bulb, P. australiensis, P. johnstoni, P. linnodynastes; and (3) far posterior to esophageal bulb, P. carinae. The more recently described Papuan species, P. andersoni, belongs to category 3, whereas P. allisoni n. sp. is most similar to the species in category 2. Inglis (1968) plotted distance of the vulva from anterior against length of esophagus for the Australian species and for category 2 species, i.e., P. australiensis, P. johnstoni, P. linnodynastes, the ratios are 1.2, 1.4, and 1.4, respectively. Parathelandros allisoni n. sp. most closely resembles P. australiensis in that in these 2 species the vulva is nearer the posterior end of the esophagus than any of the other species (vulva/esophageal ratio, 1.0 and 1.2, re­spectively). Parathelandros allisoni n. sp. is easily differentiated from P. australiensis in that males of P. australiensis have a spherical post­cloacal lobe, a character not found in P. allisoni n. sp.

ACKNOWLEDGMENTS

Peggy Firth prepared illustrations 1–9. Ezra Teodoru and Sarah Goldsberry assisted with dissections. We thank Fred Malea, Brian Urwa, Dage, Genta Sr., Genta Jr., Munda, Peter, Tanunu, and many other inhabitants of the Bunisi area for field assistance; and Aussiya, Helen Kurage, David Mitchell, Bena Seta, and the people of Bunisi for providing logistical assistance and permission to work in the Mt. Simpson area. We thank the Papua New Guinea National Museum and Art Gallery for providing in-country collaborative assistance and the Department of Environment and Conservation, National Research Institute, and Milne Bay Provincial Government for permission to conduct this research. This is contribution 2007-011 from the Pacific Biological Survey at the Bishop Museum.

LITERATURE CITED


NEW AND ALREADY KNOWN ACANTHOCEPHALANS MOSTLY FROM MAMMALS IN VIETNAM, WITH DESCRIPTIONS OF TWO NEW GENERA AND SPECIES IN ARCHAICANTHOCEPHALA

Omar M. Amin*, Nguyen Van Ha†, and Richard A. Heckmann‡

Institute of Parasitic Diseases, P.O. Box 28372, Tempe, Arizona 85285. e-mail: omaramin@aol.com

ABSTRACT: Adults of 2 new species and 2 new genera of acanthocephalans in class Archiacanthocephala, collected between 1998 and 2004 in Vietnam from the intestines of mammals, are described, i.e., Cucullanorhynchus constrictrucanus n. gen., n. sp. (Oligacanthorhynchidae) from a leopard Panthera pardus (Linnaeus) (Mammalia: Felidae) and Paraproctacanthorhynchus ornatus n. gen. n. sp. (Oligacanthorhynchidae) from the Chinese pangolin Manis pentadactyla (Linnaeus) (Mammalia: Manidae). Adult Sphaerichnorhynchus macropothispous Amin, Wongcawad, Marayong, Saehoong, Suwattanacoup, and Sey, 1998 (Plagiorhynchidae) are described for the first time from 2 females collected from a tiger Panthera tigris (Linnaeus) (Mammalia: Felidae) and from 1 male from a water monitor Varanus salvadori (Reptilia: Varanidae). Characteristic features distinguishing the new species or genera from related taxa are as follows. The trunk of C. constrictrucanus has an anterior hood in both sexes and a posterior constriction in females. The anterior trunk of P. ornatus has many small festoons and proboscis hooks are inserted in elevated papillae separated by beady, near hexagonal, ornate grids.

The material reported in this work was collected as a part of a larger faunistic research program conducted by the Institute of Ecology and Biological Sciences (IEBS), Hanoi, Vietnam, from a wide variety of terrestrial vertebrates since 1998. Free-ranging hosts, as well as those from the Hanoi Zoological Park and Museum, were examined for parasites. Acanthocephalans were made available by N.V.H. who was instrumental in carrying out the IEBS program. The number and uniqueness of the new taxa, especially those from leopard and pangolin, attest to the extraordinary status of these new and unusual acanthocephalan hosts. The present report adds new and interesting knowledge and a better understanding of the parasite fauna of Vietnam, which is just opening up for international collaborative research in parasitology sponsored by the IEBS. Few acanthocephalans have been recently reported from Vietnam (e.g., Amin et al. 2000, 2004). A total of only 28 species of Acanthocephala (17 in Echinorhynchida, 7 in Gyracanthocephala, and 4 in Neoecinorhynchida) have been reported from Vietnamese fishes to date (Arthur and Te, 2006). No species are known from reptiles or mammals.

MATERIALS AND METHODS

Collection data include host name and size, number examined, prevalence, parasite names, mean and range of parasites per examined host, plus collection localities and number of specimens available for study (Table 1). The reptilian hosts were purchased from farmers in the localities noted and subsequently examined for parasites. The mammalian hosts were collected from the Hanoi Zoological Park and Museum by N.V.H. Freshly collected specimens were relaxed for a few hours or overnight in 0.9% NaCl, then transferred to 80% ethanol. Worms were then punctured with a fine needle and subsequently stained in Mayer's acid carmine, destained in 4% HCl in 70% ethanol, dehydrated in ascending concentrations of ethanol (24 hr each), cleared in graduated (increasing) concentrations of terpineol in 100% ethanol to 100% terpineol, then 50% terpineol and 50% Canada Balsam (24 hr each), and finally whole-mounted in Canada balsam. Some thick specimens were sliced before mounting to improve visibility of internal structures.

Measurements are in micrometers unless otherwise stated. The range is followed by the mean (in parentheses). Length measurements are given before the width; the latter refers to maximum width. Trunk length does not include the neck, proboscis, or bursa. Eggs refer only to fully developed mature eggs removed from the body cavity. Specimens were deposited in the U.S. National Parasite Collection (USNPC), Beltsville, Maryland.

RESULTS

The taxa described in this work include 2 new species and 2 new genera of acanthocephalans belonging in 1 class and 1 family, as well as another species already known from immatures in another class and family, collected from 1 species of reptiles and 3 species of mammals between 1998 and 2004. This collection is organized according to the taxonomy scheme of Amin (1985).

DESCRIPTION

Cucullanorhynchus n. gen.

(Figs. 1–9)

Diagnosis: Trunk robust, elongate-spindle-shaped with no grooves or annulations (Figs. 1, 2). Body wall in both sexes are thick with anterior end modified as sinuate fold (Fig. 2) forming anterior hood when extended (Figs. 5, 9) with a rimmed ventro-terminal opening enclosing the proboscis. Posterior part of female trunk also sinuate forming prominent constriction (Figs. 2, 7). Proboscis is subcylindrical, with 12 almost straight, regularly alternating rows each with 3 simple-rooted hooks directed posteriorly (Fig. 6). Proboscis receptacle is single-walled enclosed within outer nonmuscular protrusors originating at base of neck and inserting at various points on receptacle; brain at middle of its' internal ventral surface (Fig. 9). Lemnisci very long, may reach posterior end of trunk (Fig. 2), ribbon-like, with few, usually anterior, giant nuclei (Figs. 1, 2, 4). Testes equal, oblong, lying obliquely just its' internal ventral surface (Fig. 9). Lemnisci very long, may reach posterior end of trunk (Fig. 2), ribbon-like, with few, usually anterior, giant nuclei (Figs. 1, 2, 4). Testes equal, oblong, lying obliquely just its' internal ventral surface (Fig. 9). Lemnisci very long, may reach posterior end of trunk (Fig. 2), ribbon-like, with few, usually anterior, giant nuclei (Figs. 1, 2, 4). Testes equal, oblong, lying obliquely just its' internal ventral surface (Fig. 9).

Taxonomic summary

Locality and host: Two males and 7 females of a new species were collected from 1 leopard, Panthera pardus (Linnaeus) from Hanoi Zoological Park, Hanoi, Vietnam.

Remarks

Schmidt (1972) and Amin (1985) recognized 8 genera in Oligacanthorhynchidae; Macrancanthorhynchus Travassos, 1917; Neonicola

<table>
<thead>
<tr>
<th>Hosts</th>
<th>Common name</th>
<th>examined</th>
<th>Locality†</th>
<th>Standard length (cm) mean + SD (range)</th>
<th>Prevalence</th>
<th>Intensity* specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reptilia (Varanidae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Varanus salvator</em> Laurenti</td>
<td>Water monitor</td>
<td>5</td>
<td>Vinh Phuc†</td>
<td>50.2 + 15.1 (44.5–56.2)</td>
<td>20%</td>
<td>SMO: 1.0 1</td>
</tr>
<tr>
<td>Mammalia (Felidae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Panthera pardus</em> (Linnaeus)</td>
<td>Leopard</td>
<td>1</td>
<td>Zoological Park‡</td>
<td>—</td>
<td>100%</td>
<td>CC: 9.0 9</td>
</tr>
<tr>
<td><em>Panthera tigris</em> (Linnaeus)</td>
<td>Tiger</td>
<td>1</td>
<td>Zoological Park</td>
<td>—</td>
<td>100%</td>
<td>SMO: 2.0 2</td>
</tr>
<tr>
<td>Mammalia (Manidae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Manis pentadactyla</em> (Linnaeus)</td>
<td>Chinese pangolin</td>
<td>1</td>
<td>Museum‡</td>
<td>—</td>
<td>100%</td>
<td>PO: 1.0 1</td>
</tr>
</tbody>
</table>

* SMO: *S. macropisthospinus*; CC: *Cucullanorhynchus constictruncatus n. gen., n. sp.; PO: *Paraprosthencrus ornatus* n. gen., n. sp.
† Vinh Phuc Province, Vietnam (21°13’N, 105°31’W).
‡ Hanoi Zoological Park (Zoo) and Museum, Institute of Ecology and Biological Resources, VAST, Hanoi, Vietnam (21°2’N, 105°48’W) (GPS Garmin 12).

Figures 1–9. *Cucullanorhynchus constictruncatus n. gen., n. sp. from Panthera pardus.* (1.) Holotype male. Note the protonephridial organ (arrow) just posterior to cement glands. (2.) Allotype female. Note the undulating, retracted hood at the anterior end (arrow). (3.) Reproductive system of a paratype female. Note the corrugated uterine wall, the fibrillar connective tissue extending from near the gonopore to the anterior end of the uterine bell, and the protonephridial structure (arrows). (4.) A segment of a lemniscus showing its structural organization. (5.) A dorsal view of an extended trunk hood obscuring part of the proboscis of a paratype female. (6.) The papillated proboscis of a paratype female showing hooks and roots. (7.) A detail of the subventral position of the gonopore and posterior trunk constriction in a paratype female. (8.) Eggs teased out of the body cavity of a female. (9.) The anterior end of a sliced paratype female showing the walls and other structures of the proboscis receptacle and a sectioned hood. Scale bars = 1, 2, 5.0 mm; 3, 4, 5, 1.0 mm; 5, 6, 7, 500 μm; 8, 50 μm.
DESCRIPTION

Cucullanorhynchus constrictancrus n. sp. (Figs. 1-9)

General: Oligacanthorhynchidae, with characters of the genus. Trunk is somewhat short, robust with anterior hood in both sexes (Figs. 1, 5, 9) and posterior constriction in females (Figs. 2, 7). No sexual dimorphism in size of shared structures except proboscis hooks and lemnisci being markedly larger in females than in males. Proboscis is subphreral papillated, with an internal ribbed pattern, almost obscured by dorsal extension of hood, with 12 rows of hooks each with 3 massive rooted hooks lacking bars or manubria (Fig. 6). Proboscis receptor is enclosed within nonmuscular protrusors. Lemnisci subequal, very long, ribbonlike, more slender, and not attached to body wall distally, occasionally reaching posterior end of trunk, with 7 angular, ovoid, or oblong giant nuclei within central canal in anterior half of lemmiscus (Fig. 4).

Male (based on 2 specimens with sperm): Trunk 10.32–11.05 (10.69) mm long, 2.50–2.55 (2.52) mm wide near middle. Proboscis 437–468 (452) long, 520–551 (535) wide. Proboscis hooks from anterior (n = 6 each) 88–104 (98), 82–95 (91), and 62–77 (70). Protrusors surrounding proboscis receptor 1,450–1,550 (1,500) long, 520–572 (546) wide. Lemnisci 5,000 long, 275–300 (287) wide anteriorly. Testes preequatorial; anterior testis 1,175–1,325 (1,250) long, 500–550 (525) wide; posterior testis 1,100–1,300 (1,200) long, 500–525 (512) wide. Cement glands 775–1,350 (1,050) long, 650–725 (694) wide; anterior glands largest. Protopharyndial structure capsule-type, fanlike, just behind posterior cement gland. Two major cement ducts surround Safftigen's pouch dorsoventrally and join it posteriorly. Safftigen's pouch 2,050–2,125 (2,087) long, 700–775 (737) wide. Gonopore terminal (Fig. 1).

Female (based on 7 gravid specimens): Trunk 9.17–13.12 (11.20) mm long, 2.15–3.00 (2.50) mm wide near middle. Proboscis 406–562 (462) long, 541–624 (586) wide. Proboscis hooks from anterior (n = 11 each): 125–140 (134), 83–99 (92), and 73–104 (82). Protrusors surrounding proboscis receptor 1,375–1,750 (1,621) long, 468–650 (553) wide. Lemnisci 6,375–10,750 (8,375) long, 175–400 (295) wide. Reproductive system (Fig. 3) 1.87–2.37 (2.13) mm long; 16.7–20.4 (19.0%) of trunk length. Uterus, with corrugated outer wall, attached anteriorly to subventral side of body wall with fibrillar connective tissue strands. Protopharyndial structure capsule-type, comblike, surrounding uterine bell (Fig. 3). Mature eggs removed from body cavity ovoid, 73–83 (76) long, 52–57 (54) wide with sculptured outer membrane (Fig. 8). Gonopore subterminal behind posterior constriction (Fig. 7).

Taxonomic summary

Type host: Leopard, Panthera pardus (Linnaeus).
Type locality: Zoological Park (12°2′N, 105°48′), Hanoi, Vietnam.
Type material: USNPC 983357 (holotype male), USNPC 983558 (allotype female), USNPC 983559 (paratypes).
Etymology: The genus is named for the anterior hood and the species for the constriction near the posterior end of females.

Remarks

The anterior trunk hood is considered the unique feature of C. constrictancrus. Its ability to contract and extend may produce a suction cuplike structure representing a possible adaptation for more effective attachment to the host intestinal lining.

DESCRIPTION

Paraprosthernorchis n. gen. (Figs. 10–13)

Diagnosis: Female has very long trunk, broad posteriorly, gradually narrowing anteriorly. Anterior trunk end has many festoons. Proboscis is subglobular and ornate, with hooks arising at anterior of paillae present in nearly hexagonal beady grids covering most of surface of proboscis (Figs. 10, 11). Hooks in 16 almost regularly alternating rows of 3 hooks each. Hooks and simple roots massive especially anteriorly. Hooks are in 2 anterior circles with posteriorly directed roots; posterior hooks with large horizontally oblong base (Fig. 12). Proboscis receptor is pierced dorsally with proboscis retractor muscles and is enclosed in a nonmuscular layer of protrusors originating at base of neck and inserting at various points on receptor; brain is on ventral inner surface at middle level of receptor. Lemnisci long, with giant nuclei usually in swellings along their length (Fig. 10). Reproductive system has elaborated capsaus-type Gill-like protonephridia at anterior end of uterine bell. Gonopore terminal (Fig. 13). Specimen with only ovarian balls; no eggs.

Taxonomic summary

Locality and host: One female of a new species belonging in this genus is described from the Chinese pangolin collected from the Hanoi Zoological Park, Hanoi, Vietnam (Table I).

Remarks

This oligacanthorhynchid acanthocephalan, representing the 12th genus of the family, is closest to Prosthenorchis Travassos, 1915, as restricted by Schmidt (1972) and Amin (1985) to 3 species: Prosthenorchis elegans (Diesing, 1851) Travassos 1915 from primates in South America; Prosthenorchis lemuri Machado, 1950, from lemurus in Madagascar; and Prosthenorchis fraterna (Baer, 1959) Schmidt, 1972 from Felidae in Africa. The anterior trunk of these 3 species of Prosthenorchis, as in Paraprosthernorchis n. gen., has a conspicuous, festooned collar. The many other species formerly assigned to Prosthenorchis that lacked such a collar (Meyer, 1933; Golvan, 1962; Petrochenko, 1958; Yamaguti, 1963) have been relegated to other oligacanthorhynchid genera; see Schmidt (1972), Amin (1985), and Golvan (1994). Prosthenorchis sinicus Hu, 1990 (see Golvan, 1994), from dogs in China do not belong in that genus either; its anterior trunk does not possess a festooned collar.

The new genus is distinguished from Prosthenorchis, the only other oligacanthorhynchid genus with a festooned collar, by the features presented in Table II. The most taxonomically important and generically diagnostic differences are the ornate proboscis and its armature, terminal gonopore, worm size, and host and geographical distribution.

DESCRIPTION

Paraprosthernorchis ornatus n. sp. (Figs. 10, 13)

Female (based on 1 female in ovarian ball stage): Trunk is 236.25 mm long, 3.12 mm wide near posterior end. Proboscis is 437 long, 520 wide with 16 near regularly alternating rows
FIGURES 10–13. Holotype female *Paraprosthenuorchis ornatus* n. gen., n. sp. from *Manis pentadactyla*. (10.) The proboscis, receptacle, and lemnisci (posterior portions not shown). (11.) An enlargement of the proboscis showing hooks and roots as well as the festooned trunk collar. Note the beadlike, ornate pattern of the proboscis. (12.) One row of proboscis hooks and roots. (13.) Female reproductive system. Note the fibrillar wall of the uterine bell and the protonephridial structure (arrow). Scale bars: 10, 2.0 mm; 11, 500 μm; 12, 100 μm; 13, 2.0 mm.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Paraprosthenuorchis</em></th>
<th><em>Prosthenuorchis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Trunk</td>
<td>Over 200 mm long</td>
<td>Up to 50 mm long</td>
</tr>
<tr>
<td>Proboscis</td>
<td>Ornate</td>
<td>Not ornate</td>
</tr>
<tr>
<td>Proboscis hooks</td>
<td>3 in each of 16 rows</td>
<td>3 in each of 12 rows</td>
</tr>
<tr>
<td>Hook root</td>
<td>Hook tips not barbed</td>
<td>Hook tips with barbs</td>
</tr>
<tr>
<td>Posterior hook base</td>
<td>Simple, no manubria</td>
<td>Manubria large, complex</td>
</tr>
<tr>
<td>Festoons</td>
<td>Large, oblong horizontally</td>
<td>Small, discoid</td>
</tr>
<tr>
<td>Protonephridia</td>
<td>Gill-like, capsular</td>
<td>Up to 23</td>
</tr>
<tr>
<td>Gonopore</td>
<td>Terminal</td>
<td>Unknown in females</td>
</tr>
<tr>
<td>Host and distribution</td>
<td>Manidae in Vietnam</td>
<td>Subterminal</td>
</tr>
<tr>
<td>Intermediate hosts</td>
<td>Ants and termites</td>
<td>Primates in South America</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Felidae in Africa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cockroaches and beetles</td>
</tr>
</tbody>
</table>
of 3 hooks each. Anterior 2 hooks are massive; length from anterior 93–108 (101) long and 43–50 (47) wide at base, and 80–105 (90) long and 23–40 (30) wide at base, both with simple roots directed posteriorly. Posterior hook is 70–85 (76) long and 15–23 (20) wide at base, with large horizontally oblong root. Outer nonmuscular protrusors of proboscs receptacle is 1,300 long, 575 wide. Lemnisci long subequal with giant nuclei usually in swellings, 8,700 long, 200 wide, and 9,500 long, 475 wide. Reproductive system is 4.25 mm long (only 1.8% of trunk length), with gill-like capsular protonephridia at anterior end of fibrillar-walled uterine bell. Gonopore terminal at pointed posterior end.

Taxonomic summary

**Type host:** Chinese pangolin, *Manis pentadactyla* Linnaeus.

**Type locality:** Hanoi Zoological Park (21°2'N, 105°48'W), Hanoi, Vietnam.

**Type material:** USNPC 98360 (holotype female).

**Etymology:** The new genus is named for its nearest oligacanthorhynchid genus, *Prosthenorchis*, and the species is named for its uniquely ornate proboscis.

Remarks

The female of the new species is distinguished from those of the 3 other species of *Prosthenorchis* by the ornate proboscis with different armature, very long trunk, position of the gonopore, and host species, among other characters featured in Table II.

The only other oligacanthorhynchid reported from pangolins are *Nephridiorhynchus palawanensis* Tubangui and Masilungan 1938 collected from *Manis javanica* Desmarest in Palawan Island, the Philippines, and *Oligacanthorhynchus gerberi* (Baer, 1959) Schmidt, 1972 and *Oligacanthorhynchus manisensis* (Meyer, 1931) Schmidt, 1972, from *Manis tricuspus* Raffinesque in Africa. These forms have nonornate probosces with 12 rows of 3 or 4 hooks each; the female protonephridia occur as a compact closed capsule and lack a festooned collar.

Like *M. javanica* and *M. tricuspus, M. pentadactyla* also feeds exclusively on ants and termites (Nowak, 1999), the most likely intermediate hosts for their acanthocephalan infections. The only species of *Prosthenorchis* for which the intermediate hosts are known is *P. elegans*. The intermediate hosts of that species are cockroaches and beetles (see Schmidt, 1985).

**EMENDED DESCRIPTION**

*Sphaerechinorhynchus macropisthospinus*

Amin, Wongswad, Marayong, Saeoong, Suwattanacoup, and Sey, 1998

(Figs. 14–18)

**General:** Plagiorhynchidae, with characters of genus. Shared characters are markedly larger in females than in males and in sexually mature adults than in immature subadults. Trunk is moderate in size, cylindrical, with males slender and wider anteriorly, and females more robust with parallel sides (Figs. 14, 15). Two major and many secondary lacunar canals are present. Body wall is thick with many dome-shaped nucleated cell clusters randomly distributed along its interface with body cavity (Fig. 17). Proboscis globular, slightly wider than long. Proboscis hooks in 14 or 15 alternating and widely spaced rows of 3 anterior robust hooks with simple posteriorly directed roots, and with usually 6 posterior spines (5 in a male, 7 in a female) with small discoid bases but no roots. Hooks progressively increase in size posteriorly and spines increase in size anteriorly (figs. 1, 5–8 in Amin et al., 1998). Proboscis receptacle is oblong in females but more inflated with pointed posterior extremely in males, with thick double walls and brain (cephalic ganglion) at its middle. Lemnisci subequal, long, ribbonlike, wider anteriorly.

**Male (based on 1 adult):** Trunk is 13.67 mm long, 1.42 mm wide anteriorly. Proboscis is 624 long, 634 wide with 15 rows each with 3 anterior rootless hooks and 5–6 posterior rootless spines. Hook length from anterior 72–85 (78), 113–120 (116), and 160–165 (162). Spine length from anterior 75–92 (82), 62–75 (67), 65–75 (69), 62–70 (66), 65–67 (66), 60–67 (62). Proboscis receptacle is 1,425 long, 750 wide. Lemnisci long and ribbon shaped, posterior end broken. Testes small, oblong-sli­nder, separate, equal, slightly preequatorial (Fig. 14). Anterior testis is 602 long, 177 wide; posterior testis is 603 long, 156 wide. Cement glands 4, long, tubular, beginning at posterior testes with 4 large single-nucleated cells at their junction with cement ducts posteriorly. Safftigen's pouch is 728 long, 250 wide. Diverticula of genital sphincter pronounced. Gonopore terminal (Fig. 14).

**Female (based on 2 gravid females):** Measurements are in Amin et al. (1998) table 5, figures 1 (proboscis), 4 (anterior end), and 5–8 (hooks and spines). Trunk is robust with thick walls and almost parallel sides. Eggs are oval with no polar prolongation of fertilization membrane (Fig. 16). Reproductive system is well developed, uterus long, with terminal gonopore (Fig. 18).

**Taxonomic summary**


**Location:** Intestine.

**Paratelic hosts:** House gecko, *Hemidactylus frenatus* Dumeril and Bibron (Reptilia: Gekkonidae) (type), *Kaloula pulchra* Gray and *Microhyla* sp. Tschudi (Amphibia: Microhylidae), and *Ophiocephalus striatus* Bloch (Pisces: Ophiocephalidae); see Amin et al. (1998).

**Locality (adults):** Hanoi Zoological Park (21°2'N, 105°48'W) and Vinh Phuc Province (21°13'N, 105°31'W), Vietnam.

**Other localities (immatures):** Doi Suthep–Pui National Park, suburban Chiang Mai, Thailand, and unidentified fish stream, Bangkok, Thailand; see Amin et al. (1998).

**Type specimens:** Adults: USNPC 98361 (plesiotype male from *V. salvator*), USNPC 98362 (plesiotype female from *P. tigris*). Immatures: USNPC 87621 (holotype female from *H. frenatus*), USNPC 87622 (paratype female from *Microhyla* sp.).

**Remarks**

This plagiorhynchid acanthocephalan was first described from subadult, immature females collected from the intestinal mesenteries of lizards, frogs, and fish in Thailand (Amin et al., 1998). Sexually mature adults are herein described for the first time from 2 gravid females collected from the intestine of a
tiger *Panthera tigris* (Linnaeus) from the Hanoi Zoological Park, Vietnam, and from 1 male from the intestine of a water monitor, *V. salvator* Laurenti from Vinh Phuc Province, Vietnam (Table I).

The 2 gravid females of *S. macropisthospinus* studied were morphologically similar to the subadults described from paratenic hosts (see figs. 1–8 in Amin et al., 1998), except that measurements of all structures, especially the proboscis and its armature, receptacle, lemnisci, and reproductive system, were markedly larger in the gravid specimens (Table III). In addition, gravid females have a markedly more robust trunk with almost parallel sides and a considerably more developed reproductive system (Figs. 15, 18) compared with immatures. Previous records of *S. macropisthospinus* have been only from body cavity sites of paratenic hosts from 3 classes of vertebrates in Thailand, including murrel fish, *O. striatus*; 2 amphibians, *K. pulchra* and *Microhyla* sp.; and 1 reptile, the house gecko, *H. frenatus*. “The fish, amphibian, and reptilian hosts represent a succession from aquatic to terrestrial habitat probably leading to a yet to be identified snake definitive host” (Amin et al., 1998). Definitive hosts of all other species of the genus are snakes. Three hundred and eight snakes of 3 species examined in a related study were not infected by this acanthocephalan. An aquatic crustacean intermediate host may be involved as is suggested by the recovery of *Sphaerechinorhynchus rotundocapitatus* cystacanths from the semiaquatic water skinks and lizards (Daniels, 1985, 1990).

The recovery of gravid females of *S. macropisthospinus* from a tiger is the first report of any member of *Sphaerechinorhynchus* from a mammal. The recovery of an adult male specimen from the water monitor (*Varanus* Laevi) is also a first and equally most unusual record. It suggests that both definitive host species either fed directly on an infected paratenic host or consumed an infected definitive snake host, yet to be identified, which serve as either definitive or postcyclic hosts. The monitor lizard is known to swallow anything that is remotely edible, including snakes, frogs, lizards, and fish (Gaulke, 1991; Traeholt, 1993, 1994; Vogel, 1979). Tigers also have a diverse diet, including anything that moves, from medium-sized mammals to smaller vertebrates, including frogs, reptiles, and fish (Seidensticker, 1996; Stoen and Wegge, 1996; Sankar and Johnsingh, 2002).

These above records indicate that *S. macropisthospinus* appears to be more wide spread outside of Thailand than previously thought. The reported paratenic hosts are of south and southeastern Asian distribution (Taylor, 1962; Nelson, 1976). *Microhyla* spp. is prolific and comprises small lowland forms (Zug, 1993). *Kaloula pulchra* is the more common of the 2 species in the genus and is widespread in many ponds in Thailand, Malaysia, Sri Lanka, and parts of India (Taylor, 1962). This acanthocephalan was considerably more common in the
TABLE III. Comparison between the measurements of subadult and gravid female Sphaerechinorhynchus macropisthospinus.*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Hosts</th>
<th>Site</th>
<th>Mature (subadult) females</th>
<th>Gravid females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amin et al. (1998)</td>
<td>T. variegatus, T. albinus</td>
<td>Intestine</td>
<td>9.39-28.18 (18.63) x 3.65-664 (775)</td>
<td>70-120 (70) x 76-152 (79)</td>
</tr>
<tr>
<td>Gaulke and P. Sirikanchana (2002)</td>
<td>M. variegatus</td>
<td>Intestine</td>
<td>0-104 (95) x 112-129 (121)</td>
<td>70-81 (75) x 75-120 (75)</td>
</tr>
<tr>
<td>Amin et al. (1998)</td>
<td>T. variegatus, T. albinus</td>
<td>Intestine</td>
<td>7-100 (70) x 3.65-664 (775)</td>
<td>70-120 (70) x 76-152 (79)</td>
</tr>
<tr>
<td>Gaulke and P. Sirikanchana (2002)</td>
<td>M. variegatus</td>
<td>Intestine</td>
<td>0-104 (95) x 112-129 (121)</td>
<td>70-81 (75) x 75-120 (75)</td>
</tr>
<tr>
<td>Gaulke and P. Sirikanchana (2002)</td>
<td>M. variegatus</td>
<td>Intestine</td>
<td>0-104 (95) x 112-129 (121)</td>
<td>70-81 (75) x 75-120 (75)</td>
</tr>
</tbody>
</table>

**Note:** *Subadult females (3.65-664, 775) and gravid females (70-120, 70) are measured in micrometers.*

-- gecko, *H. frenatus,* than in the 2 amphibian species, with the gecko appearing to be the more typical paratenic host in which *S. macropisthospinus* attained a larger size and further development (Amin et al., 1998). It is interesting to note that *H. frenatus* was also examined from Babe Park in Vietnam, but was not found to be infected with *S. macropisthospinus.* It is also interesting that the tiger and leopard examined in this study, who both came from Hanoi Zoological Park, were each infected with a distinct species of Acanthocephala, despite their similar carnivorous feeding behavior. Differential preference for small prey by sympatric populations of leopards and tigers (Sankar and Johnsingh, 2002) was related to activity periods and microhabitat use (Karanth and Sunquist, 2000).

LITERATURE CITED


—. P. V. LUC, AND P. N. DOANH. 2000. Revision of the Genus *Pallisentis* (Acanthocephala: Quadrigyridae) with the erection of three new subgenera, the description of *Pallisentis* (Brevitritospinus) vietnemensis subgen. et sp. n., a key to species of *Pallisentis,* and the erection of a new quadrigyrid genus, Pararaoasentis gen. n. Comparative Parasitology 67: 40–50.


2. Isdatel'stvo Akademii Nauk SSSR, Moscow, Russia. (In Russian: English translation by Israel Program for Scientific Translations Ltd., Jerusalem, Israel. 1971, 478 p.)


SANDONELLA SAN DONI (LYNSDALE, 1960), AN ENIGMATIC AND MORPHOLOGICALLY UNIQUE CESTODE PARASITIC IN THE OSTEOGLOSSIFORM FISH HETEROTIS NILOTICUS IN AFRICA

Alain de Chambrier, Jean Mariaux, Aminata Sène*, Zuheir N. Mahmoud†, and Tomáš Scholz‡

Département des Invertébrés, Muséum d’histoire naturelle, P.O. Box 6434, CH-1211 Genève 6, Switzerland.

E-mail: alain.dechambrier@villge-ge.ch

ABSTRACT: Sandonella sandoni (Lyndale, 1960) is the type and only species of the Sandonellinae, a cestode subfamily of unclear phylogenetic position. It is redescribed here on the basis of a re-examination of its syntypes, voucher specimens from museum collections, and freshly collected material from the intestine of Heterotis niloticus (Osteoglossiformes: Arapaimidae) from Benin, Nigeria, Senegal, and the Sudan. The species possesses several unique morphological characters, such as (1) a vitellarium formed by 2 compact, but deeply lobulated, postovarian masses near the posterior margin of proglottids; (2) a scolex with a highly modified apical structure formed by 4 muscular retractile lappets; (3) a well-developed circular musculature, which is external to the inner longitudinal muscles; (4) a dilated, vesicle-like proximal part of the external sperm duct; (5) the unique morphology of the uterus and its development, which represents an intermediate form between the 2 basic types recognized in the Proteocephalidea; (6) the growth of eggs during their development within the uterus; and (7) the complex proglottization with intermingled smaller and larger (wider) proglottids. The morphology of S. sandoni, including the form and distribution of microtriches, was studied by scanning electron microscopy for the first time, and the lectotype and paralectotypes of S. sandoni are designated. Sequences of the 28S rRNA gene of 4 specimens (2 from the Sudan and 2 from Senegal) were identical, which confirms conspecificity of geographically distant samples. Sandonella sandoni sequences have also shown that it actually belongs among the Proteocephalidea, being a sister taxon of a relatively derived clade of Palaearctic proteocephalideans, containing Glantinaeus osculata and Paraprotocephaeus parasitarii from catfish and Palaearctic species of the Proteocephalus aggregate.

Lyndale (1960) described the proteocephalidean tapeworm Proteocephalus sandoni from the intestine of the osteoglossiform fish Heterotis niloticus (Cuvier, 1829) (Arapaimidae) from the Sudan. On the basis of other Sudanese specimens, Khalil (1960) provided additional morphological data and erected a new genus, Sandonella, and a new subfamily, Sandonellinae, to accommodate this species because it possessed a compact vitellarium posterior to the ovary, which is a unique character among the Proteocephalidea. The validity of the monotypic subfamily Sandonellinae and its placement in the Proteocephalidea has since been widely accepted (Freze, 1965; Brooks, 1978; Schmidt, 1986; Rego, 1994).

However, the morphology of Sandonella differs so markedly from that of all proteocephalideans (Freze, 1965; Rego, 1994) that it might even be questionable whether it belongs to Proteocephalidea or not, especially because it also parasitizes a primitive fish host (Kumazawa and Nishida, 2000). In a preliminary phylogenetic analysis of proteocephalidean subfamilies based on morphological characters only (Rego et al., 1998), the Sandonellinae appeared to be a sister taxon of a relatively derived clade composed of the Corallobothriinae, Proteocephalidae, and Gangesiinae. In contrast, Bä and Marchand (1994) found differences in the ultrastructure of the spermatozoon of Sandonella sandoni from other proteocephalideans, especially in the presence of a single axoneme (vs. 2 in other taxa), and concluded that these differences may indicate a diphyletic origin of the Proteocephalidea.

Recent progress has been achieved in the systematics and phylogeny of proteocephalidean cestodes (de Chambrier and Vaucher, 1997; 1999; Mariaux, 1998; Scholz and Hanzelová, 1998; Zehnder and Mariaux, 1999; Zehnder and de Chambrier, 2000; Zehnder et al., 2000; de Chambrier et al., 2004; Hyspa et al., 2005; Scholz et al., 2007), but the phylogenetic position of S. sandoni has never been assessed, because no material was available for DNA analysis. Recently, ethanol-fixed specimens were obtained in Senegal and the Sudan. This made it possible to compare the genetic differences among 4 geographically distant samples. Sandonella sandoni sequences have also shown that it actually belongs among the Proteocephalidea, being a sister taxon of a relatively derived clade of Palaearctic proteocephalideans, containing Glantinaeus osculata and Paraprotocephaeus parasitarii from catfish and Palaearctic species of the Proteocephalus aggregate.

MATERIALS AND METHODS

The following specimens collected from the intestine of H. niloticus were studied (acronyms of museum collections are as follows: BMNH = Natural History Museum, London, U.K.; IPCAS = Institute of Parasitology, České Budějovice, Czech Republic; MHNG INVE = Natural History Museum, Geneva, Switzerland, Invertebrate Collection; U.S. National Parasite Collection, Beltsville, Maryland): (1) type specimens (syntypes); 1 slide (whole mount) with 3 specimens from the Sudan collected by J.A. Lyndale (BMNH 1998.11.2.56); (2) 5 slides (whole mounts) with 5 specimens from the Nile River, Khartoum, Sudan, collected by L.F. Khalil in 1960 (BMNH 1998.11.2.128—1 slide; MHNG INVE 34797—4 slides); (3) 4 slides (whole mounts) with 5 immature specimens from the River Ouémé, Agonlin, Benin, collected by L. Euzet on 10 May 1973 (MHNG INVE 18155); (4) 6 whole mounts and 23 transverse sections of 6 specimens from Richard Toli, Dagana Province, Senegal, collected by A. Sène in September 2005 (IPCAS C-467; MHNG INVE 37833–37836, 37854–37856) and 4 whole mounts and 11 transverse sections of 4 other specimens collected by A. Sène at the same locality in February 2006 (MHNG INVE 53515, 53516; BMNH 2008.1.11.4; IPCAS C-467); (5) 2 slides (whole mounts) with 2 specimens from Lekki Lagoon (freshwater), Lagos, Nigeria, collected by B. Akinsanya (BMNH 2005.4.13.1-3); (6) 44 slides (21 whole mounts and 23 slides with cross, longitudinal, and sagittal sections) from 17 stained

Received 29 March 2007; revised 8 August 2007; accepted 9 August 2007.

* Department of Animal Biology, Cheikh A. Diop University, Avenue Cheikh A. Diop, Dakar, Senegal.
† Department of Zoology, Faculty of Science, University of Khartoum, P.O. Box 321, 11115 Khartoum, Sudan.
‡ Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic.

© American Society of Parasitologists 2008
specimens (out of 110 specimens found) in vials from the Nile River (hosts sold in fish markets of Khartoum, Sudan, collected by A. de Chambrier and T. Scholz on 21, 22, and 23 March 2006 (BMNH 2008.1.11.3; IPCAS C-467; MNHNG INVE 48139, 48141, 48393, 49342–49343, 49347–49348, 49351, 49353, 49400, 50018); (7) 20slides (13 whole mounts and 7 slides with cross sections) from 20 stained specimens (of 32 specimens found) from the Nile River at Kosti, Sudan; and 1 specimen on 25 and 27 March 2006 (BMNH 2008.1.11.1-2; IPCAS C-467; USNPC 109994.00; MNHNG INVE 48143–46, 49355). Specimens from Senegal were fixed with 90% ethanol, whereas those from the Sudan with 4% hot formaldehyde solution, with pieces of several worms placed in 99% pure ethanol for DNA analysis. The worms for morphological studies were then stained with Mayer’s hydrochloric carmine solution, dehydrated in an ethanol series, cleared with eugenol (clove oil), and mounted in Canada balsam. For histology, pieces of strobila and several scoleces were embedded in paraffin, cross-sectioned at 12–15 μm, stained with Weigert’s hematoxylin, and counterstained with 1% eosin B (Scholz and Hanzelová, 1998; de Chambrier, 2001). Eggs were studied in distilled water.

Four specimens (2 from the Sudan and 2 from Senegal) were prepared for scanning electron microscopy (SEM) as follows: specimens were dehydrated in graded ethanol series, then transferred to a graded amyl acetate series, critical point dried in a Balzers CPD 030, and coated with gold in a Balzers Union Desk IV Sputter Coater. Adhesive discs were mounted on SEM stubs and sputter coated with gold before examination in a Hitachi S-570 scanning electron microscope at the Museum of Natural History, Geneva. Microthrix terminology follows Thompson (1982). Molecular datasets (see de Chambrier et al., 2004) were deposited in GenBank under accession number AM931032.

Sequences were edited and aligned with SequecherTM v.4.6 (Gene Codes Corp., Ann Arbor, Michigan) with default parameters (dirty data), and minor corrections were done by hand. Regions in which the alignment was unreliable were removed from the analysis. The final matrix was analyzed with PAUP* v.4.0b10 (Swofford, 2002).

REDESCRIPTION

Sandonella sundoni (Lynsdale, 1960) Khalil, 1960
(Figs. 1–15)

Prothocephalus sundoni Lynsdale, 1960

Diagnosis (unless otherwise stated, based on 37 specimens recently collected in the Sudan): Prothocephalus, Proteocephalidae, Sandonellinae, Testes, ovary, uterus and vitelline follicles medullary. Worms 51–186 mm long (x̅ = 87 mm, n = 8, TM = 46–99 mm). Strobila craspedo, with proglottids wider than long. In immature proglottids, segmentation does not coincide with proglottization, and 1 segment may be composed of up to 4 proglottids (Figs. 4, 8F). Strobila of lectotype consists of 446 proglottids: 212 immature in total (up to appearance of spermatozoa in vas deferens). 40 mature (up to appearance of eggs in uterus), 151 pre gravid (up to appearance of hooks in oncospheres) and 43 gravid. Strobila covered uniformly with small filiform microtriches, about 1.3 long and 0.1 wide at level of proliferation zone (Fig. 8A). Scolex 353–585 long (n = 17; TM = 435–475) and 300–525 wide (n = 16, TM = 390–490). Internal sperm duct thick-walled, forming ring-like marginal structure, 265–495 wide (TM = 360–440), formed by 4 retractile lappets, disposed dorsally and laterally. Fertilization occurs in the lateral testes (Figs. 2, 3, 8E). Scolex surface covered with dense, small filiform microtriches (<1 μm long) (Fig. 8H). Suckers spherical, uniciliate, 120–190 in diameter (n = 64, TM = 175), directed laterally. Proliferation zone 335–960 long (from posterior margin of suckers to ring-like marginal structure delimiting first proglottid; TM = 615–745) and 210–490 wide (TM = 360–440). Longitudinal inner musculature well developed, formed by numerous circular muscles, often forming irregularly shaped bundles. Circular muscle fibers present, external to inner longitudinal musculature, weakly developed laterally. Dorsal longitudinal musculature formed by individual muscle fibers (not figured). Subterminal segments well developed (Figs. 5, 10, 13). Posterior margin of each proglottid walled with well-developed net of transversal and dorsoventral muscles. Paired ventral oncosphere canals wider than dorsal ones, thin-walled, forming numerous anastomoses in proliferation zone and basal part of scoloces in proglottids, walled with well-developed net of transversal and dorsoventral muscles. Paired ventral oncosphere canals wider than dorsal ones, thin-walled, forming numerous anastomoses in proliferation zone and basal part of scoloces. In proglottids, ventral canals overlapped by lateralmost testes, usually forming secondary canals running laterally or dorsally and terminating just beneath surface. Dorsal osmoregulatory canals narrow, thick-walled, anastomosed in first immature proglottids, without secondary canals.

Tests medullary, spherical to oval, 45–70 in diameter, numbering 48–73 (x̅ = 60, n = 9; 58–80 according to Lynsdale [1960]), in 1 or 2 irregular layers, in 2 lateral fields confluent anteriorly, continuous between proglottids, with posterior testes dorsal to lateral lobes of vitellarium (Fig. 6). External sperm duct (vas deferens) winding, reaching almost to median line of body, dilated proximally to form voluminous, vesicle-like structure just near junction of vasa efferentia (Fig. 5); distal part of external sperm duct thick-walled, lined with chromophilic cells. Cirrus-sac elongate, gradually narrowing to distal end, thick-walled proximally, 140–215 long and 35–50 wide, representing about 13–19% of proglottid width (x̅ = 16, n = 24, CV = 10%; TM = 18–23%; n = 20, CV = 6%) (Figs. 6, 7). Internal sperm duct thick-walled, forming...
several loops in proximal third or half of cirrus-sac. Cirrus narrow, straight, lined with chromophilic cells. Genital pore irregularly alternating, situated at 37–62% (n = 14, TM = 40–60%; n = 20, CV = 11%) of proglottid length. Genital atrium narrow.

Ovary medullary, compact, bilobed, with short and wide lateral lobes connected by ventrally situated isthmus (Fig. 6). Total length of ovary 70–165, representing 31–75% of proglottid length (n = 30, CV = 18%); width of ovarian lobes 270–470, representing 28–40% (n = 24, CV = 11%) of proglottid width. Mehlis’ glands between and slightly posterior to ovarian lobes, about 100–150 in diameter, representing 8–10% of proglottid width.

Vaginal canal narrow, almost straight or slightly sinuous, thick-walled and lined with chromophilic cells, crossing dorsally cirrus-sac; terminal part of vaginal canal thin-walled, dilated to form small atrium lined with numerous chromophilic cells just before opening in genital atrium. Vagina posterior (62%, n = 286; TM = 70%, n = 50) or anterior (38%; TM = 30%) to cirrus-sac. Vaginal sphincter and seminal receptacle absent.

Vitellarium medullary, postovarian, just at posterior margin of proglottid, formed by 2 separate, transversely elongated compact masses 30–100 long, with deep lobules, especially on ventral side, connected by vitelline duct; length of vitellarium represents 16–33% of proglottid length, total width of both vitellarium 47–60% of proglottid width (r = 53, n = 20, CV = 7%) (Figs. 6, 13).

Uterus medullary, appearing as rounded concentration of numerous small, intensely stained cells in immature proglottids, with lumen lined externally with chromophilic cells in last mature proglottids (Fig. 12) and in pregravid and gravid proglottids (Figs. 9, 11, 12). Uterine sac first rounded or oval, compact, becoming branched, with numerous diverticules occupying up to 83% of width in last gravid proglottid (Figs. 9, 11, 12). In last emptied gravid proglottids, uterine diverticules reduced in size due to hypertrophy of their septa.

Primer of uterine pore already present in immature proglottids as a concentration of chromophilic cells passing from medulla through cortex to ventral surface of proglottids (Fig. 10). In gravid proglottids, uterine pore spherical or widely oval, situated medially, at equatorial level of each proglottid or slightly pre-equatorial (arrow, Fig. 8G). Small uterine reservoir present in ventral cortex, with some eggs concentrated before being discharged. In last proglottids, uterine pore may extend longitudinally.

Egg spherical, with hyaline outer membrane (collapsed in permanent formes: Arapaimidae) (vernacular name “olak” in the Sudan). Egg spherical, with hyaline outer membrane (collapsed in permanent formes: Arapaimidae) (vernacular name “olak” in the Sudan).

Diagnosis: Proteocephalidea, Proteocephalidae, Sandonellinae. Worms of medium size, cespaced. Strobila with proglottids wider than long; immature segments containing up to 4 proglottids. Scolex without metascolex, with highly modified apical structure formed by 4 retractile lappets, disposed dorsoventrally 2 by 2, with funnel-shaped cavity on lateral sides. Circular musculature present external to inner longitudinal muscle bundles. Testes, ovary, vitellarium and uterus medullary. Testes in 2 lateral fields confluent anteriorly, continuous between proglottids. Vitellarium medullary, postovarian, just at posterior margin of proglottid, formed by 2 separate, transversely elongated compact masses with deep lobules. Uterus developing as oval, unlobed sac, later forming several uterine diverticules on each side. Small uterine reservoir and uterine pore present in ventral cortex. Eggs numerous, growing within uterus. Parasite of osteoglossiform fish in Africa.

Type and only species: Sandonella sandoni (Lynsdale, 1960).

Genetic variability and phylogenetic position of Sandonella

Sequences of the 28S rRNA gene of 4 samples of S. sandoni (1,006 to 1,066 bp long) were obtained and aligned. All fragments were 100% identical. In a first stage, sequences of S. sandoni were aligned with those of 86 Proteocephalidea and 4 Tetraphyllidea (outgroups) in a 91-taxa and 1,177-character matrix, 354 of them being informative (154 characters were removed because of uncertain alignment). A total of 2,331 equally parsimonious shortest trees resulted from the heuristic search (L = 2,041, CI = 0.292, RC = 0.185). Sandonella sandoni sequences always branched as a sister group of a clade composed of G. osculata, Paraprotocephalus parassili, and all Palaearctic species of the Proteocephalus aggregate (see de Chambrier et al., 2004; Scholz et al., 2007). Remarkably, the Sandonella branch is far longer than any other on this tree. (Fig. 16). When no characters are excluded, the number of most parsimonious trees (L = 2,337) increases considerably, up to 19,600 (as limited by our computer’s available memory). The strict consensus of these trees shows nevertheless the same general structure as in Figure 16. The position of S. sandoni in relation to the Palaearctic species in particular is conserved.

In a second stage, a reduced 13-taxon matrix comprising 1 outgroup and representatives of all main Proteocephalidea lineages was analyzed. A parsimony branch and bound analysis performed on this 1,097-bp matrix (171 informative characters) returned 2 equally parsimonious shortest trees (L = 467, CI = 0.473, RC = 0.199) and confirms the position of Sandonella as a sister group of the Palaearctic Proteocephalus (P. perecar) + G. osculata. Bootstrap supports this relationship, although not very strongly (71), but all basal relationships were not supported (data not shown). A ML analysis was performed on the same dataset. The best fit model selected by the AIC in Modeltest was the GTR + I + G with the following parameters: Base = (0.1968 0.2148 0.3495), Nst = 6, Rmat = (1.4340 8.8757 2.5320 1.3017 13.1834), rates = gamma, shape = 0.6078, pinvar = 0.3978. The best tree score is 3884.60995 and its structure is fundamentally similar to the previous
FIGURE 8. Sandonella sandoi (Lynsdale, 1960), scanning electron micrographs. (A) Anterior end, dorsoventrally. (B) Scolex, dorsoventrally. (C) Scolex, laterally. (D) Lateral margins of muscular lappets of the apical part of the scolex, with lateral velum. (E) Scolex with partly invaginated lappets. (F) Differentiating immature proglottids (see Fig. 4); note the wrinkles lining the segment divided in 4 sets of genital organs; bold arrows show the margins of 1 segment, small and dotted arrows show the margins of proglottids. (G) Gravid proglottids with uterine pores; note liberated eggs (white arrow). (H) Filiform microtriches on the internal surface (cavity) of suckers. (I) Filiform microtriches of the neck. Abbreviations: vl, velum. Scale bars: A, B, C, F = 100 μm; D = 10 μm; E = 50 μm; G = 200 μm; H, I = 5 μm.
In the original description of *P. sandoni* (= *Sando nella sandoni*), Lynsdale (1960) did not observe the vitellarium and misinterpreted a cavity between muscular lappets on the apical part of the scolex as an apical sucker. Khalil (1960) supplemented the species description by adding new characters, especially data on the compact vitellarium situated near the posterior margin of proglottids, but, like Lynsdale (1960), he claimed that the vagina is always posterior to the cirrus sac. The vagina may be in fact also anterior to the cirrus sac, as observed in 38% of 286 proglottids of the voucher specimens studied and 30% of 50 proglottids of the lectotype. Khalil (1960) also incorrectly illustrated in his Figure 5 the gravid proglottids because the uterus is never unlobed (entire) in the last proglottids, but it possesses several diverticules.

On the basis of this study, the following morphological characteristics of *S. sandoni* should be listed as unique or exceptional among proteocephalideans.

1. **Vitellarium:** The vitellarium of *S. sandoni* is actually not formed by individual follicles separated from each other, as first observed by Khalil (1960). It somewhat resembles that of the Cyclophyllidea but markedly differs in shape and size because the vitellarium of cyclophyllidean cestodes is compact, oval, or semilunar in shape, not formed by 2 separated masses as in *S. sandoni*, and is almost always markedly smaller than the ovary (Fuhrmann, 1928; Khalil et al., 1994). In *S. sandoni*, both vitelline masses are compact but with deep lobes, especially on the ventral side, and are much longer than the ovary.

A somewhat similar vitellarium, reported to be compact, forming 2 lateral bands turned posteroomedially near the posterior margin and joined posterior to the ovary, was found in *Proteocephalus pentastoma* (Klaptocz, 1906), a parasite of a species of *Polypterus* in the Sudan (Jones, 1980).

2. **Scolex morphology:** The apical part of the scolex is formed by 4 retractile lappet-like muscular structures. Khalil (1960) recognized the muscular nature of this part of the scolex, which led Freze (1965) to claim it is possibly "an apical suckorial organ." This study, in which we included for the first time SEM observations and histological sections, supports this assumption. The scolex of *S. sandoni* somewhat resembles that of some cestodes parasitic in elasmobranchs, especially members of the Lecanicephalidea (Caira et al., 1999, 2001; Jensen, 2005), in possessing a well-developed apical part with attachment organs, but these apical structures are apparently non-homologous because proteocephalideans and lecanicephalideans are not closely related (Olson et al., 2001).

3. **Circular inner musculature:** Unlike all other proteocephalideans, *S. sandoni* possesses a layer of circular muscle fibers external to the inner longitudinal musculature. Circular musculature is present in some tapeworms of unrelated groups, such as gyrocothylideans, pseudophyllideans, and tetraphyllideans, but is usually internal to the inner longitudinal musculature (Fuhrmann, 1928; Caira et al., 1999, 2001), not external, as in *S. sandoni*.

4. **Sperm duct (vas deferens):** The presence of an enlargement of the proximal part of the vas deferens (external sperm duct), which forms a vesicle-like, thin-walled structure just near the connection of 2 main collecting canals (vas efferentia), observed in *S. sandoni*, is also a unique feature, in which this taxon differs from all members of the Proteocephalidea.

5. **Structure of the uterus and its development:** The structure of the uterus, which first enlarges as an unlobed, spherical, or widely oval sac and then forms numerous diverticules on all sides, differs from that described in all but 1 (*P. pentastoma*) proteocephalidean. *P. pentastoma*, the uterus arises as diverticules that grow from the end of the uterine duct in the center of proglottids (Jones, 1980). The wall of the uterus is lined externally with chromophilic cells during all its development. The development of the uterus of *S. sandoni* thus represents an intermediary form between the 2 basic types of the uterus development recognized in the Proteocephalidea (de Chambrier et al., 2004).

In immature proglottids of *S. sandoni*, a preformed uterine pore was observed, which is also an atypical feature observed in only very few proteocephalidean taxa, such as *Thaumasio-scolex didelphidis* Cafèda-Guzmán, de Chambrier and Scholz, 2001, from a mammal (opossum) and *Nomimoscolex suspectus* Zehnder, de Chambrier, Vaucher and Mariaux, 2000, from catfish (Zehnder et al., 2000; Cafèda-Guzmán et al., 2001).

6. **Intrauterine growth of eggs:** The eggs of *S. sandoni* are formed within the uterus of pregravid proglottids, as is usual in all species of the Proteocephalidea (Freze, 1965; Scholz, 1999). However, they increase in size during intrauterine development, whereas eggs of all but 1 proteocephalidean do not enlarge so markedly within the uterus before they are released. In only 1 species, *Proteocephalus hobergi* de Chambrier and Vaucher, 1999, from a doradid catfish *Oxydoras kneri* from Paraguay, the first eggs are conspicuously smaller than mature ones, which might be related to the presence of a thick supplementary layer observed in the eggs of this species (de Chambrier and Vaucher, 1999), and also found in *S. sandoni* (Fig. 15).

7. **Absence of seminal receptacle:** Unlike all other proteocephalideans, the proximal end of the vaginal canal of *S. sandoni* does not enlarge to form a thick-walled seminal receptacle. The canal is narrow up to its joining with a wide oocapt to form the fertilization canal.

**Intraspecific morphological and genetic variability**

A comparative study of the types—museum specimens from the Sudan, Nigeria, and Benin and freshly collected tapeworms from Senegal and the Sudan—has shown a great morphological homogeneity among all the populations studied. There were only negligible differences in the shape and measurements of some structures (data not shown), which were apparently caused by different methods of fixation. The conspecificity of all samples studied was confirmed by the identity of sequences of the 28S rRNA genes of all 4 isolates studied. Therefore, *S. sandoni* is considered to be a species with low morphological and genetic variability, which occurs throughout a large part of Africa.

**Host spectrum, prevalence, and distribution**

*Sando nella sandoni* is a relatively frequent parasite of *H. niloticus*. In the Sudan, this tapeworm was found in 12 of 19
(63%) fish examined and with a mean intensity of 11.8 (range 1–47 worms, abundance 7.4). In Senegal, 7 of 9 fishes (77%) examined were infected with a mean intensity of 4.8 (range 1–13, abundance 3.7). Until now, *S. sandoni* has been found in Benin, Chad, Nigeria, Senegal, and the Sudan (Khalil, 1960; Lynsdale, 1960; Bâ and Marchand, 1994; Khalil and Polling, 1997; this study). It probably also occurs in other regions of the distribution area of *H. niloticus*, which is native in all the basins of the Sahelo-Sudanese region (i.e., the Senegal, Gambia, Corubal, Volta, Ouémé, Niger, Bénoue, Chad, the Nile basins, and Lake Turkana) (Froese and Pauly, 2007).

**Phylogenetic position of *S. sandoni***

In this study, we provide the first data on the phylogenetic position of the member of 1 of the 2 proteocephalidean subfam-
Outgroups
Basal Proteocephalidea
Sandonella
Palaearctic Proteocephalidea

Mostly Neotropical Proteocephalidea

Acanthobothrium sp.
Rostellotaenia sp.
Gangesia parasiliuri
Kapsulotaenia sp.
Acanthotaenia cf. shipeyi
Postgangesia inarmata

Sandonella sandoni
Proteocephalus percae
Glanitaenia osculata
Scholzia emarginata
Rudolphiella piracatinga
Amphoteromorphus piriformis
Corallobothrium solidum

0.05 substitutions/site

FIGURE 17. ML analysis of partial 28S rDNA, 13 taxa. Best tree. Bootstrap values >50% are shown above (ML) and below branches (Parsimony).

should be very cautious when assessing the support of such long branches (e.g., Kuhner and Felsenstein, 1994).

As mentioned above, S. sandoni is unique in possessing several morphological characteristics absent in any other proteocephalidean. However, some of these characters somewhat resemble those reported by Jones (1980) in P. pentastoma from another African fish. Both taxa are found in evolutionarily ancient fish hosts (Polypteriformes and Osteoglossiformes; Kumazawa and Nishida, 2000) and in the same region of Africa. This resemblance is particularly obvious at the strobilar level, with characters such as the presence of a compact vitellarium reaching posterior to the ovary, a strongly branched uterus with numerous diverticles on each side, and the presence of up to 4 proglottids in 1 segment in the immature part of the strobila. In contrast, the scolex of both the taxa markedly differs in their morphology (i.e., that of P. pentastoma being of a shape typical for most members of the Proteocephalinae). Unfortunately, no DNA material of P. pentastoma is available for assessing its possible phylogenetic affinity to S. sandoni.

Molecular data do not seem to support results of Bå and Marchand (1994), who found that S. sandoni differs markedly from all other proteocephalideans in the presence of only 1 axoneme (2 in other proteocephalideans; see Brujianská et al., 2004). Recent ultrastructural studies have shown that the spermatozoon of S. sandoni is similar to that of other proteo-
ccephalidans in several characteristics, such as the number and thickness of the crested body and the shape of the nucleus (see Bruňánská et al., 2004). However, the presence of a single axoneme has been confirmed in the new material from the Sudan (M. Bruňánská, pers. comm.).

ACKNOWLEDGMENTS

The authors thank David I. Gibson and Eileen Harris (London) for loan of the type and comparative material of \( S. \) sandoni, Louis Euzet (Sète) for comparative material from Benin and permission to use his hand-made sketches for making a drawing of the scolex, Cheikh Tidiane Bâ (Dakar) for his collaboration in obtaining the Senegal material, and Dia-Eldin Elienaïm (Davis, California) for help in organizing the stay of the 2 authors (A.de C. and T.S.) in the Sudan. The authors are also grateful to Boyko Georgiev (Sofia) for fruitful suggestions, Magdaléna Bruňánská (České Budějovice) for providing unpublished data on the sperm ultrastructure of \( S. \) sandoni, Rossana Martini and André Pizù for providing SEM photomicrographs, and Florence Marteau and Gilles Roth (all Geneva) for their help with drawings. A research stay in the Sudan would not have been possible without the invaluable help of Ali Adam and Sayed (University of Khartoum), Khalid Bashir Abaker, and Ammar Osmar (White Nile Fisheries Research Station in Kosti). The support of the Embassy of Switzerland in Khartoum (Chargé d’Affaires Andrea Rechlin) is also acknowledged. A.de C. is also deeply indebted to the “Donation Georges et Antoine Claraz” for supporting this study. T.S. acknowledges financial support of the Grant Agency of the Czech Republic (projects 524/04/0342) and the Institute of Parasitology (project ZD022018 and LC 522); A.S. is grateful to Patrice Mugny, Head of the “Département de la Culture”, City of Geneva, for financial support to realize a research stay in Switzerland in 2006.

LITERATURE CITED


BRUŇÁNSKÁ, M., T. SCHOLZ, AND M. H. IBRAHIM. 2004. Ultrastructural characteristics of the spermatozoan of the cestode \( C. \) solidum (Fritsch, 1886) (Proteocephalidea: Corallobothriinae), a parasite of \( D. \) marsiusculepis (Sirelliformes: Malaperturidae) from the Nile, Egypt. Parasitology Research 94: 421-426.


VALIDATING THE SYSTEMATIC POSITION OF PROFILICOLLIS MEYER, 1931 AND HEXAGLANDULA PTEROCHENKO, 1950 (ACANTHOCEPHALA: POLYMORPHIDAE) USING CYTOCHROME C OXIDASE (COX 1)

Martín García-Varela and Gerardo Pérez-Ponce de León
Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, A.P. 70-153, C.P. 04510, México, D.F. México. e-mail: garciv@servidor.unam.mx

ABSTRACT: Members of the Polymorphidae (Acanthocephala) are distributed worldwide as endoparasites of marine mammals, fish-eating birds, and waterfowl. The family contains 10 genera, with approximately 127 species. Polymorphids are characterized by having a spinose trunk with a bulbous proboscis, double-walled proboscis receptacle, and long lemnisci, and 4 tubular cement glands. The taxonomic position of several genera within Polymorphidae has been controversial when considering morphological and ecological characters. The mitochondrial coding gene cytochrome-c oxidase representing species of 5 genera of polymorphids (Corynosoma, Lühe, 1904, Hexaglandula Petrochenko, 1950, Southwellina Witenberg 1932, Polymorphus Luhe, 1911, and Profilicollis Meyer, 1931) were sequenced to determine the sister-group relationships among 2 particular genera, i.e., Hexaglandula, and Profilicollis. Maximum likelihood and Bayesian analyses showed that Polymorphidae is a monophyletic assemblage, and that 3 major clades are present. Our results provide support for the idea that Hexaglandula represents an independent lineage, whereas, in the case of Profilicollis, there is no conclusive evidence that they are not members of Polymorphus. The analyses also confirm that Polymorphus is paraphyletic, suggesting that the genus represents a complex of species that should be re-molecular data. Our observations suggest that decapods (intercolonized at least twice during the evolutionary history of the group.

Adults of the Polymorphidae Meyer, 1931 are intestinal parasites of marine mammals, fish-eating birds, and waterfowl. The life cycle typically includes a crustacean (amphipods, copepods, and decapods) as an intermediate host and may include fishes, snakes, frogs, and toads as a paratenic host (Schmidt, 1985). The Polymorphidae contain 10 genera, with approximately 127 species diagnosed by having a spinose trunk, bulbous proboscis, double-walled proboscis receptacle, and usually 4 tubular cement glands (Schmidt, 1973, 1985; Amin, 1985; Aznar et al., 2006). The taxonomic position among the genera of Polymorphidae has been controversial because of the lack of a robust phylogenetic hypothesis. In particular, the validity of 2 genera, i.e., Hexaglandula Petrochenko, 1950 and Profilicollis Meyer, 1931, has been questioned when only morphological characters are considered, since species allocated to each of these genera have been considered as species of Polymorphus Luhe, 1911 (Yamaguti, 1963; Amin, 1992). Previously, Nickol et al. (1999, 2002) used ecological criteria, in addition to morphology, to demonstrate that both Hexaglandula and Profilicollis are valid and independent genera within the family. Hexaglandula includes 6 species as parasites of fish-eating birds, whereas Profilicollis is composed of 12 species that parasitize sea otters and fish-eating birds (Amin, 1985; Nickol et al., 1999; Brockerhoff and Smale, 2002).

The main objective of the present research was to provide a phylogenetic framework, on the basis of cytochrome-c oxidase (cox 1) sequences, with which to shed light on the systematic position and the sister-group relationships of Hexaglandula and Profilicollis within the Polymorphidae.

MATERIALS AND METHODS
Collection and storage of worms
Acanthocephalans used in this study were collected from naturally infected vertebrate and invertebrate hosts (Table I). Worms were washed 3 times in normal saline solution, preserved in absolute ethanol, and stored at 4 C. When available, a few specimens of some species were stained with Meyer’s pararosaniline, cleared with methyl salicylate, and mounted on permanent slides using Canada balsam for taxonomic identification. Voucher specimens were deposited at the Colección Nacional de Helminitos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (Table I).

Amplification and sequencing of DNA
For molecular work, specimens were digested overnight at 56 C in a solution containing 10 mM Tris-HCL (pH 7.6), 20 mM NaCl, 100 mM Na2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. After digestion, DNA was extracted from the supernatant using the DNAzol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer’s instructions. A partial fragment of 655 base pairs of cox 1 was amplified using the polymerase chain reaction (PCR) with the forward primer 5’-AGT TCTAATCATAA(R)GATAT(Y)GG and reverse 5’-TAAACTTCAGG GTGACCAAATAATCA (Folmer et al., 1994).

PCR reactions (25 µl) consisted of 10 mM of each primer, 2.5 µl of buffer 10X, 50 mM MgCl2, and 1 U of Taq DNA polymerase (Platinum Taq DNA, Invitrogen, Brazil). PCR cycling parameters for cox 1 amplifications included denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 1 min, annealing at 40 C for 1 min, and extension at 72 C for 1 min, followed by a postamplification incubation at 72 C for 7 min.

Each PCR product was cleaned up and filtered using Millipore columns (Amicon, Billerca, Massachusetts). PC products were cloned by ligation into pGEM-T vector (Promega, Madison, Wisconsin) and used to transform competent Escherichia coli (JM109). Positive clones were identified by blue/white selection, and target insert size was confirmed by PCR of DNA extracts prepared from bacterial (clone) colonies. Liquid cultures for miniprep reactions were grown in Luria broth containing 50 µg/ml ampicillin. Plasmids for DNA sequencing were prepared using commercial miniprep kits (Qiaprep, Qiagen, Valencia, California). Plasmids were sequenced for both DNA strands using 2 universal primers. Sequencing reactions were performed using ABI BigDye (PE Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 310 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Codoncode version 1.4.5 (Codoncode, Dedham, Massachusetts). All sequences have been deposited in Genbank data set (accession numbers in Table I).

Alignments and phylogenetic analyses
The sequences generated in this study were aligned with other sequences available in the Genbank data set. A total of 19 sequences from...
TABLE I. Specimen information and GenBank accession numbers for specimens studied in this work. Sequences marked with an asterisk were indicated by some previous phylogenetic analyses (Near et al., 1998; Swofford, 2005, 2006).

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Locality</th>
<th>Vouchers (CNHE)</th>
<th>Gen bank access</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corynosoma enhydri</td>
<td>Enhydra lutris</td>
<td>Monterey Bay, California, U.S.A.</td>
<td>3429</td>
<td>DQ089719</td>
</tr>
<tr>
<td>Corynosoma magdaleni</td>
<td>Phoca hispida saimensis</td>
<td>Lake Saimaa, Finland</td>
<td>EF467872*</td>
<td></td>
</tr>
<tr>
<td>Corynosoma strumosum</td>
<td>Phoca hispida homica</td>
<td>Baltic Sea, Finland</td>
<td>EF467871*</td>
<td></td>
</tr>
<tr>
<td>Corynosoma strumosum 1</td>
<td>Phoca vitulina</td>
<td>Monterey Bay, California, U.S.A.</td>
<td>EF467870*</td>
<td></td>
</tr>
<tr>
<td>Hexaglandula corynosoma</td>
<td>Nycitassa violacea</td>
<td>Veracruz, Mexico</td>
<td>5757</td>
<td>EF467869*</td>
</tr>
<tr>
<td>Polymorphus brevis 1</td>
<td>Nycticorax nycticorax</td>
<td>Michoacín, México</td>
<td>5777</td>
<td>DQ089717</td>
</tr>
<tr>
<td>Polymorphus brevis 2</td>
<td>Egretta thula</td>
<td>Guerrero, México</td>
<td>5766</td>
<td>EF467861*</td>
</tr>
<tr>
<td>Polymorphus minutus</td>
<td>Gammarus pulex</td>
<td>Dijon, France</td>
<td>EF467865*</td>
<td></td>
</tr>
<tr>
<td>Profillicollis almani 1</td>
<td>Enhydra lutris</td>
<td>Monterey Bay, California, U.S.A.</td>
<td>5777</td>
<td>DQ089720</td>
</tr>
<tr>
<td>Profillicollis almani 2</td>
<td>Melanita perspicillata</td>
<td>Monterey Bay, California, U.S.A.</td>
<td>5767</td>
<td>EF467863*</td>
</tr>
<tr>
<td>Profillicollis almani 3</td>
<td>Larus pipixcan</td>
<td>Caleta Lenga, Chile</td>
<td>5776</td>
<td>EF467864*</td>
</tr>
<tr>
<td>Profillicollis butulus 1</td>
<td>Somateria mollissima</td>
<td>Denmark</td>
<td>5768</td>
<td>EF467862*</td>
</tr>
<tr>
<td>Profillicollis butulus 2</td>
<td>Anas platyrhynchos</td>
<td>U.S.A.</td>
<td>DQ089721</td>
<td></td>
</tr>
<tr>
<td>Southwellina hispida 1</td>
<td>ND</td>
<td>Baltic Sea, Finland</td>
<td>EF46786*</td>
<td></td>
</tr>
<tr>
<td>Southwellina hispida 2</td>
<td>Tigrisoma mexicanum</td>
<td>Veracruz, México</td>
<td>5769</td>
<td>EF467867*</td>
</tr>
<tr>
<td>Southwellina hispida 3</td>
<td>Egretta garzetta</td>
<td>Tabasco, México</td>
<td>5770</td>
<td>EF467868*</td>
</tr>
<tr>
<td>Centrorhynchus sp</td>
<td>Falco peregrinus</td>
<td>California, U.S.A.</td>
<td>DQ089716</td>
<td></td>
</tr>
<tr>
<td>Gorgorhynchoides bulbuci</td>
<td>Eugerres plumieri</td>
<td>Quintana Roo, México</td>
<td>DQ089715</td>
<td></td>
</tr>
<tr>
<td>Plagiorynchus cylindraceus</td>
<td>Porcilio saber</td>
<td>Dijon, France</td>
<td>DQ089724</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

cox 1 was readily aligned on the basis of the inferred cox 1 protein sequences using the Clustal W software (Thompson et al., 1994). Plagiorynchus cylindraceus Goerce, 1782, Centrorhynchus sp., and Gorgorhynchoides bulbuci Cable and Mafarachisi, 1970 were chosen as outgroups because they are closely related to the Polymorphidae, as indicated by some previous phylogenetic analyses (Near et al., 1998; Near, 2002; García-Varela et al., 2000, 2002; García-Varela and Nadler, 2005, 2006). Maximum likelihood (ML) tree was inferred using PAUP* 4.0b10 (Swofford, 2002). For ML analyses, a nested likelihood ratio test was used to assess the fit of general time reversible nucleotide substitution models (Rodríguez et al., 1990) through use of Modeltest version 3.0 (Posada and Crandall, 1998). The best-fit ML model for the cox 1 data set was used for likelihood analysis. Tree searches were performed using 100 random-addition heuristic searches with tree-branch reconstruction branch swapping. The relative reliability of clades was assessed by bootstrap resampling with 1,000 bootstrap replicates. MrBayes v3.1.2a (Huelsenbeck and Ronquist, 2001) was used for the Bayesian analysis, sampling every 1,000 trees over 5,000,000 generations (burn-in determined empirically).

To compare trees representing specific alternative phylogenetic hypotheses, topological constraints were defined on trees obtained from ML analyses. Differences between unconstrained (best) and constrained trees representing alternative hypotheses were evaluated using the Kishino and Hasegawa likelihood test (Kishino and Hasegawa, 1989) as executed in PAUP*. Trees were drawn using RETREE and DRAWGRAM from PHYLIP (Felsenstein, 1999). The genetic distances among taxa were estimated using uncorrected method. The alignment has been deposited in TreeBASE under number SN3422 (Sanderson et al., 1994).

RESULTS

The genetic distances among individuals from different populations of Corynosoma strumosum Lühe 1904, Southwellina hispida Van Cleave 1925, Polymorphus brevis Van Cleave, 1916, Profillicollis almani Perry 1942, and Profillicollis butulus Van Cleave 1916 ranged from 1 to 5%, whereas the genetic divergence among species ranged from 11 to 21%, and among genera the genetic distance ranged from 22 to 30%. Nucleotide frequencies for cox 1 data set were 0.235 (A), 0.118 (C), 0.265 (G), and 0.386 (T). The heterogeneity of nucleotide frequencies across taxa was tested using the "basefreq" option implemented in PAUP* (χ² = 59.57, P = 0.28). This result indicates that the nucleotide frequencies of cox 1 were not significantly heterogeneous across taxa, which is advantageous because ML method performs optimally when nucleotide frequencies are homogeneous (Omilian and Taylor, 2001). For the alignment, the likelihood ratio test indicated that the model with best fit was the Kimura 2 parameter model (K81; Kimura, 1980) with a gamma distribution of 0.726 (+ G; Yang, 1994) and with a proportion of invariable sites of 0.3401.

ML analysis using this model yielded a single best tree with -ln(likelihood) = 5183.011 (Fig. 1). The ML tree inferred from cox 1 data set shows that Polymorphidae is a monophyletic assemblage, and 3 major clades are present. One clade was composed of Polymorphus minutus as the sister taxon of 3 populations of Profillicollis almani, plus 2 populations of P. butulus. Bootstrap support for this clade was 100%. A second clade including Hexaglandula corynosoma as the sister taxon of 3 populations of S. hispida, plus 2 populations of Polymorphus brevis, yielded a bootstrap value of less than 50%, whereas the subclade most derived composed of 3 populations of S. hispida and 2 populations of P. brevis showed a bootstrap value of 68%. The third clade, the most basal, was composed of 3 species of Corynosoma (Corynosoma enhydri Morozov 1940, Corynosoma magdalenii Montreuil 1958, and 2 populations of C. strumosum Rudolph 1802), with a bootstrap value of 86%. The Bayesian analysis yielded almost identical relationships among the polymorphs included in the analysis, and Bayesian posterior probabilities of the 3 major clades show similar results as the ML analysis (Fig. 2).

DISCUSSION

The Bayesian and ML analyses showed that Polymorphus is paraphyletic and this is probably the main reason for the con-
troversial taxonomic arrangement of the Polymorphidae. *Polymorphus* is currently considered as one of the most diverse genera within Polymorphidae, with 28 described species. Species of *Polymorphus* include amphipods as first intermediate hosts, and waterfowl as definitive hosts distributed worldwide (Connell and Corner, 1957; Denny, 1969; Podesta and Holmes, 1970; Amin, 1985, 1992). Recently, Aznar et al. (2006) suggested that *Polymorphus* is not monophyletic, even though not all the congeneric species of *Polymorphus* were included in their phylogenetic analysis. In some taxonomic treatments, *Profilicollis*, *Arythmorhynchus*, and *Hexaglandula* have been considered junior synonyms of *Polymorphus* (Yamaguti, 1963; Amin, 1992). Our results clearly indicate that the 2 species of *Profilicollis* (*P. altmani* and *P. botulus*) are sister taxa of *Polymorphus minutus*, whereas in a separate clade, *H. corynosoma* is the sister species of *P. brevis* + *S. hispida*. The 2 species of *Polymorphus* are not each other’s closest relatives, supporting the contention that the genus, as currently conceived, is not monophyletic, a conclusion that must be considered with caution since only 2 representative species of the genus were considered in the analyses. To test the paraphyly of *Polymorphus*, a constrained ML tree was generated, with *P. minutus* and *P. brevis* as monophyletic assemblages. The −ln(likelihood) score for the original hypothesis (paraphyly of *Polymorphus*) shown

---

**FIGURE 1.** Maximum likelihood tree inferred from the cox 1 data set (−ln[likelihood] = 5183.011). Branch lengths are scaled to the expected number of substitutions per site. Numbers below internal nodes show ML bootstrap support values. Values less than 50% are not shown in the nodes.
in Figure 1 was 5183.011, whereas the score for the constraint hypothesis (monophyly of Polymorphus, tree not shown) was 5238.077. On the basis of the Kishino and Hasegawa (1989) likelihood test as executed in PAUP*, the alternative topology is significantly worse (SD = 55.6 and $P < 0.05$). Therefore, the species composition of Polymorphus as currently conceived is unsatisfactory; it might be possible that the genus represents a complex of species that should be re-examined and then reclassified using morphological, ecological, and molecular characters, on the basis of the idea that has been recently elaborated by several authors (see Aznar et al., 1999; Nickol et al., 1999, 2002; Sardella et al., 2005; Aznar et al., 2006).

Hexaglandula also has had an unsettled taxonomic history. Travassos (1915) described Polymorphus corynosoma as a new species of polymorphid parasite of Nyctanassa violacea in Brazil. Petrochenko (1950) erected the subgenus Hexaglandula within Polymorphus to include it. Later, Petrochenko (1958) elevated Hexaglandula to a generic rank because the 6 species

![Bayesian tree inferred from the cox 1 data set. Numbers above internal nodes show posterior probabilities support values.](image-url)
possessed 6 tubular cement glands, instead of 4. This new genus was later accepted in some classifications in the absence of critical taxonomic studies (Golvan, 1960; Yamaguti, 1963; Schmidt, 1973; Amin, 1985). However, Amin (1992) proposed the designation of Hexaglandula as a junior synonym of Polymorphus because of the fact that the number of cement glands was not considered, in his opinion, a valid taxonomic character to distinguish Hexaglandula from Polymorphus. In contrast, Nickol et al. (2002) found that the species of Hexaglandula use decapods (fiddler crabs, Uca spinicarpa Rathbun, 1900 and Uca rapax Smith, 1870) as intermediate hosts, whereas members of Polymorphus use amphipods (Hynes and Nicholas, 1957; Hirsch, 1980; Haine et al., 2005); the combination of both sources of information, i.e., ecological and morphological, were used by Nickol et al. (2002) to support the concept that Hexaglandula represents a valid genus and that it should not be considered a synonym of Polymorphus. The molecular data generated herein also suggest that Hexaglandula is an independent genus within the family. The ML and Bayesian trees placed H. corynosoma as sister species of a group composed of 3 populations of S. hispida, plus 2 populations of P. brevis. The genetic divergence among species of Southwellina and Polymorphus is 22%, among Southwellina and Hexaglandula it is 32%, and among Polymorphus and Hexaglandula it is 32%. This level of genetic divergence demonstrates that they represent independent lineages, and this evidence supports the argument of Nickol et al. (2002) that Hexaglandula is a valid genus.

Profilicollis was erected as a new genus of Polymorphidae by Meyer (1931) to include polymorphids with long necks, fully ovoid proboscis in both sexes, and eggs with concentric membranes. Later, Webster (1948) and Golvan (1960) placed Profilicollis as a subgenus of Polymorphus because of the fact that long necks and spheroid proboscises also occur to some extent in certain species of Polymorphus. This idea was followed by Amin (1992) when he developed a taxonomic revision of Polymorphus. More recently, Nickol et al. (1999) used data regarding host–parasite associations and proposed the redefinition of Profilicollis as a valid genus in Polymorphidae. The 12 species currently allocated to this genus include decapod crustaceans as intermediate hosts, instead of amphipods as for Polymorphus (Nickol et al., 2002). The phylogenetic hypotheses generated in the present study show that the 2 species of Profilicollis (P. altmani and P. botulus) are close relatives and they are the sister taxa of Polymorphus minutus, the type species of Polymorphus. The genetic divergence among species of Profilicollis is 24%, whereas the divergence among Profilicollis and Polymorphus minutus is 27%. This level of genetic divergence in conjunction with the position of P. minutus on the cladogram generates 2 hypotheses: (1) that Profilicollis is a synonym of Polymorphus since the latter genus was described first, by Lühe (1911), which gives it nomenclatural priority; and (2) that they represent independent lineages on the basis of the premise that they have anatomical features that distinguish them and they utilize different intermediate hosts. The first scenario would support Schmidt and Kuntz (1967) and Amin (1992) that there is insufficient morphological evidence to sustain the generic status of Profilicollis, whereas the second scenario supports the suggestion of Nickol and coworkers (1999, 2002) that biological differences inherent in the use of decapods as intermediate hosts instead of amphipods, taken together with distinctive morphological traits, are evidence of generic status. In our opinion, the second scenario is more plausible and we are in favor of the position of Nickol et al. (1999, 2002). Fortunately, in this case, our molecular data generated are not conclusive and no hypothesis can be supported with the database we have generated to date, since only a few species of both genera have been sequenced, and, therefore, the inclusion of at least 3 or 4 more species of Profilicollis and Polymorphus will be necessary to resolve this taxonomic problem.

The phylogenetic hypothesis proposed in the present study is the first molecular approach to understanding the evolution of the Polymorphidae and represents the starting point for future research. Mapping the intermediate hosts used by polymorphids onto the cladogram (not shown) provides an evolutionary framework to understand the host–parasite associations. Apparently, amphipods are the plesiomorphic hosts for polymorphids, and it seems that the use of decapod crustaceans as intermediate hosts in their life cycle arose at least 2 times during the evolutionary history of the group, once in the Profilicollis clade, and the other in the Southwellina and Hexaglandula clade (postulating a reversal in Polymorphus brevis). This represents the most parsimonious explanation regarding the use of certain crustaceans as intermediate hosts. We are aware that taxon sampling is incomplete, but our data provide preliminary support for that contention. The inclusion of other genera such as Andracaantha, Bolbosoma, Atyrhyphynchus, and Diplospinifer, and more species of Polymorphus in a more comprehensive phylogenetic analysis of the Polymorphidae, will be needed, not only to produce a robust classification scheme, but also for a better understanding of the evolutionary history of this interesting group of acanthocephalans.

ACKNOWLEDGMENTS

We are grateful to Murray Dailey, Marie-Jeanne Perrot, William Font, Thomas Kjaer, Tuula Sinisalo, Dan Benesh, and Mario George for providing specimens preserved in ethanol for this study. The help of Patricia de la Torre and Laura Marquez with the molecular techniques is greatly appreciated. This research was supported by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-IN206906) (M.G.V.).

LITERATURE CITED

BROCKERHOFF, A. M., AND L. R. SMALES. 2002. Profilicollis novoae-landensis n. sp. (Polymorphidae) and two other acanthocephalan parasites from shore birds (Haematopodidae and Scolopacidae) in New Zealand, with records of two species in intertidal crabs (De-


Petrochenko, V. I. 1950. Some biological characteristics of the genus Polymorphus and the significance of these in systematics. Trudi Vsesoyuznogo Institut Gel'mintologov 4: 98–108.


A NEW SPECIES OF MYXOZOAN (MYXOSPORA) FROM THE BRAIN AND SPINAL CORD OF RAINBOW TROUT (ONCORHYNCHUS MYKISS) FROM IDAHO

Carla I. Hogge, Matthew R. Campbell, and Keith A. Johnson
Idaho Department of Fish and Game, Eagle Fish Health Laboratory, 1800 Trout Road, Eagle, Idaho 83616. e-mail: chogge@idfg.idaho.gov

ABSTRACT: A new species of Myxosporea, Myxobolus neurotropus n. sp., is described from the brain and spinal cord of rainbow trout (Oncorhyncus mykiss) from Duncan Creek, Owyhee County, Idaho. Spores are oval, have 2 pyriform polar capsules, and possess a thick spore wall (sutural rim) with a short intracapsular offshoot. The mean spore dimensions are length 11.8 μm, width 10.8 μm, and thickness 8.8 μm. This myxozoan is compared to other described Myxobolus species found in cranial tissues of salmonids in terms of spore morphology and phylogenetic analysis. Because it is found in brain and spinal cord, it is encountered while performing screening tests for Myxobolus cerebralis, the causative agent of salmonid whirling disease. Where chronic inflammation and granulomatous lesions are associated with M. cerebralis, histological examination shows no host response to M. neurotropus n. sp. A diagnostic polymerase chain reaction (PCR) test is included as an aid in properly identifying the species.

Myxobolus Butschli, 1882 (Myxozoa, Myxosporea, Myxobolidae) includes over 700 described species, primarily from fish hosts (Eiras et al., 2005). Of these, several species have been detected in the cranial tissues of salmonids, including Myxobolus cerebralis (Hofer, 1903), Myxobolus neurobius (Schuberg & Schröder 1905), Myxobolus kisutch (Yasutake & Wood 1957), Myxobolus arcticus (Pugachev & Khokhlov 1979), and Myxobolus farionis (Gonzalez-Lanza & Alvarez-Pellitero 1984). Myxobolus cerebralis is found in cartilage or bone, whereas M. neurobius, M. kisutch, M. arcticus, and M. farionis have been described in nerve tissue. While screening salmonids for M. cerebralis, the causative agent of salmonid whirling disease, we observed another Myxobolus species. Herein, we describe this myxobolid as a new species from brain and spinal cord of rainbow trout (Oncorhyncus mykiss); also included are 185 SSU rDNA sequence information and results of a diagnostic polymerase chain reaction (PCR) test.

MATERIALS AND METHODS

Sample source
Rainbow trout were obtained from Duncan Creek, Owyhee County, Idaho in 2003, 2005, and 2006. For each necropsy, fish were given a lethal dose of tricaine methanesulfonate (Argent Laboratories, Redmond, Washington). In 2003, with the use of a dermal biopsy punch (Miltex Instrument Co., Bethpage, New York) and adult fish, we removed a 6-mm punch of tissue from the area posterior to the eye encompassing the brain and including the medulla oblongata, to be used for DNA extraction. In 2005, heads of juvenile fish were removed behind the operculum to include the brain and anteriormost portion of the spinal cord. The remaining body was divided into anterior, medial, and posterior sections. All tissues were fixed in 10% neutral buffered formalin for histological examination. In 2006, adult fish were obtained. Brain tissue and the anteriormost part of the spinal cord were removed and stored at 4°C to be processed later the same day for microscopic examination. The spinal cords were dissected from these fish, and cut into quarters for pepsin–trypsin digest (PTD) to be done on each quarter.

Microscopy
The brain and spinal-cord tissue were blended with sterile saline in a Waring blender, layered on 8 ml of 55% dextrose, centrifuged at 850 g for 30 min, and pelletedized. The resulting spore pellet was washed with sterile saline and refrigerated for fresh, wet-mount preparations.

To determine spore morphology, slides were prepared from the fresh spore pellet alone, with the addition of India ink to visualize a mucous envelope, and with 2% Lugol’s iodine to observe the presence of an iodinophilic vacuole. Spores in the fresh wet mount were photographed with the use of Nomarski differential interface contrast to aid in counting the polar filament coils within the polar capsules. Measurements, based on 31 spores, were obtained according to Lorn and Arthur (1989) with a Nikon Optiphot microscope with the use of a Leica DC500 color digital camera and Image Pro Express v. 5.1 digitizing software. An aliquot was sent to the Utah Veterinary Diagnostic Laboratory, Logan, Utah, where it was processed by standard techniques and photographed with the use of a scanning electron microscope.

Pepsin–trypsin digest (PTD)
PTD was performed on each quarter of the dissected spinal cords following American Fisheries Society Bluebook protocols (MacConnell, 2003) modified in that the spinal cord was not defleshed prior to adding pepsin.

Histology
With the use of the fixed tissues, heads were processed first by standard techniques, sectioned, and then stained with May–Grunwald Giemsa and H&E stains. Slides were examined under a compound microscope for the presence of parasite developmental stages and myxospores in brain and spinal cord. The fish determined to be positive for myxospores in the brain and spinal cord were used; the anterior, medial, and posterior sections were processed as above and all tissues were examined for the presence of the parasite.

PCR, sequencing, and phylogenetic analysis
Genomic DNA was isolated from 6-mm punches of brain tissue with the use of a QIAGEN DNeasy® tissue kit (QIAGEN, Valencia, California) according to the manufacturer’s protocol.

Following partial 185 SSU rDNA sequencing of isolates from the Duncan Cr. location (Hogge et al., 2004), additional primers were designed and used in conjunction with existing primers to amplify overlapping fragments of the 185 SSU rDNA. In the 5’ region, primers 18E (CTGGTGTGATCCTGCGATT) (Hillis and Dixon, 1991) and Mc3R (CCCGTAACCGAAAACCTTGA) (Hogge et al., 2004) produced a fragment ~1,200 base pairs (bp). Primers CH1 (GGGCTCAAACACTA TCAAGG) and CH1440 (TTGCTCTACACGTCAACATA) (this study) produced a fragment ~1,400 bp. In the 3’ region Myxg3F (GGAC TAACRAATGCGAGGCGA) (Kent et al., 2000) and 18R (CTACGG AAAAATTGTTACG) (Whipp et al., 2003) were used to amplify a fragment ~1,000 bp long.

The PCR was performed in 50-μl volumes consisting of 1X PCR buffer, 2.5 mM MgCl2, 5 μM tetramethylammonium chloride, 400 μM deoxyribonucleotide triphosphates, 20 pmol of each primer, 2 U Taq, and 2 μl of template DNA. The DNA was denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56–59°C for 1 min, and 72°C for 1–2 min. Amplification products were sequenced by Nevada Genomics Center (University of Nevada, Reno, Nevada) with the use of primers listed above and primer Mc5L683 (AACAACTGGAGGGCA AGTCT) (Hogge et al., 2004), then aligned and checked using Sequencer® 4.6 (Gene Codes Corporation, Ann Arbor, Michigan).

For comparison, SSU rDNA sequences of other representative myxozoans were retrieved from GenBank, including M. cerebralis (Gen-
Myxobolus neurotropus n. sp. Line drawing of the myxospore. Scale bar = 5 μm.

Bank accession number U96492), M. neurobius (AF085180), M. arcticus (AF085176), Myxobolus insidiosus (U96494), Myxobolus squamalis (U96495), M. kisutchi (EF431919), and Ceratomyxa shasta (AF001579). These sequences were truncated to match the sequence of the new species and aligned with the use of ClustalW (Thompson et al., 1994) in the San Diego Supercomputer Center (SDSC) Biology Workbench (http://workbench.sdsc.edu). The software program PAUP version 4.0b10 (Swofford, 1998) was used to estimate uncorrected pairwise genetic distances between aligned sequences. A maximum-likelihood estimation (MLE) phylogram was constructed, also with PAUP, under the settings for the General Time Reversible plus Gamma model as identified with the use of the software program FINDMODEL (http://www.hiv.lanl.gov/content/hiv-db/findmodel/findmodel.html). Support for nodes were assessed with the use of 100 bootstrap replications, with C. shasta included as an outgroup.

Diagnostic PCR

In the SDSC Biology Workbench, the M. neurotropus n. sp. sequence and GenBank sequences from the various Myxobolus species listed above were aligned with the use of ClustalW. Primer 3 (SDSC Biology Workbench) was used to identify primer pairs in regions that would only amplify M. neurotropus n. sp. PCR was performed as above with the use of the resulting primers CH260L (TTACCGTGAACTGACTCAOC) and CH260R (GATGTTGACTACCCACAGCTA) amplifying at 94 C for 5 min, followed by 35 cycles of 94 C for 1 min, 59 C for 45 sec, and 72 C for 1 min to produce a 260-bp amplicon. This diagnostic PCR was then tested for specificity with 9 isolates of the new species from separate locations throughout Idaho representing all fish host species known to date, cutthroat trout (Oncorhynchus clarki), brook trout (Salvelinus fontinalis), bull trout (S. confluentus), Chinook salmon (O. tsawytscha), and sockeye salmon (O. nerka). Amplification products were electrophoresed through a 2% agarose gel, stained with 1% ethidium bromide, and visualized under ultraviolet light.

DESCRIPTION

Myxobolus neurotropus n. sp.
(Figs. 1–4)

Plasmodia: Cysts in brain and spinal cord not present. No trophozoites observed.

Mature spores: Oval, occasionally almost circular in valvular view. Thick spore wall (sutural rim) with short intracapsular offshoot (Fig. 1). Two polar capsules pyriform, 1 slightly longer than the other, extending past the midlength of spore, 6–8 polar filament coils (Fig. 2). Mean spore measurements in micrometers ± SD (n = 31); length, 11.8 ± 0.48; width, 10.8 ± 0.49; thickness (n = 5) 8.8 ± 0.31. Polar capsule

Figure 1. Myxobolus neurotropus n. sp. Line drawing of the myxospore. Scale bar = 5 μm.

Figure 2. Myxobolus neurotropus n. sp. spore in wet mount, Nomarski differential interface contrast. Scale bar = 5 μm.

Figure 3. Scanning electron microscope (SEM) image of Myxobolus neurotropus n. sp. Scale bar = 4.29 μm.

Figure 4. Histological section of anterior spinal cord. Myxobolus neurotropus n. sp. spores lying along white matter tracks. Giemsa. Scale bar = 10 μm.
**TABLE II.** Comparison of mean measurements (in micrometers) and ranges (in parentheses) of *Myxobolus neurotropus* n. sp.  

<table>
<thead>
<tr>
<th>Species</th>
<th>Length</th>
<th>Width</th>
<th>Thickness</th>
<th>Shape</th>
<th>PC,* length</th>
<th>Site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. neurotropus</em></td>
<td>11.8† (11.2–13)</td>
<td>10.8 (10.4–12.3)</td>
<td>8.8 (8.4–9.1)*</td>
<td>Oval to circular</td>
<td>5.6 and 5.9 (5.0–6.9)</td>
<td>Fresh brain and spinal cord. No cyst formation.</td>
<td>This study</td>
</tr>
<tr>
<td><em>M. kisutchi</em></td>
<td>(7–8.5)</td>
<td>(6.5–7)</td>
<td>(3.5–3.8)</td>
<td>Oval</td>
<td>(3.8–5.5)</td>
<td>Fixed spinal cord. No cyst formation.</td>
<td>Yasutake and Wood (1957)</td>
</tr>
<tr>
<td><em>M. cerebralis</em></td>
<td>(7.4–9.7)</td>
<td>(7–10)</td>
<td>(6.2–7.4)</td>
<td>Oval to circular</td>
<td>(4.2–6)</td>
<td>Fresh cartilage</td>
<td>Lom and Dykova (1992)</td>
</tr>
<tr>
<td><em>M. neurobius</em></td>
<td>(10–12)</td>
<td>8</td>
<td>6</td>
<td>Oval, tapered anterior</td>
<td>(6–7)</td>
<td>Fixed nerve tissue. Cyst formation.</td>
<td>Schuberg and Schröder (1905)</td>
</tr>
<tr>
<td><em>M. neurobius</em></td>
<td>(13.4–14)</td>
<td>(8.5–9.2)</td>
<td>(6.8–7.4)</td>
<td>Oval, tapered anterior</td>
<td>(7.8–8.5)</td>
<td>Fresh nerve tissue</td>
<td>Pugachev and Khokhlov (1979)</td>
</tr>
<tr>
<td><em>M. arcticus</em></td>
<td>(14.3–16.5)</td>
<td>(7.6–7.7)</td>
<td>(6.8–7.4)</td>
<td>Pyriform</td>
<td>(6.6–9)</td>
<td>Brain and spinal cord</td>
<td>Pugachev and Khokhlov (1979)</td>
</tr>
</tbody>
</table>

* Polar capsule.  
† Mean (micrometers), (range).  

The inability to consistently distinguish the morphologically similar spores of *M. cerebralis* and *Myxobolus neurotropus* n. sp. was the impetus for this research. At times, the *M. neurotropus* n. sp. spores observed in PTD appeared larger than the range for *M. cerebralis*. Other times, the spore size was similar to those of *M. cerebralis*. Despite this morphological similarity, phylogenetic analysis indicates that *Myxobolus neurotropus* n. sp. does not exceed 15% (Hedrick et al., 1991). Therefore, even with shrinkage considered, *M. kisutchi* is smaller than *M. neurotropus* n. sp. The last species to consider is *M. neurobius*.

An effort was made by the authors to obtain samples of *M. neurobius* from trout in the Gutach, Germany (type host and type location) for current morphological and molecular comparison. Not only was reference material for *M. neurobius* unavailable, German colleagues report that they no longer see infected fish despite considerable routine diagnostic screening (M. El-Matbouli, pers. comm.). Thus, comparisons are based on the original description of *M. neurobius* by Schuberg and Schröder (1905) from alcohol-fixed nerve tissue and a later description by Pugachev and Khokhlov (1979), presumably from fresh material. Although the latter authors indicate a spore size for *M. neurobius* that is larger than *M. neurotropus* n. sp., clearly the difference, reported in both descriptions of *M. neurobius*, is that the spore width and breadth is much less than those of *M. neurotropus* n. sp. (see Table II). Line drawings and text describing the *M. neurobius* spore report a rounded posterior end, but a tapered anterior. The *M. neurotropus* n. sp. spore is oval or occasionally almost circular, but not pyriform. Schuberg and Schröder (1905) indicate 8–10 polar filament coils in their original description of *M. neurobius*, whereas drawings given by Shulman (1966) indicate 13 coils both figures higher than the 6–8 coils we report for *M. neurotropus* n. sp.

![Figure 6. Two percent agarose gel showing the 260-bp rDNA amplicon from the Myxobolus neurotropus n. sp. diagnostic PCR. In the top row lanes 1–9 are M. neurotropus n. sp. positive samples from host species (see text). Oncorhynchus mykiss (lanes 1, 2, 8, and 9). Salvelinus confluentus (lane 3). Oncorhynchus clarki (lanes 4 and 5). Oncorhynchus tschawytscha (lane 6), and Oncorhynchus nerka (lane 7). Lanes 10–17 are Myxobolus cerebralis positive samples (PCR confirmed; Andree et al. 1998). In the bottom row lanes 18–23 are isolates of Myxobolus kisutchi (WA), Myxobolus squamalis (CA), M. squamalis (OR), Myxobolus insidiosus (OR), Henneguya sp. (ID), and Henneguya salminicola (ID) respectively; and lanes 24–29 are pepsin-trypsin digest confirmed Myxobolus sp. negative O. mykiss, O. tschawytscha, O. nerka, O. clarki, Salvelinus fontinalis, and S. confluentus. Lane 30 is a master mix negative control.](image-url)
A NEW DICYEMID FROM SEPIELLA JAPONICA (MOLLUSCA: CEPHALOPODA: DECAPODA)

Hidetaka Furuya
Department of Biology, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan. e-mail: hfuruya@bio.sci.osaka-u.ac.jp

ABSTRACT: A new species of dicyemid mesozoan is described from Sepiella japonica Sasaki, 1929, collected from the eastern area of Inland Sea, Osaka Bay, and Kii Strait in Japan. Dicyema sepiellae n. sp. is a large species that reaches about 4,000 μm in length. This species lives in folds of the renal appendages. The vermiform stages are characterized as having 24–29 peripheral cells, a conical calotte, and an axial cell that extends to the middle of the metapolar cells, but to the base of propolar cells in vermiform embryos. Infusoriform embryos consist of 39 cells; 2 nuclei are present in each urn cell and the refringent bodies are solid. This is the first description of a dicyemid in any species of Sepiella.

Dicyemids mesozoans (phylum Dicyemida) are the most common and characteristic endosymbionts that typically are found in the renal sac of benthic cephalopod molluscs. Dicyemids are distributed in a variety of geographical localities, i.e., Okhotsk Sea, Japan Sea, western and northeastern Pacific Ocean, New Zealand, northern Indian Ocean, Mediterranean Sea, northwestern and eastern Atlantic Ocean, Gulf of Mexico, and Antarctic Ocean. About 108 species of dicyemids have so far been reported in at least 40 species of benthic cephalopods of the world. The majority of the dicyemid species studied were found to be host specific. Typically, 2 or more species of dicyemids live in each host species or each host individual (Furuya, 1999). Dicyemids insert the distinct anterior region termed a “calotte” into renal tubules or crypts of the renal appendages or attach to the surface of the renal appendages of the host (Furuya et al., 2003a).

In Japan, the first record of dicyemids was published by Nouvel and Nakao (1938). They described Dicyema misakiense Nouvel & Nakao, 1938 from Octopus vulgaris Lamarck, 1798, and Dicyema orientale Nouvel & Nakao, 1938 from Sepioteuthis lessoniana Lesson, 1830. Nouvel (1947) later described Dicyema acuticephalum Nouvel, 1947 from O. vulgaris and identified a dicyemid species from Sepia esculenta Hoyle, 1885 as Pseudicyema truncatum Whitman, 1883, which had been described earlier in Europe. Subsequently, 2 dicyemid species, Dicyema japonicum Furuya & Tsuneki, 1992 and Dicyema clavatum Furuya & Koshida, 1992, were described from O. vulgaris and Callistoctopus minor (Sasaki, 1920), respectively (Furuya et al., 1992a). Furuya (1999) also reported 14 new species of dicyemid from 6 cephalopod species caught off the coasts of Japan, i.e., Amphioctopus fangsiao (d’Orbigny, 1840), C. minor, Octopus hongkongensis Hoyle, 1885, Enteroctopus dofleini (Wülker, 1910), S. esculenta, and Sepia lycidas Gray, 1849. More recently, a new dicyemid was described from S. lessoniana by Furuya and Tsuneki (2005) and 9 new dicyemids from Amphioctopus kagoshimensis (Ortmann, 1888), Amphioctopus areolatus (de Haan, 1840), A. fangsiao (d’Orbigny, 1840), and Sepia latimanus Quoy & Gaimard, 1811 by Furuya (2005, 2006a, 2006b, 2006c).

Hochberg (1982, 1990) reviewed genera of cephalopod hosts that harbored dicyemids. In Sepiioidea, dicyemids were detected from 5 genera, Rondeletiola, Rossia, Sepieta, Sepiola, and Sepia, but not from Sepiella. Recently, Furuya and Tsuneki (2003) have recorded the dicyemids in Sepiella japonica Sasaki, 1929. In present paper, I described this new species from S. japonica collected from the eastern area of Inland Sea, Osaka Bay, and Kii Strait in Japan.

MATERIALS AND METHODS

In this study, 43 individuals of S. japonica were examined for dicyemids from 1989 to 2006. Host specimens were obtained from fishermen, who collected them in Osaka Bay (off Akashi, Hyogo and Izumi-Sano, Osaka), Inland Sea (off Ushimado, Okayama), and Kii Strait (off Saigasagi, Wakayama), Honshu, Japan. Small pieces of the renal organ with attached dicyemids were removed and smeared on glass microscope slides. The smears were fixed immediately in Bouin’s fluid for 24 hr and then stored in 70% ethyl alcohol. Most of them were stained in Ehrlich’s hematoxylin and counterstained in eosin. Stained smears were mounted with Entellan (Merck, Germany). Dicyemids were observed with a light microscope (Olympus BX-51, Olympus, Tokyo, Japan) at magnifications up to ×2,000. Measurements and drawings were made with the aid of an ocular micrometer and a drawing tube (Olympus U-DA), respectively.

The terminology for cell names used in the description of infusoriform larvae is based on Nouvel (1948), Short and Damian (1966), Furuya et al. (1992b, 1997), and Furuya (1999).

Specimens of the dicyemids are deposited in the Osaka University Museum, Toyonaka, Osaka, Japan (OUM) and in the author’s collection. The cuttlefish specimen harboring the dicyemids is deposited in the OUM.

DESCRIPTION

Dicyema sepiellae n. sp. (Figs. 1–3; Tables I, II)

Diagnosis: Large dicyemid; body lengths reaching 4,000 μm. Calotte shape conical. Vermiform stages with 24–29 peripheral cells; 4 propolar cells + 4 metapolar cells + 2 parapolar cells + 14–19 trunk cells. Infusoriform embryos with 39 cells; refringent bodies solid; 2 nuclei present in each urn cell.

Nematogens (Figs. 1a, 2a, c, 3): Body length 500–4,000 μm, width 30–60 μm; widest in region of diapolar; trunk width mostly uniform. Peripheral cell number 24–29 (Table II, Fig. 3): 4 propolar cells + 4 metapolar cells + 2 parapolar cells + 12–17 diapolar cells + 2 uropolar cells. Calotte conical in shape, rounded anteriorly; cilia on calotte about 6 μm long, oriented anteriorly. Propolar cells and their nuclei smaller than metapolar cells and their nuclei, respectively. Propolar cells occupy anterior 50–60% of calotte length when viewed laterally (Fig. 1a, b). Cytoplasm of propolar cells more darkly stained by hematoxylin than cytoplasm of other peripheral cells. Axial cell cylindrical, rounded anteriorly; cell extends forward to middle of metapolar cells. About 20 vermiform embryos present in an axial cell of large individuals.

Vermiform embryos (Figs. 1c, 2e, f, 3): Full-grown vermiform embryos range from 150 to 180 μm in length, from 15 to 20 μm in width. Peripheral cell number 24–29 (Table II, Fig. 3); trunk cells arranged in opposed pairs. Anterior end of calotte bluntly pointed. Axial cell pointed anteriorly; extends to base of propolar cells; nucleus usually located...
**FIGURE 1.** *Dicyema sepiellae* n. sp., photographs of syntype specimens on slide OUM-00014. (a) Anterior region of nematogen. (b) Anterior region of rhombogen. (c) Vermiform embryos within axial cell. (d) Infusorigen. (e, f) Infusiform embryos within axial cell: (e) optical horizontal section; (f) optical sagittal section. Scale bars represent 5 μm. Abbreviations: A, apical cell; AG, agamete; AX, axial cell; C, couvercle cell; CL, calotte; D, diapolar cell; DC, dorsal caudal cell; DI, dorsal internal cell; DV, developing vermiform embryo; M, metapolar cell; NI, nucleus of the axial cell of infusorigen; O, oogonium; P, propolar cell; PA, parapolar cell; PO, primary oocyte; R, refringent body; S, spermatogonium; SP, sperm; U, urn cell; UC, urn cavity; UP, uropolar cell; VC, ventral caudal cell; V1, first ventral cell.

in center of axial cell. Axial cell of full-grown embryos often with as many as 2–5 agametes.

**Rhombogens** (Figs. 1b, 2h, d, 3): Body similar in length to nematogens, length 500–4,000 μm, width 30–60 μm. Peripheral cell number typically 24–29 (Table II, Fig. 3). Calotte conical, rounded anteriorly. Axial cell shape and anterior extent similar to nematogens. From 2 to 5 infusorigens present in axial cell of each parent individual. About 30 infusiform embryos present in an axial cell of large individuals. Accessory nuclei usually present in trunk cells (Fig. 2d).

**Infusorigens** (Fig. 1d; n = 20): Mature infusorigens small-sized; composed of 6–10 (mode 7) external cells (oogonia and primary oocytes) + 3–6 (mode 4) internal cells (spermatogonia, primary spermatocytes, and secondary spermatocytes) + 4–12 (mode 6) spermatozoa. Mean diameter of fertilized eggs, 17.1 μm; that of spermatozoa, 3.8 μm. Axial cell round or ovoid, range in diameter 20–36 μm.

**Infusiform embryos** (Figs. 1e, f, 2g–i; n = 20): Full-grown embryos large, average 36.9 ± 1.5 μm in length (excluding cilia; mean ± SD); length–width–height ratio 1.0:0.75:0.70; shape ovoid, bluntly rounded.

**FIGURE 2.** *Dicyema sepiellae* n. sp., drawn from syntype specimens on slide OUM-00014. (a, b) Vermiform stages, entire: (a) nematogen; (b) rhombogen. (c) Anterior region of nematogen. (d) Anterior region of rhombogen. (e, f) Vermiform embryos within the axial cell: (e) cilia omitted; (f) optical section. (g–i) Infusiform embryos: (g) dorsal view (cilia omitted); (h) ventral view (cilia omitted); (i) sagittal section. Abbreviations: A, apical cell; AG, agamete; AL, anterior lateral cell; AN, accessory nucleus; AX, axial cell; C, couvercle cell; CA, capsule cell; CL, calotte; D, diapolar cell; DC, dorsal caudal cell; DI, dorsal internal cell; DV, developing vermiform embryo; E, enveloping cell; G, germinal cell; L, lateral cell; LC, lateral caudal cell; M, metapolar cell; MD, median dorsal cell; P, propolar cell; PA, parapolar cell; PD, paired dorsal cell; PVL, posteroventral lateral cell; R, refringent body; U, urn cell; UC, urn cavity; UP, uropolar cell; V, vermiform embryo; VC, ventral caudal cell; V1, ventral internal cell; V1, first ventral cell; V2, second ventral cell; V3, third ventral cell.
FIGURE 3. Frequency distribution of trunk peripheral cell numbers in vermiform individuals of *D. sepiellae* (n = 150).

to pointed posteriorly; cilia at posterior end 7 μm long. Refractive bodi­ies present, solid; occupy anterior 30% of embryo length when viewed laterally (Fig. 1f). Cilia project from ventral internal cells into urn cavity (Fig. 2i). Capsule cells contain small granules. Mature embryos with 39 cells: 35 somatic + 4 germinal cells. Somatic cells of several types present: external cells that cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surfaces (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells), external cells with refringent bodi­es (2 apical cells); external cells without cilia (1 couvercle cell + 2 anterior lateral cells + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 2 nuclei. All somatic nuclei appear pycnotic in mature infusoriform embryos.

**Taxonomic summary**

Type host: Sepiella japonica Sasaki, 1929 (Mollusca; Cephalopoda: Decapodidae), male (mature), 150 mm ML. (OUM-MO-00012).

Other hosts: None

Type locality: Japan, Honshu, Osaka Prefecture, Osaka Bay, off Izumi-Sano, 34°30′N, 135°10′E, 30 m.

Additional localities: Japan, Honshu, Hyogo Prefecture, Osaka Bay, off Akashi, Okayama Prefecture, Inland Sea, off Ushimado, and Wa­kayama Prefecture, Kii Strait, off Saigasakı.

Site of infection: Anterior ends (calottes) inserted into crypts of the renal appendages within the renal sacs.

Prevalence: In 28 of 43 hosts examined.

Specimens deposited: A syntype slide deposited in Osaka, Japan (OUM-ME-00014).

Etymology: The species name refers to the host cuttlefish.

**Remarks**


*Dicryema madrasensis* and *D. nouveli* were described from Octopus sp. and Loligo duvauceli d’Orbigny, 1839, respectively, in the eastern area of Bay of Bengal (Kalavati et al., 1984). *Dicryema sepiellae* is distinguishable from *D. madrasensis* in the number of urn cell nuclei of infusoriform embryos (2 vs. 1). *Dicryema sepiellae* is also distinguishable from *D. nouveli* in the number of agametes at eclosion of vermiform embryos (2–5 vs. 5–8) and the size of infusoriform embryos (37 μm vs. 75–90 μm).

*Dicryema lycidocecum* and *D. rhadinum* were described from *S. ly­cidas* and *S. esculenta*, respectively, in the Japanese waters (Furuya, 1999). *Dicryema sepiellae* is easily distinguishable from these two spe­cies in the cellular composition and the number of cells of infusoriform embryos. The infusoriform embryos of *D. sepiellae* are of the typical type among embryos consisting of 39 cells (Furuya et al., 2004). How­ever, the infusoriform embryos of *D. lycidocecum* and *D. rhadinum* consist of 37 cells that lack the anterior lateral cells (Furuya, 1999).

The size and number of infusorigens are diagnostic characteristics of the dicyemid species (Furuya et al., 1999). There is a negative curvi­linear relationship between the number of infusorigens per rhombogen and the number of gametes (egg-line and sperm-line cells) per infuso­ragen (Furuya et al., 2003b; Furuya, 2005, 2006a, 2006b, 2006c). Irrespective of genera, 4 distinct groups of reproductive strategy are clas­sified within the dicyemid species: (1) rhombogens form a relatively small number of medium- to large-sized infusorigens (less than 5) and produce a relatively large number of gametes (more than 20) per infus­orgen; (2) rhombogens produce a large number of infusorigens (more than 5), each of which has at most 20 gametes; (3) rhombogens produce large numbers of large-sized infusorigens with a large number of gam­etes; and (4) rhombogens form a relatively small number of small-sized infusorigens with a few gametes (at most 10). Rhombogens of *D. se­piellae* have a medium number of small-sized infusorigens, and thus this species is located between the first type and the second type.

**DISCUSSION**

Six species of *Sepiella* have been reported from the eastern Atlantic Ocean, Indian Ocean, and western Pacific Ocean (Khromov et al., 1998; Lu, 1998). The most distinctive feature of this genus is the presence of a large subcutaneous gland that opens by a pore at the posterior end of the mantle between the fins. *Sepiella japonica* inhabits areas from the coastal waters down to continental shelf and has a relatively wide distribution in the northern Pacific Ocean from the Philippines to Russia (Nesis, 1982).

In the present study, a new dicyemid species, *D. sepiellae*, was found in 28 of 43 examined individuals of *S. japonica* caught in the eastern area of the Inland Sea, Osaka Bay, and Kii Strait in Japan. These data revealed geographical variations in prevalence, 47.8% (11/23) in Osaka Bay, 40.0% (2/5) in Kii Strait, and 100% (15/15) in the Inland Sea. There is a direct relationship between host size and dicyemid occurrence (Furuya et al., 1992a), i.e., smaller or younger cephalopods of a host species generally do not harbor dicyemids. Depending on the species of host cephalopod, there is probably a specific size at which the species is infected with dicyemids. For instance, dicyemids are not detected in small individuals of the medium­sized octopus, *O. vulgaris*, which is similar in size to the small­sized octopus, *A. fangsiao*, which harbors dicyemids. In *S. les­soniana*, however, the relationship between the host size and dicyemid presence differs on the basis of geographical location (Furuya and Tsuneki, 2005). In *S. japonica*, the 15 examined individuals that harbored no dicyemids were medium and large sized and the absence of dicyemids in them cannot be attributed to host size, but to geographical location.
Chromidinid ciliates were found in 2 cuttlefishes of *Sepia japonica*. Chromidinids, as well as dicyemids, exist in folds of the renal appendage and the pancreatic appendage. Co-occurrence of both dicyemids and chromidinids was occasionally reported in *Eledone cirrhosa* (Lamarck, 1798), *Octopus salutii* Véran, 1839, *Todarodes pacificus* (Steenstrup, 1880), *Sepia kohiensis* Hoyle, 1885, *Sepia peterseni* Appellöf, 1886, and *Euprymna morsei* (Verrill, 1881) (Nouvél, 1937; Furuya and Hochberg, 1999; Furuya, Ota et al., 2004). In contrast to dicyemids, chromidinids characteristically infect oceanic cephalopods (epi- and mesopelagic squids and octopods). Infection of benthonic or epibenthic hosts has been occasionally reported, but in all cases the chromidinids were found only in octopods that produce planktonic larvae, i.e., *O. salutii*, *O. vulgaris*, *Scapharopus unicirrhus* (Chiaie, 1839), and *E. cirrhosa* or in cuttlefishes whose young feed in surface waters, i.e., *Sepia elegans* Blainville, 1827, *S.

### Table I. Dicyemid species from the cuttlefish *Sepiella japonica*.

<table>
<thead>
<tr>
<th>Host no.</th>
<th>ML* (cm)</th>
<th>Sex†</th>
<th>Locality‡</th>
<th>Date of examination</th>
<th>Dicyemids</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ62</td>
<td>17.8</td>
<td>♂</td>
<td>1</td>
<td>3 April 1989</td>
<td>None</td>
</tr>
<tr>
<td>SJ1143</td>
<td>13.3</td>
<td>♂</td>
<td>2</td>
<td>18 January 1993</td>
<td>None</td>
</tr>
<tr>
<td>SJ225</td>
<td>12.6</td>
<td>♂</td>
<td>2</td>
<td>22 November 1994</td>
<td>None</td>
</tr>
<tr>
<td>SJ292</td>
<td>17.2</td>
<td>♂</td>
<td>3</td>
<td>28 March 1995</td>
<td>None</td>
</tr>
<tr>
<td>SJ293</td>
<td>19.4</td>
<td>♂</td>
<td>3</td>
<td>28 March 1995</td>
<td><em>Dicyema sepiellae</em></td>
</tr>
<tr>
<td>SJ294</td>
<td>17.7</td>
<td>♂</td>
<td>3</td>
<td>28 March 1995</td>
<td>None</td>
</tr>
<tr>
<td>SJ295</td>
<td>16.9</td>
<td>♂</td>
<td>3</td>
<td>28 March 1995</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ496</td>
<td>14.3</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ497</td>
<td>14.7</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ498</td>
<td>15.2</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ499</td>
<td>14.0</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ500</td>
<td>13.3</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ501</td>
<td>13.9</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ502</td>
<td>13.8</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ503</td>
<td>13.6</td>
<td>♂</td>
<td>2</td>
<td>2 April 2000</td>
<td>None</td>
</tr>
<tr>
<td>SJ600</td>
<td>18.3</td>
<td>♂</td>
<td>4</td>
<td>8 June 2000</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ601</td>
<td>17.1</td>
<td>♂</td>
<td>4</td>
<td>8 June 2000</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ602</td>
<td>16.5</td>
<td>♂</td>
<td>4</td>
<td>8 June 2000</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ603</td>
<td>16.8</td>
<td>♂</td>
<td>4</td>
<td>8 June 2000</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ604</td>
<td>19.8</td>
<td>♂</td>
<td>4</td>
<td>8 June 2000</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ847</td>
<td>15.7</td>
<td>♂</td>
<td>3</td>
<td>20 February 2003</td>
<td>None</td>
</tr>
<tr>
<td>SJ1453</td>
<td>15.7</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1454</td>
<td>16.2</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1455</td>
<td>15.9</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1456</td>
<td>14.3</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1457</td>
<td>15.5</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1458</td>
<td>15.3</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1459</td>
<td>16.2</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1460</td>
<td>15.1</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1461</td>
<td>14.8</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1462</td>
<td>15.6</td>
<td>♂</td>
<td>4</td>
<td>11 May 2004</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1573§</td>
<td>15.0</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1574</td>
<td>13.7</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1575</td>
<td>17.5</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1576</td>
<td>12.8</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1577</td>
<td>13.6</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1578</td>
<td>13.3</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1579</td>
<td>15.4</td>
<td>♂</td>
<td>1</td>
<td>3 April 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1766</td>
<td>14.5</td>
<td>♂</td>
<td>1</td>
<td>7 December 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1767</td>
<td>12.5</td>
<td>♂</td>
<td>1</td>
<td>7 December 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1768</td>
<td>14.6</td>
<td>♂</td>
<td>1</td>
<td>7 December 2006</td>
<td>None</td>
</tr>
<tr>
<td>SJ1769</td>
<td>11.7</td>
<td>♂</td>
<td>1</td>
<td>7 December 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
<tr>
<td>SJ1770</td>
<td>12.5</td>
<td>♂</td>
<td>1</td>
<td>7 December 2006</td>
<td><em>D. sepiellae</em></td>
</tr>
</tbody>
</table>

* Dorsal mantle length.
† All specimens were matured.
‡ 1, Osaka Bay (off Izumi-Sano); 2, Osaka Bay (off Akashi); 3, Kii Strait (off Saigasaki); 4, Inland Sea (off Ushimado).
§ The host (symbiotype, OUM-MO-00012) for the syntype of *D. sepiellae*.
¶ Chromidinid ciliates were found found.

symbiont. A total of 30 species of cephalopods representing 22 genera harbors chromidinids (Hochberg, 1982, 1983, 1990; Furuya, Ota et al., 2004). In contrast to dicyemids, chromidinids are the most frequently encountered...
pia orbignyana Féruccac, 1826, and Sepiola rondeleti Leach, 1834 (Hochberg, 1983). The larvae of S. japonica are planktonic (pers. obs.); thus, it is possible that the planktonic larvae are infected with chromidinids before they settle to the bottom and pick up dicyemids.

Most dicyemid species are composed of a constant number of calotte and trunk peripheral cells in vermiform stages (Furuya et al., 2007). However, intraspecific variations have been observed in the number of trunk peripheral cells of several dicyemid species, e.g., *Dicyema bilobum* Couch & Short, 1964; *Dicyema benthocotopi* Hochberg & Short, 1970, *D. madrasensis*, *D. lycideaecum*, *D. rhadinum*, *Dicyema erythrum* Furuya, 1999, and *Dicyema koshidai*, Furuya & Tsuneki, 2005 (Couch and Short, 1964; Hochberg and Short, 1970; Kalavati et al., 1984; Furuya, 1999; Furuya and Tsuneki, 2005). *Dicyema sepiellae* also showed a range of variation in cell numbers of trunk peripheral cells. In this case, the trunk peripheral cells usually varied from 5 to 8 in number. The extent of variations in trunk peripheral cell numbers was expressed by the coefficient of variation (CV) (Furuya et al., 2007). Values of CV were classified into 3 patterns, i.e., no statistical variations (CV < 0.05), moderate variations (0.05–0.10), and large variations (0.10 < CV). More than 70% of species had no variations and the rest showed moderate variations. Only 3 species, *D. madrasensis*, *D. benthocotopi*, and *Dicyemennea canadensis* Furuya, Hochberg & Short, 2002, had large variations (Furuya et al., 2007). *Dicyema sepiellae* has a relatively large variation of the trunk peripheral cells that range from 16 to 21. However, the value of CV is 0.54 and, therefore, possesses moderate variation. This is due to the particular peripheral cell distribution that has a relatively large range with similar frequencies; approximately 30 individuals are found in each 18–21 trunk peripheral cells (Fig. 3).

In contrast to vermiforms, in infusoriform embryos of *D. sepiellae* intraspecific variations are not observed in the number of somatic cells. Generally, in infusoriform embryos, such intraspecific variations are rarely found in the number of somatic cells. Differences in cell numbers among vermiform embryos of the same species are attributed to the number of terminal divisions in particular cell lineages (Furuya et al., 1994, 2001). They cause interspecific variations in trunk peripheral cell numbers. This suggests that the development of infusoriform embryos may be more highly programmed than that of vermiform embryos.

**ACKNOWLEDGMENTS**

I express my thanks Dr. Kazuhiko Tsuneki of Osaka University for his critical reading of the manuscript and valuable advice. I also thank Masaaki Yoshida and Kazutaka Suzuki of Osaka University for their assistance in collecting host sepiolids and preparing smears. This study was supported by grants from the Japan Society for the Promotion of Science (research grants 14540645 and 18570087).

**LITERATURE CITED**


**TABLE II.** Number of peripheral cells in new species of dicyemid.

<table>
<thead>
<tr>
<th>Cell no.</th>
<th>Vermiform embryos</th>
<th>Nematogens</th>
<th>Rhombogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>25</td>
<td>6</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>26</td>
<td>12</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>27</td>
<td>11</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>28</td>
<td>8</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>29</td>
<td>11</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

**CITED LITERATURE**


AN EXPLORATION OF DIVERSITY AMONG THE OSTERTAGIINAE (NEMATODA: TRICHOSTRONGYLOIDEA) IN Ungulates FROM SUB-SAHARAN AFRICA WITH A PROPOSAL FOR A NEW GENUS

Eric P. Hoberg, Arthur Abrams, and Vanessa O. Ezenwa

U.S. National Parasite Collection and Animal Parasitic Diseases Laboratory, Agricultural Research Service, United States Department of Agriculture, BARC East 1180, 10300 Baltimore Avenue, Beltsville, Maryland 20705. e-mail: eric.hoberg@ars.usda.gov

ABSTRACT: Abomasal nematodes (Ostertagiinae: Trichostrongyloidea) representing a previously unrecognized genus and species are reported in African buffalo (Syncerus caffer caffer) from Kenya, Uganda, and South Africa. Africanastrangylosis buceros gen. nov. et sp. nov. is characterized by a symmetrical tapering synloph in the cervical region and a maximum of 60 ridges in males and females. Bursal structure is 2–2–1, with subequal Rays 4/5, massive Rays 8, and Rays 9/10, and a massive dorsal lobe that is reduced in length, ventrally and dorsally inflated, and positioned ventral to externodorsal rays. Spicules are tripartite, and the gubernaculum is broadly alate in the anterior. A proconus is present. Among ostertagiines with a 2–2–1 bursa (Cervicoaprastrongylosis, Hyostrongylosis, Mazamastrongylosis, Sarvaria, Spiculostrongylosis, and Teladorsagia) specimens of Africanastrangylosis are differentiated from respective genera based on the structure of the cervical synloph, patterns of dorsal, externodorsal, lateral, and ventral rays, and configuration of the genital cone, gubernaculum, and spicules. Among 13 genera of the Ostertagiinae in the global fauna, 3 are entirely limited in distribution to Africa, including Africanastrangylosis, Longistromylosis, and Pseudomarshallagia. Another 5 genera including Cervicoaprastrongylosis, Hyostrongylosis, Marshallagia, Ostertagia, and Teladorsagia are represented as mosaics, with diversity centered in Eurasia or the Holarctic. Genera not represented in the African fauna include Camelostromylosis among Caprinae and some Antelopeinae from Eurasia, Mazamastrongylosis and Spiculostrongylosis in Cervidae from the Holarctic and Eurasia, respectively, Orloffia in Cervidae and Bovidae from the Holarctic, and Sarvaria among Tragulidae and Bovinae in southern Asia. The diverse nature of the ostertagiine fauna, with a disproportionate number of endemic genera relative to other regions of the northern hemisphere, may reflect the timing of episodic expansion events for artiodactyls into Africa from Eurasia during the Tertiary and Quaternary.

Ostertagiinae nematodes represent a monophyletic group within the Trichostrongyloidea with a primary geographic distribution centered in Eurasia and the Holarctic Region (Durette-Desset, 1985; Lichtenfels and Hoberg, 1993; Hoberg and Lichtenfels 1994; Durette-Desset et al., 1999). Across Africa, diversity for species of Ostertagiinae, primarily abomasal nematodes among artiodactyls, appears relatively limited. Extensive survey and inventory over the past century among Bovinae, Antelopinae, and other pecoran artiodactyls have revealed relatively few endemic species, except for those in the genus Longistromylosis Le Roux 1931, and among the otherwise geographically widespread Ostertagia Ransom, 1907 (e.g., Mönnig, 1932; Round, 1968; Gibbons, 1977; Gibbons and Khalil, 1980; Boomker and Durette-Desset, 2003).

Among the diverse ungulate fauna characteristic of sub-Saharan Africa, there are relatively few reports of ostertagiine or other abomasal parasites in African buffalo (Syncerus caffer (Sparrman)). These have been limited to Longistromylosis mayeri Le Roux, 1931; species of Ostertagia Ransom, 1907 from Uganda (Dinnik et al., 1963; Bwangamoi, 1968; Ashworthius lerouxi Diaouré, 1964 from Congo (Diaouré, 1964), Haemonchus bedfordi Le Roux, 1929 from Uganda (Dinnik et al., 1963) and South Africa (Le Roux, 1929; Gøllepp, 1961); Haemonchus contortus (Rudolphi, 1803) from Kenya and South Africa (Curson, 1928; Ezenwa, 2003); and Haemonchus placei Place, 1893 from Kenya (Ezenwa, 2003).

Surveys in the late 1960s and more recent collections of wild African buffalo, or Cape buffalo (Syncerus caffer caffer (Sparrman)) from Uganda, Kenya, and South Africa have now revealed a previously unrecognized genus and undescribed species of ostertagiine nematode. We provide a generic diagnosis and the first description of these ostertagiines. Concurrently, we explore (1) the limits and criteria for genera within the Ostertagiinae (see also Hoberg and Abrams, 2007), and (2) aspects of the structure of the ostertagiine fauna among African ungulates. Faunal discovery, inventory, and characterization of biodiversity for complex faunas are cornerstones necessary for understanding, documenting, and predicting biotic responses to ecological perturbation under an expanding regime of global climate change (Brooks and Hoberg, 2000, 2006; Hoberg and Brooks, 2008).

MATERIALS AND METHODS

Specimens examined

Abomasal nematodes in African buffalo or Cape buffalo, S. caffer caffer (Sparrman), were collected from widely separated localities in Africa (Table I). Specimens in 2 hosts from localities in Uganda (Field 11 at Anaka Village, West Acholi District and 33 at Queen Elizabeth National Park, Toro District) were collected by J. Bindernagle during 1964–1967 and originally studied at the U.S. National Parasite Collection in the late 1960s by W. W. Becklund and M. L. Walker, who noted the distinctive morphology of these nematodes. Additional specimens were collected by 1 of us (V,E.), from 1 adult female host (Field BN1-200 on 2 February 2000) at the Mpala Ranch, Laikipia, Kenya and 2 subadult females (Field B13 on 29 May 2006; C72 on 30 May 2006) at Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. All specimens were archived permanently at the U.S. National Parasite Collection and stored in a mixture of 70% ethanol, 5% glycerin, and 3% formalin.

Other specimens examined

Specimens and sources of other species of ostertagiine nematodes used in comparative morphological studies are listed (Table I).

Microscopy

Nematodes were prepared as temporary whole mounts cleared in phenol–alcohol (80 parts melted phenol crystals and 20 parts absolute ethanol) and examined with interference contrast microscopy. The synloph was studied in whole mounts with particular attention to the pattern of ridge systems in the cervical zone and their extent posterior in males and females consistent with prior studies among the ostertagiines (Lich-
FIGURE 1. Africanastrongylus buceros gen. nov. et sp. nov., showing line drawings of the cervical synlophe in ventral and right lateral views of a female paratype (USNPC 66322). The excretory pore (exp) is on the ventralmost ridge consistent with a Type-B ventral pattern. Laterally the synlophe describes a Type-I pattern; note relative positions for the subventral gland orifices (svgo), cervical papillae (cp), and esophageal–intestinal junction (ei). Orientation is indicated by v = ventral, d = dorsal, and l = lateral.

tenfels et al., 1988). Thick transverse sections were hand cut with a cataract knife and mounted in glycerin jelly; methods were based on those developed by Durette-Desset (1983). Sections were used to count the number of ridges in a single male and female at the esophageal–intestinal junction (EIJ), 1/4, midbody, and 3/4 of total body length as determined from the anterior. Sectioning was completed for only 2 specimens due to the limited number of worms that had been collected. Additional counts of ridges were based on reconstructions from whole mounted specimens.

The male specimens were evaluated on the basis of the copulatory bursa, spicules, and genital cone. Bursal ray patterns were determined and described under the system of Durette-Desset and Chabaud (1981) and Durette-Desset (1983). Papillae of the genital cone and rays of the bursa followed the numbering system of Chabaud et al. (1970). The structure of the ovjectors was evaluated in the context of recent definitions and descriptions among related nematodes (Lichtenfels et al., 2003). All measurements are given in micrometers, unless specified otherwise. In the description and tables the sample size (n =) is followed by the range and mean ± 1 SD in parentheses.

Host nomenclature

Taxonomy for hosts follows Wilson and Reeder (1993) in all of the text and tables. Host listings have been modified from those reported in the original literature to reflect current usage and understanding of ungulate taxonomy.

RESULTS

Field collections for survey of helminth diversity in ungulates from eastern and southern Africa revealed the occurrence of abomasal nematodes. Wild Cape buffalo from the West Acholi District of Uganda and the Queen Elizabeth National Park, Toro District, Uganda, Laikipia, Kenya and the Hluhluwe-iMfolozi Park in KwaZulu-Natal, South Africa were naturally infected with a previously undiagnosed genus and undescribed species of ostertagiine with a 2–2–1 bursal form.

DESCRIPTION

Africanastrongylus gen. nov.

Diagnosis: Trichostrongylidae. Small uncoiled nematodes with well-developed bilateral tapering synlophe, miniscule thornlike cervical papillae and prominent esophageal–intestinal valve in males and females. Males monomorphic. Bursal structure 2–2–1, symmetrical, membrane lacking discrete fields of bosses. Rays 2/3 curved, divergent through midlength, convergent distally; Rays 4/5 parallel throughout length, highly divergent distally at tips; relatively narrow, subequal with Rays 4 ≤ Rays 5. Accessory bursal membrane simple, bilobed, containing filamentous papillae "7." Rays 8, massive curved mediad. Dorsal lobe massive, reduced in length, laterally and dorsally inflated, positioned ventral to externodorsal rays. Dorsal ray, or Rays 9/10, massive with stout base proximally, positioned ventral relative to Rays 8. Genital cone with weakly developed proconus; paired "0" papillae miniscule, positioned posterior to proconus on ventral aspect of cloaca. Cloaca with telamon and cuticularized support structures at orifice. Spicules alate, trifurcate, subequal. Gubernaculum present, proximal. Females amphidelphic with transverse vulva in posterior quarter lacking cuticular fans or inflations.

Taxonomic summary

Type species: Africanastrongylus buceros gen. nov. et sp. nov. Host: African buffalo, S. caffer (Sparrman).
TABLE I. Specimens of *Africanastrongylus buceros* gen. nov. et sp. nov. and other ostertagiines examined.

<table>
<thead>
<tr>
<th>Accession*</th>
<th>Field†</th>
<th>Species</th>
<th>Host</th>
<th>Locality</th>
<th>δ‡</th>
<th>V‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>USNPC 995455</td>
<td>11</td>
<td><em>Africanastrongylus buceros</em></td>
<td>Syncerus caffer</td>
<td>Uganda</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>USNPC 995468</td>
<td>33</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>Uganda</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>USNPC 86939</td>
<td>33</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>Uganda</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>USNPC 99551</td>
<td>33</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>Uganda</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>USNPC 66322.02</td>
<td>11</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>Uganda</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>USNPC 99547</td>
<td>BNI-200</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>Kenya</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>USNPC 99548</td>
<td>B13</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>South Africa</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>USNPC 99549</td>
<td>C72</td>
<td>A. buceros</td>
<td>S. caffer</td>
<td>South Africa</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

Other species of ostertagiines

<table>
<thead>
<tr>
<th>Accession*</th>
<th>Field†</th>
<th>Species</th>
<th>Host</th>
<th>Locality</th>
<th>δ‡</th>
<th>V‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>OHC 23666[1]</td>
<td>—</td>
<td>Teladorsagia hamata</td>
<td>Antidorcas marsupialis</td>
<td>South Africa</td>
<td>Not available</td>
<td>—</td>
</tr>
<tr>
<td>UP T-2053#</td>
<td>—</td>
<td>T. hamata</td>
<td>A. marsupialis</td>
<td>South Africa</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>USNPC 81213</td>
<td>—</td>
<td>Longistrongylus curvicipiuncul[†]</td>
<td>Ovis aries</td>
<td>Texas, USA</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>USNPC 77484</td>
<td>—</td>
<td>Longistrongylus sabie*[‡]</td>
<td>Aepyceros melampus</td>
<td>South Africa</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>USNPC 66325</td>
<td>—</td>
<td>Longistrongylus schrenki[††]</td>
<td>Ourebia ourebi</td>
<td>Uganda</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>USNPC 66323</td>
<td>—</td>
<td>L. schrenki</td>
<td>Kobus kob</td>
<td>Uganda</td>
<td>2</td>
<td>—</td>
</tr>
</tbody>
</table>

* Collection numbers from the U.S. National Parasite Collection (USNPC), Onderstepoort Helminthological Collection (OHC), the Natural History Museum, London (BNH), and University of Pretoria (UP).
† Field number at time of original collection.
‡ Number of male and female specimens examined.
§ Holotype male and allotype female.
|| Paratypes.
# Syntype, from original collection by H. Ö. Mönning, on 1 August 1931 at Houtkraal Farm, Karoo, Cape Province, derived from host following transport to Pretoria Zoo (Mönning, 1932).
† Longistrongylus curvicipiuncul represents a species previously referred to Bigalkenema; material examined represents an introduced population in western Texas and was from an experimental infection in domestic sheep based on larvae recovered from *Oryx bieta* (data from Craig, 1993).
** Longistrongylus sabie represents a species previously referred to *Bigalkenema*.
†† Longistrongylus schrenki represents the species previously referred to *Kobasinaema*.

orifices (SVGO), and situated slightly posterior to level of excretory pore (EXP). Cuticular ornamentation at level of EXP lacking. Esophagus with prominent valve at esophageal–intestinal junction (EIJ). Males and females monomorphic.

Bilaterally symmetrical synlophe similar in males and females. Cervical zone (n = 3) with 29–30 ridges extending to level of cephalic expansion increasing to (n = 5) 50–58 ridges at the EIJ. Laterally, synlophe tapers, consistent with Type I pattern; 1–4 pairs of ridges terminate on lateralmost ridge anterior to EIJ; cervical papillae adjacent to lateralmost ridges in right and left fields. Ventral/dorsal ridge systems parallel, consistent with Type B pattern. Sublateral/ventral fields in cervical zone with 2–4 continuous ridges (n = 5) that may assume lateral or ventral orientation; continuous ridges may diverge or remain parallel; 4 of 5 specimens with 2 continuous ridges in sublateral/ventral fields. Lateral ridges and ventral/dorsalmost ridge (n = 3) extend anterior to level of cephalic expansion. Tapering pattern extends (n = 13) 38–61% (52% ± 5.6%) of total length from anterior. In transverse section (1 male and 1 female, respectively) ridges number from the anterior 56 and 51 at EIJ (51–58 based on counts of fields in cervical reconstructions in 3 whole nematodes); 60 and 53 at 1/4; 56 and 46 at midbody; 58 and 49 at 3/4, with initial loss in dorsal/ventral fields; and 44 ridges in the male at a level anterior to the spicules. Synlophe terminates anterior to bursa in males and anus in females. In females, ridges terminate laterally 62–212 and dorsoventrally 102–220 anterior to anus. In males ridges terminate laterally at 75–250 and dorsoventrally at 192–525 anterior to the prebursal papillae.

** Figures 2–7. *Africanastrongylus buceros* gen. nov. et sp. nov., showing structure of synlophe based on photomicrographs of transverse sections in a male (2–4) and a female (5–7) paratype (series USNPC 66322); the general orientation is perpendicular and a gradient in size is not evident, although the lateralmost ridges are miniscule relative to those in adjacent fields. (2) Synlophe in male at esophageal–intestinal junction showing 56 ridges. (3) Synlophe in male at midbody showing 56 ridges. (4) Synlophe at beginning of third quarter in male showing 58 ridges. (5) Synlophe in female at esophageal–intestinal junction showing 51 ridges. (6) Synlophe in female at midbody showing 46 ridges. (7) Synlophe at beginning of third quarter in female showing 49 ridges.
Figures 8–9. *Africanastronylus buceros* gen. nov. et sp. nov., showing cervical and cephalic attributes based on photomicrographs. (8) Cervical zone in ventral view of a male paratype (USNPC 66322) denoting the position of the subventral gland orifices (svgo), cervical papillae (cp), esophageal–intestinal valve (eiv) and esophageal–intestinal junction (eij); note slight bulbous expansion of basal valve and esophagus. (9) Cervical synlophe in a female paratype (USNPC 99549) showing lateral view from near base of cephalic expansion in anterior to base of esophagus in posterior, showing Type-I tapering lateral pattern relative to miniscule lateralmost ridge and cervical papilla (cp).
Figures 10–13. *Africanastrongylus buceros* gen. nov. et sp. nov., cervical and cephalic attributes based on photomicrographs in a female paratype (USNPC 99551). (10) Cephalic extremity in left lateral view. (11) Excretory pore in lateral view in a female specimen, showing absence of ornamentation. (12) Cervical papilla, showing thornlike structure and position relative to the lateralmost ridge and the cervical synlophe. (13) Excretory pore in ventral view in a female specimen, showing absence of ornamentation, and position on ventralmost ridge.
Figures 14–16. *Africanastrongylus buceros* gen. nov. et sp. nov., showing female attributes as depicted in line drawings from paratype specimens (USNPC 99551). (14) Cephalic and cervical zone in left lateral view. (15) Ovijectors in right lateral view (same scale as Figure 14). (16) Tail and anus in left lateral view.
Figures 17–21. *Africanastronylus buceros* gen. nov. et sp. nov., showing structural characters of females based on photomicrographs. (17) Ovijectors in right lateral view of a paratype (USNPC 66322), showing form and relative dimensions of the infundibula (inf, between dotted arrows), sphincters (sp) including the bulblike sphincter-1 (s1) and elongate sphincter-2 (s2), vestibule (ve), and transverse vulva (vu), lacking ornamentation. (18) Vulva, ventral view in a paratype (USNPC 99551), showing transverse structure and adjacent synlophe. (19) Eggs with thin shell *in utero* from a paratype (USNPC 99551). (20) Tail and anus in ventral view of a paratype (USNPC 99548). (21) Tail and anus in lateral view, showing slight bulbous expansion of apex.
proconus; paired “0” papillae with broadened bases proximally, minicule, positioned posterior to proconus on ventral aspect of cloaca. Cloaca with telamon and cuticularized support structures surrounding orifice. Spicules subequal, left spicule longer in 12 of 13 specimens; left (n = 14) 195–246 (212 ± 14.1); right (n = 13) 190–240 (207 ± 15.2). Spicules, alate, narrow, weakly curved, filamentous in lateral view. Spicules triruncate with acutely pointed main process, curved mediad, terminating distally in simple bulbous membrane; ventral and dorsal processes originating at level of “ostertagian window” 76–83% of total length from anterior. Ventral process terminating in triangular barb; dorsal process terminating in narrow rounded point; length of dorsal < ventral process. Gubernaculum alate, shieldlike, concave ventrally, strongly cuticularized, with hornlike extensions on proximal margin, maximum width in dorsoventral view (n = 10) 35–42 (38 ± 1.8), tapering distally; in lateral view weakly S shaped, length (n = 12) 60–82 (67 ± 5.9).

Female: Small nematodes lacking prominent cuticular ornamentation other than synlophe. Total length (n = 18) 9,712–12,610 (11,217 ± 909.5); maximum width 140 attained at level anterior to vulva. Esophagus (n = 18) 775–905 (834 ± 33.7) long; 6.4–8.7% (7.5 ± 0.7) total body length. Valve at EU (17) 92–112 (101 ± 5.8) long, (17) 45–74 (66 ± 8.2) in maximum width. VGVO (17) 285–342 (306 ± 13.9), EXP (18) 305–482 (394 ± 54.6), and CP (18) 320–545 (421 ± 63.5) from cephalic extremity. Ovaries didelphic. Vulva opens as ventral transverse slit (n = 18) 78–85% (82 ± 2.0) of body length from anterior; cuticular inflations and fans absent. Perivulvar pores bilateral, located 195–205 posteralateral to vulva in subventral fields. Anterior infundibulum (n = 11) 185–292 (240 ± 30.8), anterior sphincter, including s1 and s2 (n = 13) 110–192 (149 ± 24). Posterior infundibulum (n = 11) 170–267 (231 ± 26.1), posterior sphincter, including s1 and s2 (n = 13) 98–162 (140 ± 16.7). Vestibule length (n = 13) 70–205 (144 ± 42.3, Total ovicocoon length (n = 10) 795–1,016 (911 ± 77.1). Eggs ovoid, with thin shell (n = 90 in 9 specimens) 62–82 (72 ± 5.9) long by 30–50 (41 ± 4.2) wide, oriented in single rows in anterior and posterior uterine limbs. Tail digitate, weakly inflated distally, lacking prominent annulations adjacent to tip, lacking synlophe, 142–218 (167 ± 19.8) in length.

**Taxonomic summary**

**Host:** African buffalo, S. caffer caffer (Sparrman), type and only known host.

**Localities:** Type locality: In type host at Anaka Village, West Acholi District, Uganda; ca. 02°25′N, 032°10′E. Additional localities: (1) Queen Elizabeth National Park, Toro District, Uganda; ca. 00°19′N, 032°058′E; (2) Mpalas Ranch, Kenya, 00°17′N, 036°52′E; (3) Hluhluwe–iMfolozi Park, KwaZulu-Natal, South Africa, 28°00′S, 031°43′E.

**Specimens:** Holotype male, USNPC 99545, in host No. 11 from type locality. Allotype female, USNPC 99546 in host No. 33 from Queen Elizabeth National Park, Uganda. Paratypes include (1) USNPC 66322.02, 7 males and 7 females in host No. 11; (2) USNPC 99551, 5 males and 2 females in host No. 33; (3) USNPC 99547, 5 females in host BN1–200, from the Mpalas Ranch, Kenya; (4) USNPC 99548, 2 males and 1 female from host B13 at Hluhluwe–iMfolozi Park, South Africa; (5) USNPC 99549, 2 females from host C72 at Hluhluwe–iMfolozi Park, South Africa. Vouchers, USNPC 86939, include 2 female nematodes in host No. 33.

**Etymology:** *Africanastrongylus* is derived from the Latin, *Afer* for African, and from the Greek strongylus for round, denoting a nematode or roundworm from Africa. The species name, *buceros*, is derived from the New Latin and Greek *boukeros* for oxlike horns, denoting the hornlike extensions on the anterior margin of the gubernaculum of the male, and a host in the subfamily Bovinae.

**Remarks**

Hoberg and Lichtenfels (1994) provided the first phylogenetic hypothesis for the monophyly of the Ostertagiinae and its relationship to the Haemonchinae within what was named the Graphidiinae clade. Conclusions from this study were corroborated by Durette-Desset et al. (1999) in demonstrating monophyly for the subclade, but with inclusion of *Graphidium* Railliet and Henry, 1909 as the basal taxon in the Ostertagiinae. In this interpretation, the previously recognized Graphidiinae subclade is equivalent to the proposed Haemonchinae for the sister taxa Ostertagiinae + Haemonchinae (Hoberg and Lichtenfels, 1994; Durette-Desset et al., 1999). We would suggest that inclusion of *Graphidium* remains problematic and is not otherwise compatible with Ostertagiinae.

A morphological and phylogenetic diagnosis for the Ostertagiinae within Trichostrongylidea and relative to their haemonchina sister group includes: (1) tripartite spicule tips; (2) spicules with an “ostertagian window” (a foramenlike structure that is visible at point of tri­furcation for the primary, dorsal and ventral processes of the spicule tips); (3) paired “0” papillae; (4) membranous and simple accessory bursal membrane containing filamentous “7” papillae (modified in minor morphotypes for males of polymorphic species, e.g., Drözdz, 1995); and (5) prominent esophageal valve separating the basal esophagus from the intestine. Additionally, other diagnostic characters exhibit some level of homoplasy, including (1) a vulva with cuticular ornamentation in the form of irregular inflations (Hoberg et al., 1993a); (2) genera characterized by species with polymorphic males (Drözdz, 1995); and (3) certain tapering patterns of the cervical synlophe appear limited to taxa within the subfamily, but overall are not indicative of monophyly (e.g., Lichtenfels et al., 1988; Lichtenfels and Hoberg, 1993; Lichtenfels et al., 1993; Hoberg, 1996). A suite of putative synapomorphies proposed for the Ostertagiinae is not represented in *Graphidium*, and placement of this taxon may require further consideration, but is beyond the scope of the current study.

Currently, a maximum of 12 genera, diagnosed by a suite of attributes outlined above, are represented among the Ostertagiinae. Clarification for generic-level taxonomy of the ostertagiines was recently proposed (Hoberg and Abrams, 2007) in the context of a revision involving *Sar­waria caballeroi* (Chabaud, 1977). The basis for the taxonomy in the current article in part adopts facets of different proposals for synonymies and the validity of certain taxa (e.g., Andreeva, 1956; Drözdz, 1965; Durette-Desset and Chabaud, 1981; Durette-Desset, 1982; Gibbons and Khalil, 1982a; Durette-Desset, 1983, 1985, 1989; Jansen, 1989; Durette-Desset et al., 1999). Fundamental differences in bursal structure and the patterns for Rays 2/3, Rays 4/5, and Rays 6 serve to distinguish larger inclusive groups within the subfamily (Durette-Desset, 1983; Durette-Desset et al., 1999).

Among the Ostertagiinae, 6 genera are characterized by a bursal pattern of 2–1 (2) (Cameleostromylus Orloff, 1933; Longistromylus Le Roux, 1931; Marshallagia Orloff, 1933, Orloffia Drözdz, 1965; Os­tertagia Ransom, 1907; and Pseudomarshallagia (Roetti, 1941)). Alternatively, a 2–2–1 pattern is typical among 6 genera (Cervicapras­trongylus Gibbons and Khalil, 1982; Hyostromylus Hall, 1921; Mazanastrongylus Cameron, 1935; Sarwaria Drözdz, 1965; Spiculop­
Africanastrongylus (Orloff, 1933); and Teladorsagia Andreeva and Satubaldin, 1954); further criteria for, and validity of, *Cervicopneumastrangylus*, *Ma-

*zamastrongylus*, and *Sarwaria*, are reviewed elsewhere (Gibbons and Khalil, 1982b; Lichtenfels et al., 1993; Hoberg, 1996; Lichtenfels et al., 1996; Hoberg and Abrams, 2007).

**Africanastrongylus** gen. nov. is immediately distinguished from all species of *Camelostrongylus, Longistrongylus, Marshallagia, Orloffia, Ostromastacum*, and *Zamastrongylus* by the structure of the 2–2–1 bursa in males (Durette-Desset, 1983); note that the concept for *Camelostrongylus* as proposed by Durette-Desset (1989) that subsumes many species of *Ostertagia* within this genus is not accepted here. Among this group of genera, species of *Longistrongylus* typically possess narrow filamentous spicules and a substantially reduced dorsal lobe and ray (Gibbons, 1972, 1973, 1977) that appear superficially similar to *A. buceros*. Among spe-
cies of *Longistrongylus*, based on descriptions and examination of some representatives (Table I), the reduced lobe is not strongly inflated lat-

erally or dorsally and remains in a dorsal position relative to the exter-
nodorsals or Rays 8. The dorsal ray, although stout, is narrow at the base and the bursa contains numerous and discrete fields of bosses. The “0” papillae are filamentous, of constant diameter, highly divergent, disposed in an arcuate pattern, emanate in bulbous expansions, and are enclosed in a bilobed membrane; a proconus is consistently absent. Further, the accessory bursal membrane in species of *Longistrongylus* is highly reduced or modified, and is not simple or membranous, as seen in *Africanastrongylus*. Females of all species of *Longistrongylus* are characterized by irregular cuticular inflations at the level of the vulva (Hoberg et al., 1993a).


proaches a 2–2–1; however, the distal tips of Rays 4, 5, and 6 are all highly divergent, the dorsal lobe is not strongly defined, and the bases of Rays 8 and the dorsal ray are not massive (Mönig, 1933; Ortlepp, 1963; Gibbons, 1973, 1977).

Among ostertagines with a 2–2–1 bursa, *Africanastrongylus buceros* can be distinguished in the following manner. In *Spiculopteragia* and *Mezamastrongylus*, the absence of a proconus, Rays 4 < 5 in length, robust Rays 4, presence of a unique hood-ridge system in the ventral cervical synlophe, and a liphike and protruding excretory pore (Andreева, 1958; Lichtenfels et al., 1993; Hoberg, 1996; Hoberg and Khrus-
talev, 1996) differentiate these genera from *Africanastrongylus*. Further, among species of *Spiculopteragia*, males are polymorphic and spicules are adorned with prominent fan-like membranes. In *Cervicopneumastrangylus* and *Hyostosstrongylus*, the structure of the parallel cervical synlophe (Type 2 lateral), absence of a proconus, a bursa with Rays 4/5 parallel and not divergent distally, elongate Rays 8, and an elongate dorsal ray (Gibbons and Khalil, 1982a, 1982b; Durette-Desset et al., 1992; Hoberg et al., 1993b) contrast with this suite of attributes in *Africanastrongylus*. Compared to *Teladorsagia*, polymorphism among males, a robust Rays 4, an elongate dorsal ray and lobe, elongate and relatively straight Rays 8, and absence of a proconus (Andreeva, 1956, 1958; Drötzå, 1965, 1995; Hoberg et al., 1999) represent consistent differences relative to *Africanastrongylus*.

**Africanastrongylus buceros** is morphologically similar but distinct from species of *Sarwaria*. Species of both genera are characterized by a tapering, Type 1, lateral synlophe, miniscište but thornlike cervical papillae, and a reduced but laterally inflated dorsal lobe disposed ven-

trally to Rays 8 (Lichtenfels et al., 1996; Hoberg and Abrams, 2007). In *Africanastrongylus*, Rays 2/3 are initially divergent and distally con-

vergent, whereas Rays 4/5 are subequal in length, parallel through their length, and divergent distally; Rays 8 are massive and medially curved, and both a proconus and gubernaculum are present. *Sarwaria*, including *Sarwaria bubalis* (Swar, 1956) and *S. caballeroi* (Chabaud, 1977), however, contrasts in having Rays 2/3 weakly divergent along their entire length, Rays 4 < 5 in length, a robust Rays 4, a relatively elongate, narrow and straight Rays 8, and both a proconus and gubernacu-

lum are absent (Drötzå, 1965; Chabaud, 1977; Hoberg and Abrams, 2007). We propose *Africanastrongylus* as a previously unrecognized genus that is morphologically consistent with placement among the Ost-
tergaiinae.

Among a diverse global assemblage, including 24 species and 7 genera of ostertagines known from the African fauna (Table II), *A. buceros* gen. nov. et sp. nov. must also be differentiated from 2 problematic species, namely, *Ostertagia kenynensis* Gibbons and Khalil, 1980 in Da-

mara Dik Dik (*Madaqua kiriki Günther*) and Grant’s gazelle (*Gazella granti Brooke*) and *Teladorsagia hamata* (Mönig, 1932) in Springbok (*Antidorcas marsupialis* (Zimmerman)) and Bontebok (*Damaliscus py-gargas* (Pallas)). The latter species, originally described in *Ostertagia Ransom, 1907*, was later transferred to *Spiculopteragia Orloff, 1933* by Travassos (1937), to *Apteragia Jansen, 1958* by Jansen (1958), and most recently to *Teladorsagia Andreeva and Satubaldin, 1954* by Durette-

desset (1989). Gibbons and Khalil (1980) recognized the similarity of these nematodes, both with a 2–2–1 bursal formula, and distinguished *O. kenynensis* based on the configuration of the dorsal process of the spicules (lacking a prominent hooklike structure), and weakly curved and parallel Rays 4/5.

**Paratype specimens of *O. kenynensis*, and a syntype male specimen of *T. hamata*, were in general agreement with original descriptions (Mönig, 1932; Gibbons and Khalil, 1980). Observations of the structure of the synlophe and other attributes in *T. hamata* are limited to the single specimen available to us and the original description (Mönig, 1932). Other type and voucher specimens of *T. hamata* were unfortunately lost in transit to the USNPC from the Onderstepoort Helminthological Collection.

New data on structural attributes of the synlophe, bursa, and spicules are partially described based on these specimens of *O. kenynensis* and *T. hamata*. The lateral synlophe in the cervical region is parallel and Type 2 and the cervical papillae are massive and thornlike; a greater number of ridges characterize *T. hamata* (Mönig, 1932; Gibbons and Khalil, 1980). Overall, the structure and configuration of the bursa and bursal rays and dorsal lobe is similar; “7’’ papillae are contained in an accessory bursal membrane that is reduced and inconspicuous. The spicules are robust and massive, resembling those characteristic of mi-

nor morphotypes among the ostertagines (Drötzå, 1995) and have a simple ventral process and modified dorsal process. Additionally, spic-

ules in paratypes of *O. kenynensis* were characterized by a weakly de-

veloped barb on the curved dorsal process, which is not visible in all orientations. Although these species exhibit extensive overlap in some meristic characters (Mönig, 1932; Gibbons and Khalil, 1980; Tables III, IV), they can be unequivocally distinguished. We conclude the *O. kenynensis* and *T. hamata* are morphologically similar congeners repre-

senting an undetermined genus among the ostertagines; a taxonomic decision regarding these species is deferred, and is considered beyond the scope of the current study.

Together with *A. buceros*, specimens of *O. kenynensis* and *T. hamata* share a suite of characters, including a bursal formula of 2–2–1, where Rays 4/5 are subequal to equal in length, parallel, relatively straight and narrow, and which diverge distally at the tips adjacent to the bursal margin; Rays 2/3 are divergent throughout and become convergent dis-

tally. The dorsal lobe is strongly reduced, and curves ventrally relative to Rays 8 and the dorsal ray, or Rays 9/10, bifurcate in the distal half. In *O. kenynensis* the bursal margin adjacent to the dorsal lobe is thick-


---

**Figures 29–33.** *Africanastrongylus buceros* gen. nov. et sp. nov., showing spicules and gubernaculum depicted in line drawings in the male holotype and paratypes. (29) Spicules in ventral view of holotype (USNPC 99545) showing alate structure with medially curved main processes, triangular ventral processes and simple, weakly pointed to rounded dorsal processes. (30) Spicule, left, in medio-lateral view of paratype (USNPC 66322) showing bent or kinked main shaft, trifurcation of dorsal and ventral processes and the ostertagine window. (31) Spicule, left, in dorsal view of a paratype (USNPC 66322) showing rounded, weakly pointed dorsal process. (32) Gubernaculum in ventral view of paratypes (USNPC 66322, 99548, 99551) showing shieldlike structure in anterior and hornlike processes consistent among all male specimens. (33) Gubernaculum and spicule in right lateral view of paratype (USNPC 66322) showing weakly S-shaped structure and relative positions.
FIGURES 34–37. *Africanastrongylus buceros* gen. nov. et sp. nov., showing male bursal attributes based on photomicrographs of paratypes. (34) Bursa in left lateral view (USNPC 66322) showing position of proconus (pc), “0” papillae (0), accessory bursal membrane and “7” papillae (7), and ventrally disposed dorsal lobe (dl). (35) Bursa in lateral view (USNPC 99548) showing bend in spicules and S-shaped gubernaculum. (36) Bursa in dorsal view (USNPC 66322) showing disposition of narrow, filamentous spicules, shieldlike anterior of gubernaculum, dorsal lobe, and lateral thickening of bursal membrane (arrows). (37) Dorsal lobe in ventral view (USNPC 66322) showing laterally inflated form and incision.
FIGURES 38–42. *Africanastrongylus buceros* gen. nov. et sp. nov., showing genital cone in male based on photomicrographs of holotype (USNPC 99545). (38) 0 papillae paired, ventral view (Figs. 38–40 are sequential from ventral to dorsal through single specimen). (39) Accessory bursal membrane in ventral view showing straight, filamentous “7” papillae (7) and bilobate or incised structure. (40) Dorsal lobe and Rays 9/10 in ventral view showing ventrally directed papillae near terminus of short, stout ray. (41) Spicule tips in ventral view showing triangular structure at termination of ventral processes and medially curved main shafts capped with hyaline tips. (42) Gubernaculum and dorsal processes of spicules in dorsal view; note plate or shieldlike structure of anterior gubernaculum and simple termination of dorsal processes.
### TABLE II. Diversity for genera and species of Ostertagiinae in African ungulates and other mammalian hosts, with a listing of geographic localities and host records for the sub-Saharan region.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host species</th>
<th>Geographic localities</th>
<th>Authors*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cervicaprastrongylus</em> Gibbons and Khalil, 1982†</td>
<td>Hyemoschus aquaticus</td>
<td>Gabon</td>
<td>(14)</td>
</tr>
<tr>
<td><em>C. gabonensis</em> (Durette-Desset and Chabaud, 1974)‡</td>
<td>Lepus timidus</td>
<td>Mali</td>
<td>(15)</td>
</tr>
<tr>
<td><em>C. moreli</em> (Durette-Desset and Denke, 1978)‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hyostongylus</em> Hall, 1921</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. kigeziensis</em> Durette-Desset et al., 1992‡</td>
<td>Gorilla gorilla beringei</td>
<td>Uganda</td>
<td>(16)</td>
</tr>
<tr>
<td><em>H. okapiae</em> (Berghe, 1937)‡§</td>
<td>Okapia johnstoni</td>
<td>Congo</td>
<td>(1)</td>
</tr>
<tr>
<td><em>H. rubidus</em> (Hassall and Stiles, 1892)∥</td>
<td>Cephalophus natalensis</td>
<td>South Africa</td>
<td>(7)</td>
</tr>
<tr>
<td><em>Longistongylus</em> Le Roux, 1931‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. albifrontis</em> (Mönnig, 1931)‡</td>
<td>Acelaphus buselaphus, Antidorcas marsupialis, Damaliscus pygargus, Kobus ellipsiprymnus</td>
<td>Chad, South Africa</td>
<td>(24, 31, 40, 41, 42, 43)</td>
</tr>
<tr>
<td><em>L. banagiense</em> (Gibbons, 1972)‡</td>
<td>Aepyceros melampus, Alcelaphus buselaphus, Damaliscus lunatus, Gazella granti, Gazella thomsonii, Oryx gazelle, Redunca sp.</td>
<td>Kenya, Tanzania, Uganda</td>
<td>(18, 19)</td>
</tr>
<tr>
<td><em>L. curvispiculum</em> (Gibbons, 1973)‡</td>
<td>Aepyceros melampus, Antidorcas marsupialis, Connochaetes taurinus, Damaliscus lunatus, Damaliscus pygargus, Gazella granti, Gazella thomsonii, Neotragus moschatus, Pelea capreolus, Capra hircus, Ovis aries</td>
<td>Kenya, Tanzania, South Africa</td>
<td>(6, 19, 22, 28, 29, 31, 32, 40, 41, 42, 43)</td>
</tr>
<tr>
<td><em>L. meyeri</em> Le Roux, 1931‡</td>
<td>Aepyceros melampus, Alcelaphus buselaphus, Connochaetes taurinus, Gazella granti, Gazella thomsonii, Hippotragus equinus, Kobus ellipsiprymnus, Madoqua kirki, Syncerus caffer, Capra hircus</td>
<td>Chad, Kenya, South Africa, Tanzania, Uganda</td>
<td>(2, 13, 20, 22, 24, 36, 43, 45, 52)</td>
</tr>
<tr>
<td><em>L. namaquensis</em> (Ortlepp, 1963)‡</td>
<td>Antidorcas marsupialis, Damaliscus pygargus, Pelea capreolus, Ovis aries</td>
<td>South Africa</td>
<td>(6, 28, 29, 31, 44)</td>
</tr>
<tr>
<td><em>L. sabie</em> (Mönnig, 1932)‡</td>
<td>Aepyceros melampus, Gazella granti, Gazella thomsonii, Raphicerus melanotis, Redunca arundinum, Sylvicapra grimmia, Bos taurus</td>
<td>Kenya, South Africa, Tanzania</td>
<td>(10, 20, 27, 39, 41, 42, 43, 46)</td>
</tr>
<tr>
<td><em>L. schrenki</em> (Ortlepp, 1939)‡</td>
<td>Cephalophus natalensis, Hippotragus equinus, Kobus ellipsiprymnus, Kobus kob, Kobus sp., Madoqua kirki, Ourebia ourebi, Pelea capreolus, Redunca arundinum, Redunca sp.,</td>
<td>Kenya, Mozambique, South Africa, Tanzania, Uganda</td>
<td>(6, 7, 10, 11, 13, 20, 24, 43, 52)</td>
</tr>
<tr>
<td><em>L. thalae</em> (Troncy and Graber, 1932)‡</td>
<td>Acelaphus buselaphus, Hippotragus equinus, Ourebia ourebi</td>
<td>Central Africa, Kenya</td>
<td>(21, 48)</td>
</tr>
<tr>
<td><em>Ostertagia</em> Ransom, 1907</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>O. angusdunni</em> (Gibbons and Khalil, 1980)‡</td>
<td>Taurotragus oryx</td>
<td>Kenya</td>
<td>(23)</td>
</tr>
<tr>
<td><em>O. harrisi</em> (Le Roux, 1930)‡</td>
<td>Cephalophus natalensis, Tragelaphus angasii, Tragelaphus scriptus, Capra hircus (Angora Goat, Boer Goat)</td>
<td>South Africa</td>
<td>(4, 7, 8, 10, 35, 43, 50)</td>
</tr>
<tr>
<td><em>O. neveulemairei</em> Gutterres, 1947‡</td>
<td>Acelaphus sp., Hippotragus equinus, Ourebia ourebi, Bos taurus, Ovis aries</td>
<td>Congo</td>
<td>(26)</td>
</tr>
</tbody>
</table>
### Table II. Continued.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host species</th>
<th>Geographic localities</th>
<th>Authors*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. ostertagi</em> Ransom, 1972‡#</td>
<td><em>Reduncu arundinum, Trachelaphus strepsiceros, Bos taurus, Capra hircus</em> (Angora Goat, Boer Goat)</td>
<td>South Africa</td>
<td>(9, 10, 27, 30)</td>
</tr>
<tr>
<td><em>O. sissokoi</em> Diaoure, 1964‡</td>
<td><em>Sylvicapra grimmia</em></td>
<td>Congo</td>
<td>(12)</td>
</tr>
<tr>
<td><em>O. triquetra</em> Boomker and Durette-Desset, 2003‡</td>
<td><em>Pelea capreolus</em></td>
<td>South Africa</td>
<td>(3, 5, 6, 29)</td>
</tr>
<tr>
<td><em>Ostertagia</em> sp.</td>
<td><em>Aepyceros melampus, Syncerus caffer, Tragelaphus angasii</em></td>
<td>South Africa, Uganda</td>
<td>(2, 4, 13, 42)</td>
</tr>
</tbody>
</table>

**Pseudomarshallagia** (Roetti, 1941)‡

*P. elongata* Roetti, 1941‡

*Marshallagia* (Orloff, 1933)

*M. marshalli* (Ransom, 1907)\]

*Marshallagia* sp.\ | *Ovis aries, Capra hircus* | Ethiopia | (25, 47, 53) |
| *Teladorsagia* (Stadelman, 1894)\ | *Cephalophus maximus, Cephalophus natalensis, Damaliscus alibrons, Gazella thomsonii, Pelea capreolus, Raphicerus melanotis, Sylvicapra grimmia, Taurotragus oryx, Tragelaphus angasii, Tragelaphus strepsiceros, Bos taurus, Ovis aries* | Kenya, South Africa, Zambia | (2, 7, 10, 17, 27, 30, 33, 34, 35, 37, 38, 40, 42) |

**Teladorsagia** Andreeva and Satubaldin, 1954**

*T. circumcincta* (Stadelman, 1894)\ | *Gazella granti, Madoqua kirkii* | Kenya | (23) |

**Ostergiaines of undetermined affinities**

*Ostertagia kenensis* Gibbons and Khalil, 1980†‡

*Teladorsagia hamata* (Mönig, 1932)†‡

*Africanastronygus* gen. nov.‡

*A. buceros* n. sp.‡ | *Syncerus caffer* | Kenya, Uganda, South Africa | Current study

---

* Authors: (1) Berghe (1937); (2) Bwangarnoi (1968); (3) Boomker (1990); (4) Boomker et al. (1996); (5) Boomker and Durette-Desset (2003); (6) Boomker and Horak (1992); (7) Boomker et al. (1991); (8) Boomker et al. (1991b); (9) Boomker et al. (1991); (10) Boomker, Horak, and Maclvor (1989); (11) Cruz e Silva (1971); (12) Diaoure (1964); (13) Dinnik et al. (1963); (14) Durette-Desset and Chabaud (1974); (15) Durette-Desset and Denke (1978); (16) Durette-Desset et al. (1992); (17) Gebauer (1932); (18) Gibbons (1972); (19) Gibbons (1973); (20) Gibbons (1974); (21) Gibbons (1981); (22) Gibbons and Khalil (1976); (23) Gibbons and Khalil (1980); (24) Graber (1969); (25) Graber and Delavenay (1978); (26) Gutterres (1947); (27) Horak (1981); (28) Horak, Brown, et al. (1982); (29) Horak, de Vos, and De Klerk (1982); (30) Horak et al. (1991); (31) Horak, Meltzer, and de Vos (1982); (32) Khalil and Gibbons (1976); (33) Keep (1971); (34) Le Roux (1929); (35) Le Roux (1931); (36) Le Roux (1932); (37) Le Roux (1950); (38) Meester (1952); (39) Mönig (1931); (40) Mönig (1932); (41) Mönig (1933); (42) Ortlepp (1961); (43) Ortlepp (1963); (44) Ortlepp (1963); (45) Pester and Laurence (1974); (46) Pletcher et al. (1984); (47) Roetti (1941); (48) Troncy and Graber (1973); (49) Verster et al. (1975); (50) Vincent et al. (1968); (51) Yeh (1956); (52) Unpublished records established by M. Kinsella and V. Ezenwa include *Madoqua kirkii* from Kenya as a host for *L. schrenki* and *L. meyeri*; (53) Tembley et al. (1997).

† Considered a synonym of *Hyostrongylus* by Durette-Desset (1983), and as an independent genus by Gibbons and Khalil (1982a, 1982b) and Hobart et al. (1993b).

‡ Considered to be endemic to Africa.

§ Originally described in *Ostertagia*, later transferred to *Hyostrongylus* by Jansen (1958), and then to *Bergeria Drózd (1965)*; see history for this species outlined by Jansen (1958), Drózd (1965) and Gibbons and Khalil (1982a).

# Records for *O. ostertagi* in domestic ungulates are not exhaustive.

‡‡ Teladorsagia circumcincta here includes *T. trifurcata* and *T. davitiani*, which in many reports may have been considered as separate, rather than as morphotypes within a single polymorphic species (e.g., Drózd, 1995). Records reported are not exhaustive for geographic and host distribution in domestic ungulates.

†† Teladorsagia hamata and *Ostertagia kenensis* are morphologically similar and likely to be congeneric, but are not consistent with any of the known genera of the Ostergiaines.

## Notes

- *Considered a synonym of *Longistrongylus* by Durette-Desset (1983), and independent by Gibbons (1981); records listed here for *P. elongata* are not exhaustive.
- *Teladorsagia circumcincta* here includes *T. trifurcata* and *T. davitiani*, which in many reports may have been considered as separate, rather than as morphotypes within a single polymorphic species (e.g., Drózd, 1995). Records reported are not exhaustive for geographic and host distribution in domestic ungulates.

- *Teladorsagia hamata* and *Ostertagia kenensis* are morphologically similar and likely to be congeneric, but are not consistent with any of the known genera of the Ostergiaines.
TABLE III. Morphometric comparisons for male specimens of *Africanastrongyulus buceros* gen. nov. et sp. nov., *Teladorsagia hamata* and *Ostertagia kenyensis*.

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>Africanastrongyulus buceros</em></th>
<th><em>Teladorsagia hamata</em></th>
<th><em>Ostertagia kenyensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number examined‡</td>
<td>12</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Body length</td>
<td>(10) 6,350–8,555 (7,471 ± 642.70)</td>
<td>—</td>
<td>6,600–7,850</td>
</tr>
<tr>
<td>Esophagus length§</td>
<td>(11) 688–825 (757 ± 49.65)</td>
<td>710–800</td>
<td>776–943</td>
</tr>
<tr>
<td>Esophagus % of body length</td>
<td>(10) 9.4–11.3 (10.1 ± 0.69)</td>
<td>10.2–10.7</td>
<td>7.8–8.0</td>
</tr>
<tr>
<td>Esophageal-intestinal valve length</td>
<td>(10) 80–90 (91 ± 5.48)</td>
<td>71</td>
<td>—</td>
</tr>
<tr>
<td>Esophageal-intestinal valve width</td>
<td>(10) 48–75 (56 ± 7.93)</td>
<td>39</td>
<td>64</td>
</tr>
<tr>
<td>Nerve ring§</td>
<td>(8) 250–335 (303 ± 25.61)</td>
<td>240–290</td>
<td>—</td>
</tr>
<tr>
<td>Subventral esophageal gland orifices§</td>
<td>(10) 230–310 (276 ± 25.15)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Excretory pore§</td>
<td>(10) 305–421 (370 ± 31.16)</td>
<td>315–378</td>
<td>—</td>
</tr>
<tr>
<td>Spicule, length, left</td>
<td>(14) 195–246 (212 ± 14.08)</td>
<td>161–191</td>
<td>186–210</td>
</tr>
<tr>
<td>Spicule, left, % trifurcation</td>
<td>(13) 76–83 (79 ± 2.2)</td>
<td>60</td>
<td>—</td>
</tr>
<tr>
<td>Spicule, right, % trifurcation</td>
<td>(13) 190–240 (207 ± 15.19)</td>
<td>161–191</td>
<td>186–210</td>
</tr>
<tr>
<td>Gubernaculum length</td>
<td>(12) 60–82 (67 ± 5.9)</td>
<td>112</td>
<td>95–129</td>
</tr>
<tr>
<td>Gubernaculum width</td>
<td>(10) 35–42 (38 ± 1.8)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

§ Measured from anterior, cephalic extremity.

* Based on original description by Monnig (1932), and observations from a single male syntype.
† Based in part on original description by Gibbons and Khalil (1980), and examination of 2 male paratype specimens.
‡ Numbers of individual specimens examined.

end. Additionally it appears that males of these species are monomorphic, although this requires confirmation through assessment of larger numbers of specimens in individual hosts.

*Africanastrongyulus buceros* is distinguished, however, from *O. kenyensis* and *T. hamata* in the following manner: (1) tapering Type 1 synlophe (parallel Type 2 in *O. kenyensis* and *T. hamata*); (2) miniscule and thornlike cervical papillae; (3) midbody ridges numbering >56 in males and >45 in females (about 25–29 in *O. kenyensis*; about 35 in *T. hamata*); (4) presence of a proconus (absent in *O. kenyensis* and *T. hamata*); (5) membranous accessory bursal membrane containing divergent "7" papillae; (6) massive Rays 8 and Rays 9/10 (in *O. kenyensis* and *T. hamata* these rays have bases that are not inflated); (7) ..

TABLE IV. Morphometric comparisons for female specimens of *Africanastrongyulus buceros* gen. nov. et sp. nov., *Teladorsagia hamata* and *Ostertagia kenyensis*.

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>Africanastrongyulus buceros</em></th>
<th><em>Teladorsagia hamata</em></th>
<th><em>Ostertagia kenyensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number examined‡</td>
<td>18</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Body length</td>
<td>(18) 9,712–12,610 (11,217 ± 909.5)</td>
<td>—</td>
<td>8,090–11,020</td>
</tr>
<tr>
<td>Esophagus length§</td>
<td>(18) 775–905 (834 ± 33.7)</td>
<td>710–860</td>
<td>893–1,057</td>
</tr>
<tr>
<td>Esophagus % of body length</td>
<td>(18) 6.4–8.7 (7.5 ± 0.7)</td>
<td>7.8–8.8</td>
<td>6.7–7.0</td>
</tr>
<tr>
<td>Esophageal-intestinal valve length</td>
<td>(17) 92–112 (101 ± 5.8)</td>
<td>—</td>
<td>112</td>
</tr>
<tr>
<td>Esophageal-intestinal valve width</td>
<td>(17) 46–78 (66 ± 8.2)</td>
<td>—</td>
<td>57</td>
</tr>
<tr>
<td>Nerve ring§</td>
<td>(10) 270–362 (317 ± 31.6)</td>
<td>240–290</td>
<td>—</td>
</tr>
<tr>
<td>Subventral esophageal gland orifices§</td>
<td>(17) 285–342 (306 ± 13.9)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Excretory pore§</td>
<td>(18) 305–482 (394 ± 54.6)</td>
<td>306–381</td>
<td>—</td>
</tr>
<tr>
<td>Cervical papillae§</td>
<td>(18) 320–545 (421 ± 63.5)</td>
<td>320–420</td>
<td>320–410</td>
</tr>
<tr>
<td>Vulva position§</td>
<td>(18) 8,075–10,275 (9,239 ± 648)</td>
<td>Near cervical papillae</td>
<td>6,750–9,260</td>
</tr>
<tr>
<td>Vulva % body length</td>
<td>(18) 79–85 (82 ± 2.0)</td>
<td>83–84</td>
<td>83–84</td>
</tr>
<tr>
<td>Ovejector total length</td>
<td></td>
<td>(10) 795–1,016 (911 ± 77.1)</td>
<td>—</td>
</tr>
<tr>
<td>Anterior infundibulum length</td>
<td>(11) 185–292 (240 ± 30.8)</td>
<td>—</td>
<td>129–229</td>
</tr>
<tr>
<td>Anterior sphincter length#</td>
<td>(13) 110–192 (149 ± 23.9)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vestibule length</td>
<td>(13) 70–205 (144 ± 42.3)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Posterior infundibulum length</td>
<td>(11) 170–267 (231±26.1)</td>
<td>—</td>
<td>133–219</td>
</tr>
<tr>
<td>Posterior sphincter length#</td>
<td>(13) 98–162 (140 ± 16.7)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Egg length</td>
<td>(90) 62–82 (72 ± 5.9)</td>
<td>71</td>
<td>70–84</td>
</tr>
<tr>
<td>Egg width</td>
<td>(90) 30–50 (41 ± 4.2)</td>
<td>39</td>
<td>41–54</td>
</tr>
<tr>
<td>Tail length</td>
<td>(15) 142–218 (167 ± 19.8)</td>
<td>176–190</td>
<td>143–219</td>
</tr>
</tbody>
</table>

* Based on original description by Monnig (1932). Type or voucher specimens of females of this species were not available for examination.
† Based in part on original description by Gibbons and Khalil (1980), and examination of 2 paratype specimens.
‡ Numbers of individual specimens examined.
§ Measured from anterior, cephalic extremity.
¶ Complete ovejector, combining infundibula, sphincters, and vestibule.
# Combining Sphincter s1 and s2, consistent with Lichtenfels et al. (2003).
absence of numerous fields of bursal bosses (numerous in *O. kenyensis*; absent in *T. hamata*); (8) the structure and dimensions of the alate gubernaculum with anteriorly directed horns (in *T. hamata* and *O. kenyensis* the gubernaculum is irregularly narrow); (9) structural differences in the spicule tips including the barbed and triangular ventral process and simple dorsal process of near equal length; (10) narrow, filamentous spicules; (11) substantially longer spicules; and (11) triradication of the spicule tips at 76–83% from the anterior (60% in *O. kenyensis* and *T. hamata*) (Tables III, IV). Differences in the synlophe, genital cone, and bursal structure relative to *A. buceros* are those that separate genera. The generic placement of *T. hamata* and *O. kenyensis* remains undetermined, as neither species conforms to known ostertagiines with a 2–2–1 bursal pattern.

*Africanastrongylus buceros* is somewhat unusual among the ostertagiines in that males appear to have consistently greater numbers of ridges than females at all levels of the body. The only other report of this phenomenon of which we are aware is in *Longistrongylus thalae* (Troncy and Graber, 1973). In multiple specimens of *L. thalae* examined by Boomker and Durette-Desset (1997), there were 44–51 ridges in males and 42–45 in females at the level of the midbody. Males of *L. thalae* have a bursal formula of 2–1–2 and differ in other structural attributes relative to *A. buceros* (Troncy and Graber, 1973; Gibbons, 1981; Boomker and Durette-Desset, 1997).

**DISCUSSION**

**Dilemma of ostertagiine generic taxonomy**

Recognition of *A. buceros* gen. nov. et sp. nov. represents a dilemma for generic taxonomy among the ostertagiines, and highlights the continuing difficulty in establishing taxonomic limits and in defining unequivocal phylogenetic criteria for species groups within the subfamily. Although we have a reasonable understanding of phylogenetic criteria for the subfamily and hypotheses for a suite of synapomorphies that diagnose this larger taxon (Durette-Desset, 1983; Hoberg and Lichtenfels, 1994; Durette-Desset et al., 1999), the problematic nature of generic taxonomy remains. It appears accepted that differences in the 2–2–1 and 2–1–2 bursa, the configuration of lateral rays, dorsal lobe, and the structure of the synlophe, represent fundamental criteria in diagnosing genera and assemblages of genera within Ostertagiinae (e.g., Andreeva, 1956, 1958; Dróżdż, 1965; Durette-Desset and Chabaud, 1981; Gibbons and Khalil, 1982a; Durette-Desset, 1982, 1983, 1985, 1989; Jansen, 1989; Lichtenfels and Hoberg, 1993; Lichtenfels et al., 1993; Hoberg, 1996; Durette-Desset et al., 1999). It remains uncertain, however, when generic diagnoses should be emended to recognize the discovery of previously unknown diversity.

In establishing *Africanastrongylus*, we had 2 options: (1) extensively emend one or another of the existing genera to accommodate this species, or (2) recognize the apparent distinct nature of these nematodes relative to what we currently know about ostertagiine diversity. In the absence of a generic-level phylogeny, these alternatives each represent introduction of potential errors in a system where the goal should be to delineate monophyletic taxa or lineages as a basis for taxonomy. Thus, an incorrect inclusion of *A. buceros* among *Longistrongylus* or *Sarvaria* would confuse our potential interpretations of character evolution, biogeography, and host association if this species is actually not associated with either of these lineages. As a consequence, we consider our decision to recognize the distinct nature of *A. buceros* by establishing the new genus as conservative. As genetic, molecular, and morphological criteria increasingly become established with more extensive taxon sampling within this group, it will become possible to fully evaluate the hypothesis that *Africanastrongylus* represents. Concurrently, the problematic nature and incompatibility for the current taxonomy of *O. kenyensis* in *Ostertagia* and *T. hamata* in *Teladorsagia* remains apparent.

**Ostertagiines in African buffalo**

Wild African buffalo, or Cape buffalo, from the West Acholi District and the Queen Elizabeth National Park, Toro District, Uganda in the late 1960s, from Latkipia, Kenya in 2000, and from Hluhluwe-iMfolozi Park, South Africa in 2006, were infected with a previously undescribed species of ostertagiine nematode. We have established *A. buceros* for this unique abomasal nematode. There are relatively few reports of ostertagiine or trichostrongylid nematodes as abomasal parasites in African buffalo (Table II), and these have been limited to *L. meyeri* and *Ostertagia* sp. from Uganda (Dinnik et al., 1963; Ngamai, 1968); *A. lerouxi* Diaoüre, 1964 from Congo (Diaoüre, 1964); *H. bedfordi* Le Roux, 1929 from Uganda (Dinnik et al., 1963) and South Africa (Le Roux, 1929; Ortlepp, 1961; V.O. Ezenwa, data not shown); *H. contortus* (Rudolph, 1803) from Kenya and South Africa (Curson, 1928; Ezenwa, 2003); and *H. placei* Place, 1893 from Kenya (Ezenwa, 2003).

**Ostertagiine diversity in Africa**

Ostertagiines in the African fauna now include 25 species, representing 8 genera (Table II); among these, 21 species in 7 genera are apparently endemic to Africa, whereas 4 species in 4 genera have been introduced. Species diversity for *Longistrongylus* (8 species), *Africanastrongylus* (1), and *Pseudomarshallagia* (1) is restricted to Africa, with primary distributions among Antelopinae, Bovinae, Cephalophinae, and Hippotraginae. Although the latter genus has thus far only been reported in domestic caprines, species of *Longistrongylus* are also known as incidental parasites in sheep (Gibbons and Khalil, 1976). Endemic species from Africa are represented among *Hyostro­ngylus* (2) in gorillas and okapi (Bergh, 1937; Durette-Desset et al., 1992) and among *Cervicaprastrongylus* (2) in leporids and chevrotains (Durette-Desset and Chabaud, 1974; Durette-Desset and Denke, 1978), but additional diversity in these genera is distributed in Eurasia (Gibbons and Khalil, 1982b; Hoberg et al., 1993). Durette-Desset (1983, 1989) reduces *Bergheia Dróżdż*, 1965 and *Cervicaprastrongylus* as synonyms of *Hyostro­ngylus*. The status of *Hyostro­ngylus* okapi (Bergh, 1937), although retained here in *Hyostro­ngylus*, remains to be determined and will require additional and new specimens from okapi (Gibbons and Khalil, 1982b).

In Africa, *Ostertagia* constitutes a mosaic of endemic species (5, with exclusion of *O. kenyensis*) among Antelopinae, Bovinae, Cephalophinae, and Hippotraginae, and a single introduced species (*Ostertagia ostertagi*) found in domestic and wild ungulates. Additionally, *Hyostro­ngylus rubidus*, *Marshallagia marshalli*, and *Teladorsagia circumcincta*, including minor morphotypes for the latter, have been introduced and distributed in Africa coincidental with independent translocations and establishment of domestic swine, cattle, sheep, or goats (e.g., Daubney, 1933).

Placement for either *O. kenyensis* or *T. hamata* remains unresolved. Neither appears morphologically consistent with any known genus attributed to the Ostertagiinae. Among the group...
of 7 genera having a 2–2–1 bursa and either a tapering or a parallel lateral synloph, a suite of structural characters would negate an unequivocal diagnosis for either species. Interestingly, specimens of both *T. hamata* and *O. kenensis* are most similar to those attributed to minor morphotypes among the ostertagiines (e.g., Dróżdż, 1995) with robust spicules which trifurcate near 60% from the anterior, and a cuticularized and reduced accessory bursal membrane. Specimens of *T. hamata* have not been found in association with a putative major morphotype (Mönig, 1932; Orlepp, 1961; Verster et al., 1975; Horak et al., 1982), whereas *O. kenensis* has not been reported since the original description (Gibbons and Khalil, 1980). A proposal to establish and diagnose another genus among the ostertagiines for *O. kenensis* and *T. hamata* is deferred until such time as sufficient specimens become available for comparative studies.

Round (1968) includes a record for *Camelostrongylus mentulatus* (Ralllet et Henry, 1909) in *Gazella dama* (Pallas), but this represents specimens collected from captive animals in a zoo; other records from Africa are lacking. Additionally, specimens referred to as *Camelostrongylus harrisi* (Le Roux, 1930) and *Camelostrongylus* sp. by Boomker et al. (1996) are correctly placed in *Ostertagia*. *Camelostrongylus* should be retained only for *C. mentulatus*, and confusion over the taxonomy of *Ostertagia* and *Camelostrongylus* emanates from nomenclatural decisions proposed by Durette-Desset (1989).

**Structure of the African ostertagiine fauna**

The African ostertagiine fauna is a complex mosaic reflecting historical processes across relatively deep to shallow temporal scales. Endemic faunas have origins associated with dispersal and biotic expansion from Eurasia into Africa and subsequent radiation for ungulates and their parasites extending from the late Tertiary. In Africa, structure of the fauna was likely to have been influenced by the differential timing of expansion events from Eurasia and periods of occupation for respective pecoran groups, including Antelopinae, Bovinae, Hippopotaginae, Reduncinae, and others since the Miocene, in parallel to radiation among species of *Haemonchus* (Vrba 1985, 1995; Vrba and Schaller, 2000; Hoberg et al., 2004). Subsequently, domestication and later translocations during the Holocene for cattle and sheep (Ryder, 1984; Loftus et al., 1994) influenced distribution and diversity for trichostrongylid faunas (e.g., Daubney, 1933). Mosaic faunas among ungulate nematodes have now been demonstrated for all biogeographic regions (e.g., Hoberg et al., 1999, 2001, 2004; Hoberg, 2005).

Among 13 genera of the Ostertagiiinae in the global fauna, 3 are entirely limited in distribution to Africa, including *Africanastronyx*, *Longistonglyus*, and *Pseudomalabtalaia* (Table II); the number would increase to 4 of 14 genera if a new taxon were established for *T. hamata* and *O. kenensis*. *Spiculapteragia* among Cervidae and *Camelostrongylus* among antelopes and Caprinae, are the sole genera limited in distribution to Eurasia and the Paleartic; *Varvaria* among Tragulidae and Bovinae appears limited to southern Asia. All other recognized genera are distributed in 2 or more biogeographic regions (discounting the influence of recent translocation). Among these, *Cervicaprastrongylus* among Tragulidae, Antelopinae, and Le- poridae and *Hyostonglyus* among Suidae, Giraffidae, and Pon-}
```
disease (Hoberg, 1997; Brooks and Hoberg, 2006; Hoberg and Brooks, 2008).

ACKNOWLEDGMENTS

We thank E. A. Harris of the Parasitic Worms Group, Department of Zoology, National History Museum, London, United Kingdom, for loan of specimens of *O. kenyensis*. We thank J. Boomer from the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa, for access to African ostertagines, including *T. hamata*. We thank M. Kinsella for referral of specimens that initiated the current study and M. Kinsella for providing the image represented in Figure 35. We also thank P. Pilitt of the USNPC for reviews and comments on various drafts of this manuscript. Field collections for hosts and parasites by VOE in South Africa were funded by the National Science Foundation (DEB-0541762) and by the Division of Biological Sciences, University of Montana.

LITERATURE CITED


---. 1975. *Trichosomoides* (Trichostrongylidea) from East African game animals, with a redescription
atodes that cause ostertagiasis in domestic and wild ruminants in North America: An update and key to species. Veterinary Parasitology 46: 33–53.


DETECTION AND SPECIES IDENTIFICATION OF CYTOSPORIDIUM FROM TAIWAN FEEDING ANIMALS

Bing-Mu Hsu, Hau-Yang Wun*, and Chih-Li Lilian Hsu†
Department of Earth and Environmental Sciences, National Chung Cheng University, 168, University Road, Min-Hsiung, Chiayi, Taiwan, R.O.C.
E-mail: bmhsu@eq.ccu.edu.tw

ABSTRACT: In this study, 107 fecal specimens were collected from 40 sampling sites in Taiwan livestock and avian farms to test for Cryptosporidium spp. oocysts. Ten of 107 samples analyzed by enzyme-linked immunosorbent assay showed the presence of Cryptosporidium spp., among which 6 samples were simultaneously confirmed by immunofluorescence assay and polymerase chain reaction. Nucleic acid sequencing of the 18S rRNA gene identified 3 clusters of Cryptosporidium spp. Three Cryptosporidium parvum isolates were from cattle and sheep feces. One Cryptosporidium andersoni isolate was detected from pig feces. The other 2 novel Cryptosporidium genotypes were not similar to any known Cryptosporidium spp. according to the DNA sequences of the 18S rRNA gene.

Cryptosporidium spp. are common pathogenic protozoans of the gastrointestinal tract in vertebrates (Cook, 1995). Species of Cryptosporidium cause gastroenteritis in humans and wild and farm animals and are often responsible for waterborne outbreaks (Xiao et al., 1999). For instance, a significant outbreak in Milwaukee, Wisconsin, in 1993, infected 403,000 people. A probable cause of this outbreak was a decline in the performance of a water treatment plant that occurred when treatment chemicals were being changed and the source water was possibly contaminated by storm water runoff from a nearby cattle feedlot (MacKenzie et al., 1994). In fact, the usual source of Cryptosporidium spp. oocysts seems to be runoff that contaminates water supplies (Johnson et al., 1995; Smith and Rose, 1998).

At present, Cryptosporidium parvum and 9 other Cryptosporidium species—C. felis, C. muris, C. andersoni, C. wrairi, C. baileyi, C. meleagris, C. serpentis, C. surophilum, and C. nasorum—are regarded as valid on the basis of differences in oocyst morphology, site of infection, vertebrate class specificity, and genetic differences (Ong et al., 2002; Ryan et al., 2005). The most frequently identified pathogens in humans are C. parvum human genotype (Cryptosporidium hominis) and C. parvum bovine genotype. The remaining genotypes of C. parvum are found in other animals, including the dog, mouse, bear, pig, deer, and marsupial genotypes (Ong et al., 2002). A recent study suggested that all Cryptosporidium spp., including those from lower vertebrates, should be considered hazardous to humans (Tzipori and Griffiths, 1998). At least 3 species of Cryptosporidium (i.e., C. meleagris, C. felis, and C. canis) have been detected from immunocompromised persons or children (Xiao et al., 2001; Zhou et al., 2004; Ochiai et al., 2005). More complete data on the epidemiology of infection with individual Cryptosporidium genotypes might enhance the clinical significance of detection and aid in outbreak investigation.

The traditional method for the detection of Cryptosporidium spp. is microscopic examination of stool preparations. However, this procedure requires considerable time, as well as technologists well versed in the identification of fecal parasites (Morris et al., 1992). Over the last decade, a variety of highly sensitive and specific assays have been developed. The most commonly used laboratory protocols for identifying Cryptosporidium spp. oocysts in environmental samples are enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and polymerase chain reaction (PCR) (Leng et al., 1996). ELISA is a popular method in diagnostic laboratories for the detection of soluble antigens of Cryptosporidium spp. Although these assays are rapid, with detection within 1–2 hr, they are qualitative and do not distinguish between genotypes. Additionally, they are not sensitive enough to detect low levels of infection (Hsu et al., 1999). Another method for detection of intact oocysts involves the use of IFA, which is currently the methodology used for identifying Cryptosporidium spp. oocysts in water. Molecular techniques such as PCR provide alternative methods for specific detection of pathogens and, in combination with techniques such as restriction fragment length polymorphism and nucleic acid sequencing methods, have been used to classify the species of Cryptosporidium.

In Taiwan, Cryptosporidium spp. oocysts are generally found in water samples and fecal specimens (Wang and Liew, 1990; Hsu et al., 2000; Hsu, 2003), and surveys have shown that 17–50% of avian species and 0–26% of pasturing animals in Taiwan are infected with Cryptosporidium spp. However, such results have been seldom confirmed by molecular techniques (Watanabe et al., 2005). In our study, 107 fecal specimens from farm animals were obtained from 40 farms in Taiwan during 2005. The aims were to evaluate the differences and effects of ELISA, IFA, and PCR while determining the occurrence of Cryptosporidium spp. in the fecal specimens. In addition, we used nucleic acid sequencing methods to complete the diagnosis and for taxonomic identification of the Cryptosporidium spp. in Taiwan feeding animals.

MATERIALS AND METHODS

Sampling sites and sampling procedures

Fecal specimens (107) were randomly collected from livestock and avian farms located in the main 5 farming regions in Taiwan (Fig. 1). The tested specimens were from watery feces of cattle, sheep, pigs, horses, chickens, ducks, and other birds. All of the fecal samples were screened for the presence of Cryptosporidium spp. by ELISA. When positives were detected, these samples were confirmed by IFA and PCR. Finally, nucleic acid sequencing was used to identify Cryptosporidium spp.

Immunolabeling of Cryptosporidium spp.

ELISA was used to detect the presence of the organisms in fecal samples. RIDASCREEN® Cryptosporidium (C1201) was purchased

Received 27 November 2006; revised 7 March 2007, 10 July 2007; accepted 12 July 2007.
* Institute of Seismology, National Chung Cheng University, 168, University Road, Min-Hsiung, Chiayi, Taiwan, R.O.C.
† Department of Parasitology, Medical College National Cheng Kung University, Tainan, Taiwan, R.O.C.
from R-Biopharm AG (Darmstadt, Germany). Sample preparation and immunoreaction procedures followed the supplier's protocols. Readings of absorbance at 450 nm (A<sub>450</sub>) ≥ 0.05 were interpreted as positive reactions and A<sub>450</sub> < 0.05 as negative. The primary antibodies used in the ELISA are specific to Cryptosporidium spp.

IFA was used to detect Cryptosporidium spp. oocytes in fecal specimens as a confirmatory test. The fecal specimens were labeled with monoclonal antiserum (Aqua-Glo<sup>®</sup> G/C Direct, New Orleans, Louisiana) and examined with an epifluorescent microscope at ×200, ×400, or ×1,000 magnification (Olympus, Tokyo, Japan). Oocytes were identified according to the following parameters: size (4–6 μm), shape (ovoid or spherical), and surface features (bright apple green–highlighted edges). Candidates exhibiting the right size and shape were further identified by epifluorescent microscopy under a bright field according to their internal morphological features.

**Detection and identification of Cryptosporidium spp. by molecular techniques**

Molecular techniques were used to detect Cryptosporidium spp. in fecal specimens to complete the test. DNA was initially extracted according to the instructions provided with the NucliSens Isolation kit (bioMerieux, Durham, North Carolina). Aliquots of fecal specimens were added to 900 μl of lysis buffer and the components were mixed by vortexing. The sample was then allowed to lyse for 1.5 hr, with inversion of the microcentrifuge tube every 0.5 hr, and was centrifuged at 300 g for 1 min to separate unlysed and coarse material from the rest of the specimen. Free silica was then added to the sample, and the bound DNA was washed. A 50-μl volume of elution buffer was added, and after centrifugation, the PCR template was removed and used immediately or stored at −20 C.

A PCR reaction solution was prepared with 5.0 μl of PCR template and PCR mixture to create a total volume of 50 μl. The PCR mixture contained 5.0 μl of 10× PCR Buffer (20 mM MgCl<sub>2</sub>), 1.0 μl of dNTP Mix (10 mM of each dNTP), 100 pmol each of the oligonucleotide primers, and 0.5 μl of VioTaqTM DNA Polymerase (5 U/μl; Viogene, Taipei, Taiwan), as well as DNase-free deionized water.

The primers to amplify the genes of 18S rRNA, CpR1 (a Cryptosporidium spp. protein gene) and HSP-70 (heat shock protein 70) in this study were designed by Ryan, Xiao et al. (2003), Lally et al. (1992), and Khramov et al. (1995), respectively. The 2-step nested PCR primers to amplify 763- and 587-bp fragments of the 18S rRNA gene of Cryptosporidium spp. had the following sequences: 5'-GAC ATCA TCT AAC TTT CTG ACC-3' (forward primer), 5'-CTG AAG GAG TAA GGA ACA ACC-3' (reverse primer), 5'-CTC ATC AGC TTT AGA CGG TAG G-3' (nest forward primer), and 5'-TCT AAG ATT TCT ACC TCT GAC TG-3' (nest reverse primer). The 1-step primer sequences were 5'-GCC CAC CTG GAT ATA CAC TTT C-3' (forward primer) and 5'-TCC CCC TCT CTA CGG ACA GGA-3' (reverse primer) to amplify a 358-bp fragment of CpR1. The other 2-step nested PCR in this study was used to amplify the HSP-70 gene. For the primary amplification, a PCR product of 448 bp was amplified with the forward primer 5'-GTT GGT GGT ACT TTT GAT GTA TC-3' and reverse primer 5'-GCC TGA ACC TTT GGA ATA CG-3' for the secondary PCR, a product of 325 bp was amplified with the use of 2.5 ml of primary PCR product and nest forward primer 5'-GCT GAT ACT CAC TTG GGT GG-3' and nest reverse primer 5'-CTC TTG TCC ATA CCA GCA TCC-3'.

For the first 2-step nested PCR reaction to amplify the genes of 18S rRNA, the conditions for the primary PCR and secondary PCR were identical. Forty PCR cycles (94 C for 30 sec, 58 C for 30 sec, 72 C for 60 sec) were carried out in a PX2 Thermo Cycler (Thermo Electron, Dreieich, Germany) with an initial denaturation (94 C for 5 min) and a final extension (72 C for 10 min). For the second 2-step nested PCR reaction to amplify the genes of HSP-70, the conditions for the primary PCR and secondary PCR were identical. Forty PCR cycles (94 C for 30 sec, 56 C for 30 sec, 72 C for 60 sec) were carried out in the above PX2 Thermo Cycler with an initial denaturation (94 C for 5 min) and a final extension (72 C for 10 min). To attain an adequate reaction to amplify the genes of CpR1 in this study, the reaction solution was initially denatured at 94 C for 5 min and then subjected to 40 cycles of 94 C for 30 sec, 55 C for 30 sec, 72 C for 60 sec. The final extension was at 72 C for 10 min.

PCR products were identified by gel electrophoresis on a 2% agarose gel (BioBasic Inc., Toronto, Canada), performed with 5 μl of the reaction solution. The DNA fragments were confirmed by ethidium bromide staining (0.5 μg/ml, 10 min). A 100-bp DNA ladder was used as a DNA size marker. We used a Bio-Dye Terminator Cycle Sequencing Kit (5' Dye Terminator, Biosystem, Foster City, California) for sequence analysis. The gene sequences were aligned by use of the DNAMAN software program (Lynnon Biosoft, Vaudreuil, Canada).

**RESULTS**

The results of Cryptosporidium spp. monitoring of fecal samples from the livestock and avian farms in Taiwan are shown in Table 1. Primers were used to amplify 2 Cryptosporidium spp. gene loci, the partial 18S rRNA as well as HSP-70 genes, and 1 C. parvum gene locus, the CpR1 gene. Of the 107 samples collected, 10 (9.3%) were found to be positive for Cryptosporidium spp. by ELISA; 6 (5.6%) were confirmed as positive by both IFA and PCR. All the ELISA false positives were found in bovine specimens and might have occurred as a result of the presence of antigens in the stool rather than Cryptosporidium spp. Of the samples confirmed to be positive by IFA and PCR, 3 were bovine, 1 pig, and 2 sheep. No Cryptosporidium spp. were detected in any specimens from horses, ducks, chickens, or other birds.

Nucleotide sequence identification of the 18S rRNA and CpR1 genes in 6 Cryptosporidium spp. isolates are shown in...
Table I. Detection of Cryptosporidium spp. by ELISA, IFA, and PCR in fecal specimens from Taiwan.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>ELISA</th>
<th>IFA</th>
<th>18S rRNA</th>
<th>HSP-70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>75</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pigs</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sheep</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Horses</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ducks</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chickens</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Birds</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Table II. Primers for amplifying 18S rRNA nucleotide genes have been used by Ryan, Xiao et al. (2003), Ryan et al. (2005) and Geurden et al. (2006), successfully identifying a variety of different Cryptosporidium spp. The phylogenetic relationships of our Cryptosporidium isolates and additional Cryptosporidium spp. inferred by neighbor-joining analysis from pairwise comparisons of the 18S rRNA nucleotide genes are shown in Figure 2. The following additional Cryptosporidium spp. 18S rRNA sequences were obtained from GenBank: C. parvum human genotype (C. hominis, AF093489), C. parvum human isolates (DQ054817), C. parvum bovine genotype (AF093490), C. parvum rabbit genotype (AY458612), C. parvum Bovine genotype (AF093490), C. meleagrindis (AF112574), C. felis (AF108862), C. baileyi Chicken genotype (AF093495), C. muris Calf genotype (AF093496), C. andersoni (AB089285).
C. wrairi (AF115378), C. meleagrisids (AF112574), C. felis (AF108862), C. baileyi (AF093495), C. serpentis (AF093502), C. andersoni (DQ060422 and AB089285), and C. mursis calf genotype (AF093496). Analysis of our nucleotide sequence data identified 3 major clusters we grouped into C. parvum, C. andersoni, and 2 novel genotypes. The most frequently detected species sequence was C. parvum, found in 3 samples (2.8% of all samples). Cryptosporidium andersoni was found in 1 sample, and 2 novel species of Cryptosporidium were found in the other 2 specimens.

The primers used to amplify the CpRl protein gene of C. parvum specifically were designed by Lally et al. (1992). In our study, PCR for amplification of the CpRl gene was performed with the use of only 3 samples. The derived isolates were from cattle and sheep.

**DISCUSSION**

The first survey of Cryptosporidium spp. in Taiwan was done in 1990. More than 50% of the fecal specimens of avian species were diagnosed by the Ziehl–Neelsen method in Cryptosporidium spp. In one of our previous studies (Hsu et al., 2000), prevalences for Cryptosporidium spp. analyzed by ELISA in the fecal specimens of pigs, cattle, sheep, and avian species were 26.2% (11/42), 17.6% (3/17), 14.3% (1/7), and 18.2% (6/33) respectively. Watanabe et al. (2005) found a higher prevalence of Cryptosporidium spp. in cattle and sheep in Taiwan than we do in this study. Their fecal survey, which used a modified Ziehl–Neelsen method and IFA for Cryptosporidium spp., showed positive prevalences of 37.6% and 35.8% in cattle and sheep, respectively. These differences in detection were mainly caused by differences in sampling locations and detection methods.

In this study, C. andersoni identified in a pig fecal specimen (B2-6) was 98.0% (on the basis of 528 bases) similar to the C. andersoni (GenBank AB089285, DQ060422) and C. muris cattle genotypes (GenBank AF093496). Recent studies have reported that pigs are generally infected with a genetically distinct form of Cryptosporidium (pig genotype I and pig genotype II), as well as C. parvum cattle genotype (Morgan et al., 1999; Ryan, Samarasinge et al., 2003). However, C. andersoni is hard to find in pig specimens. Genetically confirmed C. andersoni infection has so far been found only in cattle, Bactrian camels, horses, and sheep (Xiao et al., 1999; Morgan et al., 2000; Ryan, Xiao et al., 2003). Pigs also be infected with C. andersoni, indicating that they can potentially serve as reservoirs of infection for feeding animals.

Cryptosporidium parvum is known to infect mainly ruminants and humans. Indeed, C. parvum is the most frequently reported species in mammals, with >150 species identified as hosts (Xiao et al., 2004). In our study, B2-12 (cattle-derived isolate) was identified as a C. parvum bovine genotype. B2-7 (cattle-derived isolate) and A-2 (sheep-derived isolate) were identified as C. parvum rabbit genotype and has high similarity (99%, on the basis of 528 bases) with C. parvum human genotypes (GenBank AF093489, DQ054817). This similarity indicates it has a wide host range and that it could possibly emerge as an important human pathogen with increasing contact between humans and feeding animals.

A novel sheep genotype (A-4) and cattle genotype (B2-21) of Cryptosporidium spp. were identified in this study. These 2 genotypes were 96% similar to each other and are most closely related to C. andersoni (79% similarity for the 18S rRNA locus). The DNA sequences of the 18S rRNA gene of these 2 isolates, sheep genotype (A-4) and cattle genotype (B2-21), were not identical to those of any known Cryptosporidium spp. and have been deposited under the GenBank accession numbers DQ904453 and DQ904454, respectively.

Our previous and present studies have shown Cryptosporidium spp. to be widely distributed in Taiwan farm animals, illustrating the need for molecular characterization in epidemiological studies. The procedures proposed in this study provide a reliable and sensitive method of detecting and identifying Cryptosporidium spp. in stool samples. In this study, we identified C. parvum, C. andersoni, and 2 novel genotypes of Cryptosporidium in the feces of livestock in Taiwan, and expanded the host range of C. andersoni. The study highlights the importance of analyzing a wide range of Cryptosporidium spp. isolates from different hosts, as well as geographic environments, and indicates that livestock farms should be considered potential sources of water contamination in Taiwan.

**ACKNOWLEDGMENTS**

This work was funded by the National Science Council of Taiwan, R.O.C. (NSC94-2211-E-194-001). We are grateful to Mr. James Steed for his editing of this manuscript.

**LITERATURE CITED**


Molecular and biological characterisation of Cryptosporidium in pigs. Australian Veterinary Journal 77: 44–47.


DESCRIPTION OF A NEW SPECIES OF CRASSICUTIS MANTER, 1936, PARASITE OF CICHLASOMA BEANI JORDAN (OSTEICHTHYES: CICHLIDAE) IN MEXICO, BASED ON MORPHOLOGY AND SEQUENCES OF THE ITS1 AND 28S RIBOSOMAL RNA GENES

Gerardo Pérez-Ponce de León, Ulises Razo-Mendivil, Rogelio Rosas-Valdez, Berenit Mendoza-Garfías, and Hugo Mejía-Madrid

Instituto de Biología, Universidad Nacional Autónoma de Mexico, Laboratorio de Helmintología, Ap. Postal 70-153, C.P. 04510, Mexico D.F., Mexico. e-mail: ppdleon@servidor.unam.mx

ABSTRACT: A new species of Crassicuts Manter, 1936 is described from the Sinaloan cichlid Cichlasoma beani (Jordan) (Osteichthyes: Cichlidae) in the upper Río Santiago basin. Crassicuts choudhuryi n. sp. differs from most of the other nominal species by having testes located in a symmetrical position. The only other species of the genus that includes some specimens exhibiting this trait is Crassicuts intermedius (Sztat 1954), a species found in 5 species of siluriforms and 1 species of characiform in South America. However, this species differs from Cr. choudhuryi n. sp. by having testes almost half of the size, and vitelline follicles extending anteriorly to the region between the acetabulum and the intestinal bifurcation. The new species is morphologically very similar to Crassicuts cichlasomae Manter, 1936, but clearly differs from this species because of the constantly symmetrical position of the testes. Additionally, Cr. choudhuryi n. sp. is found in the Santiago River basin on the Pacific slope of Mexico, parasitizing specifically the endemic Ci. beani that does not co-occur with any other cichlid. Cr. cichlasomae exhibits more hosts (about 25 species of cichlids only in Mexico) and a wider distribution range that extends from northeastern Mexico southward to Central America, Cuba, and Brazil. To corroborate that our specimens were not conspecific with Cr. cichlasomae, sequences of the internal transcribed spacer (ITS1) and the 28S ribosomal RNA genes of individuals from several populations (recently collected in southeastern Mexico) were obtained and compared to the species described herein. Sequence divergence (1.3% for the 28S and 4.0% for the ITS1) gives further support to the erection of a new species.

Cribb and Bray (1999) redefined Crassicuts Manter, 1936, while conducting a revision of the Apocreadiidae Skrjabin, 1942. This genus is characterized by consistently lacking tegumental spines, being less elongate, and having an excretory pore opening distinctly dorso-subterminally rather than terminally. The combination of these morphological traits restricts the genus to 7 species, 6 of which are parasites of freshwater fish: Cr. cichlasomae (the type species); Crassicuts bravoae Jiménez and Caballero y Caballero, 1974; Crassicuts chuscoi Pearse, 1920, Cr. intermedius; Crassicuts opisthoseminis Bravo Hollis and Arroyo, 1962; and Crassicuts wallini Pearse, 1920. A single species, Crassicuts archosargi Sparks and Thatcher, 1960, parasitizes a brackish water fish. Host and geographic distribution of members of this genus include 3 species from South America, 2 exclusively found in cichlids (Cr. chuscoi and Cr. wallini from Venezuela), and 1, Cr. intermedius, in siluriforms and characids from Argentina, Paraguay, and Brazil. Additionally, 1 species was found in a Central American cichlid (Cr. opisthoseminis from northwestern Costa Rica), and 1 species found in cichlids allocated to the Nearctic region in northeastern Mexico, Cichlasoma bravoae. The latter species, however, was not consider as valid by Vidal-Martinez et al., (2001), suggesting that “most probably” it is a synonym of Cr. cichlasomae. Finally, the genus contains 1 widespread species, Cr. cichlasomae, whose host and distributional range include about 25 species of cichlids in 10 states of the Mexican Republic along the Gulf of Mexico slope and the Yucatán Peninsula (see Pérez-Ponce de León et al., 2007, and references therein), southwards to several localities in Central America (Costa Rica and Nicaragua, see Watson, 1976; Aguirre-Macedo et al., 2001; Rodríguez-Ortiz et al., 2004), Cuba (Moravec and Barus, 1972; Vinjoy et al., 1985), and South America (Brazil, see Fernandez and Kohn, 2001). The only brackish water representative of the genus is Cr. archosargi that is a parasite of the spurdar, Archosargus probatocephalus (Walbaum), in Louisiana, Mississippi, and Texas (Sparks and Thatcher, 1960; Joy, 1971; Overstreet, 1976).

In the last 10 yr, a great effort has been made to document the biodiversity of helminth parasites of freshwater fishes in Mexico. As a part of this ongoing survey, an undescribed species of Crassicuts was found and is described herein based on morphological characters, as well as on data on sequence divergence by using ITS1 and 28S ribosomal RNA genes.

MATERIALS AND METHODS

Nineteen specimens of the Sinaloan cichlid, Cr. beani, were collected in a tributary of the Río Santiago basin, in the locality of Jesús María Corte, State of Nayarit, in September 2001. The Río Santiago runs from the Lago de Chapala westward to the Pacific coast of Nayarit. Hosts were collected by angling, and examined for helminths 4 hr after capture. Digeneans were collected from the intestines of fish and placed in saline (0.65%). Some worms were relaxed in hot (near boiling) tap water, and fixed in 70% ethanol, while others were immediately placed in 100% ethanol for DNA extraction. Preserved specimens were stained with Mayer’s paracarmine and Gomori’s trichrome, cleared in methyl salicylate, dehydrated in graded ethanol series, and mounted as permanent slides using Canada balsam. Drawings were made with the aid of a drawing tube attached to the microscope. Measurements are presented in micrometers (μm) with the mean followed by the range in parentheses. Specimens were deposited in the Colección Nacional de Helminitos (CNHE), Instituto de Biología, UNAM.

For morphological comparisons, specimens deposited at the CNHE and at the Harold W. Manter Laboratory of Parasitology (HWML) were examined. CNHE specimens include Cr. cichlasomae ex Vieja fenes-trata (Günther), Cichlasoma sp., Cichlasoma urophthalmus (Günther), and Thorichthys meeki Brind from the following localities in south-eastern Mexico: Lake Catemaco (1266, 1734, 5274) and Los Tuxtlas (5271, 5272), Veracruz State; Puerto Morelos (1297) and Laguna Bakalar (2841), Quintana Roo State; Celestún Lagoon (2845, 2841), Yucatán State; Cr. bravoae ex Herichthys cyanoguttatus Baird and Girard, Presa Rodrigo Gómez, Nuevo León State (1584); Cr. opisthoseminis ex Cichlasoma sp., Guanacaste, Costa Rica (763). HWML specimens include Cr. cichlasomae ex Cichlasoma mayorum Hubbs, Canton Xioles, Chichen-Itza, (801), Yucatán, and Cichlasoma labiatum (= Amphilo­plus labiatus Günther) from Nicaragua (800). We also observed specimens from the following regional parasite collections: Colección Hel­míntológica del CINVESTAV, Mérida, Yucatán, Mexico, (CHCM), 70

Received 14 July 2007; revised 6 July 2007; accepted 9 July 2007.

© American Society of Parasitologists 2008

257
specimens; Colección Helmintológica del Sureste de México (CIHU-JAT), Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco, México, (211 specimens, ex Cl. urophthalmus, Vieja sinsyilla, and V. fenestra). Serapio López provided specimens from his personal collection (20 specimens, ex Cl. urophthalmus, Petenia splendida, Thorichthys helleri, V. fenestra).

Genomic DNA was extracted from individual gravid worms according to the protocols proposed by Hillis et al. (1996). The primer pairs BDI (5'-GTCGTAACAGGTTTCGTTAG-3') and BD2 (5'-ATGCTTTAAATCCGAGGGT-3') (Bowles et al., 1995), 28s (5'-AACAGGTGCTGTTAATGCTTTAAATCCGAGGGT-3') and 5.8S (5'-CTTACACTGGTGTTGAAATTCAGCGGGTTG-3') (Palumbi, 1996), and LO (5'-GGTGAAACCGCTCTGTCGTAACAGGTTTCGTTAG-3') (Tkach et al., 2000) were used to amplify approximately 1000 bp of the internal transcribed spacer 1, and approximately 900 bp of the 28S ribosomal RNA gene, respectively. The amplification program for both molecular markers consisted of 1 min at 94 C followed by 35 cycles of 30 sec at 92 C, 45 sec at 55 C, and 1 min at 72 C; reactions were held at 72 C for 10 min to complete elongation and then dropped to 4 C. The PCR products were purified by using QIAquick PCR Purification Kit (Qiagen, Valencia, California). Purified products were sequenced on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, California) using Big Dye Terminator chemistry, and incorporating the same primers as those used in previous PCRs. For both molecular markers genes sense and antisense strands were sequenced, subsequently assembled, and aligned by eye using the computer program BIOEDIT (v. 3, Hall, 1999).

Pairwise distance matrices were obtained for each molecular marker using PAUP (v. 4b10, Swofford, 2002). For comparison, specimens of 2 species of Crassicutis were obtained from the United States and Mexico. Specimens of Cr. cichlasomae were collected in July, 2006 from Cl. urophthalmus in 4 localities, 1 in the State of Tabasco (El Espino), 1 in Campeche (Uumlan), and 2 in Yucatan (El Corchito and Cenote X'laich) in southeastern Mexico (Table I). The 28S and ITS1 ribosomal genes of individual specimens of all those populations were also sequenced following the same protocols. Sequences obtained in this study were submitted to GenBank (accession numbers are presented in Table I). Representative specimens of Cr. cichlasomae and Cr. archosargi from those collections aforementioned were also deposited at the CNHE.

DESCRIPTION
Crassicutis choudhuryi n. sp.  
(Figs. 1–3)

Based on measurements of 18 specimens (12 of them gravid) unless sample size (n) otherwise specified. Body oval, 2,181 (1,040–2,571) long by 1,000 (576–1,351) wide. Tegment smooth. Oral sucker subterminal, spherical, 217 (128–258) long by 224 (142–281) wide. Dispersed eye-spot pigment present near pharynx. Acetabulum pre-equatorial, 236 (167–291) long by 248 (162–306) long. Ratio of oral sucker subterminal, spherical, pretesticular, posterolateral to acetabulum, constant or nearly so, smooth to slightly lobed in shape, 134 (41–207) long by 141 (52–188) wide. Seminal receptacle sacculate, preovarian, longer than and overlapping ovary. Mehlis' gland compact, adjacent to the ovary. Laurer's canal inconspicuous. Vitelline follicles mainly extracecal, with few follicles caecal and intercecal, extending from mouth to the pharynx and the intestinal bifurcation level to short distance 146 (63–194) from the posterior extremity. Vitelline follicles confluent in preacetabular and posttesticular regions. Uterus pretesticular, intercecal, short, with few operculated eggs. Eggs large,


**Taxonomic summary**

*Type host:* Cichlasoma beani (Jordan) (Osteichthyes: Cichlidae).
*Site of infection:* Intestine.
*Prevalence of infection:* 68.4%.
*Mean intensity, and intensity range:* 5.8, 1–16.
*Type locality:* Tributary of the Río Santiago basin, in the locality of Jesús María Corte, State of Nayarit, Mexico (21°43'40"N, 104°53'04"W).
*Specimens deposited:* Holotype: CNHE 5603. Paratypes: CNHE 5604 (17 specimens).
*Etymology:* The species is named after Anindo Choudhury in recognition of his important contribution to freshwater fish parasitology in North and Central America.

FIGURES 2–3. (2) Detail of the ovarian complex and testes of *Cr. choudhuryi* n. sp. (paratype). Scale bar = 200 μm. (3) Egg of *Cr. choudhuryi* n. sp. (paratype). Scale bar = 50 μm.

**Remarks**

We compared our specimens with those of *Cr. archosargi* and *Cr. cichlasomae* from several localities in Mexico (Table 1). *Crassicutis choudhuryi* n. sp. is readily distinguished from all but 1 congeneric species, *Cr. intermedius*, by having testes constantly located in a symmetrical position. *Crassicutis intermedius* occurs in siluriform (*Cochliodon cochliodon* Kner, *Hypostomus pirata* Weber, *Hypostomus commersoni* Valenciennes, *Hypostomus boulengeri* Eigenmann and Kennedy, and *Hoplosternum littorale* Hancock) and characiform fishes (*Le-porius copelandi* Steindachner) from South America in the River Plate at Buenos Aires, Argentina; the Guaiba estuary, Brazil; and the Paraguay and Paraná rivers in Paraguay (see Bray et al., 1996, and refer-
Table II. Pairwise distance matrix of ITS1 (above), 405 bp and 28S (below), 789 bp, of 6 isolates belonging to 3 species of Crassicutis.

<table>
<thead>
<tr>
<th></th>
<th>1. C. choudhuryi n. sp.</th>
<th>2. Cr. cichlasomae (XIach)</th>
<th>3. Cr. cichlasomae (El Corchito)</th>
<th>4. Cr. cichlasomae (El Espino)</th>
<th>5. Cr. cichlasomae (Ulumal)</th>
<th>6. Cr. cichlasomae (Tres Palos)</th>
<th>7. C. archosargi (Ocean Springs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. C. choudhuryi n. sp.</td>
<td>—</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>12.0</td>
</tr>
<tr>
<td>2. Cr. cichlasomae (XIach)</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>—</td>
</tr>
<tr>
<td>3. Cr. cichlasomae (El Corchito)</td>
<td>1.4</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>4. Cr. cichlasomae (El Espino)</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>5. Cr. cichlasomae (Ulumal)</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>6. Cr. cichlasomae (Tres Palos)</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>7. C. archosargi (Ocean Springs)</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>—</td>
</tr>
</tbody>
</table>

**DISCUSSION**

To further corroborate the validity of the new species and make sure that it does not represent a morphological variation of *Cr. cichlasomae*, we sequenced the ITS1 and the 28S ribosomal RNA genes of individual specimens of *C. choudhuryi* n. sp. and also from specimens of other 2 species, i.e., *Cr. cichlasomae* collected within the allegedly “typical” distributional range of the species in southeastern Mexico, in the states of Tabasco, Campeche, and Yucatán; and *C. archosargi* from the brackish water fish *A. probatocephalus* from Mississippi. Needless to say, *C. archosargi* is the only congenic species that occurs in brackish water fishes (Cribb and Bray, 1999), and is characterized by having testes located in tandem. Additionally we sequenced specimens of *Crassicutis* collected from the cichlid *Cichlasoma trimaculatum* in the Tres Palos Lagoon, a brackish water system near Acapulco, Guerrero State. All specimens collected from this particular locality exhibited testes placed in tandem. Sequence divergence as shown in the pairwise distance matrix (Table II) indicate that all the individuals of *Cr. cichlasomae* (including the specimens from Tres Palos Lagoon) are identical for both molecular markers, and that they differ from the ones corresponding to the new species by 1.4% for the 28S gene, and by 4.0% for the ITS1 gene. With respect to *C. archosargi*, the new species exhibits an even much larger divergence level, by 6% for the 28S gene and by 12% for the ITS1 gene. In addition, *C. archosargi* differs from *Cr. cichlasomae* in 6% for the 28S, and 4.0 for the ITS1. This level of divergence in both ribosomal gene sequences has been used in other studies on digenetic trematodes to establish species differentiation (see van Herwerden et al., 1998, 1999; León-Ré­gagnon et al., 1999; Jousson et al., 2000; Tkach et al., 2000; León-Régagnon and Paredes Calderón, 2002; Razo-Mendi­vill et al., 2004; Miura et al., 2005; Nolan and Cribb, 2005; Olson and Tkach, 2005). These studies have proved that such molecular markers are useful not only in establishing species limits, but ences therein). However, as noted by Bray et al. (1996), the position of testes is quite variable in specimens collected from different host species, and that species is defined as having testes symmetrical to oblique. In the new species we describe herein, symmetrical position of testes is a constant trait, and none of the specimens we studied showed evidence of a different position of testes. In addition, the new species can be distinguished from *Cr. intermedius* by having much larger testes (almost double sized), and by having vitelline follicles that extend anteriorly to midway between the pharynx and the intestinal bifurcation and are not confluent in that region. Further, the species we describe herein was found in *C. beani*, a species that does not occur with any other cichlid. *Cichlasoma beani* represents the cichlid species with the northernmost distribution along the Pacific slope, an area restricted to river basins from the lower Rio Yaqui basin in the State of Sonora, southward to lower Rio Arneca in Jalisco State (Miller et al., 2005).

We determined 3 reasons to examine numerous specimens of *Cr. cichlasomae*. First, this is the type species for the genus, and the one most commonly found infecting cichlids in Mexico (25 species in localities along 10 states). Second, *Cr. cichlasomae* shows a wide distributional range. According to published records, it has been found in cichlids from Brazil, Costa Rica, Nicaragua, Cuba, and Mexico (Manter, 1936; Bravo and Arroyo, 1962; Watson, 1976; Moravec and Barus, 1972; Vinjoy et al., 1985; Aguirre-Macedo et al., 2001; Vidal-Martínez et al., 2001; Pérez-Ponce de León et al., 2007). Third, this species exhibits some degree of morphological intraspecific variability (Figs. 4A–D). The new species closely resembles *Cr. cichlasomae*. However, of the approximately 120 specimens examined from the CNHE, only 2 exhibited a morphology that suggested either a symmetrical or tandem position of the testes, which definitively shows that testes are constantly located in an oblique position in this species. To further corroborate our observations, we studied another 301 specimens of *Cr. cichlasomae* from regional and personal collections (see Materials and Methods). Of the 301 specimens, 267 exhibited oblique testes, 32 exhibited testes in tandem and only 2 were symmetrical. We observed that the 2 specimens with symmetrical testes were immature. We assume that the tandem or oblique position of the testes is a characteristic of *Cr. cichlasomae* and that some slight difference may occur, most probably as a result of fixation procedures. Scholz et al. (1995) studied the life cycle of *Cr. cichlasomae* in a small swamp in a lagoon factory near Mérida, in the Yucatán Peninsula. The developmental stages of this digenean are very well described in that paper, in which Figure 13 (p. 73) illustrates that the newly formed testes in young metacercariae are apparently symmetrical in position, while testes are characteristically oblique in adults, a condition that seems to be modified during development. Therefore, a second assumption could be made that the new species we describe here retains the ontogenetic trait in the adult stage.

There is another species of apocreadiid trematode, *Trematothorax hapatrichromos* Dollfus, 1950, a parasite of the cichlid *Haplochromis molflat* (Castelnau) from the Congo Republic in Africa (Dollfus, 1950), that might be related, and should be compared with the new species (S. Curran, pers. comm.). The African species is characterized by having a thick cuticle, and it is also found in cichlids (see Manter, 1962; Cribb and Bray, 1999). This species is morphologically very similar to *C. choudhuryi* n. sp. but it possesses a distinctive character, i.e., ceca are fused posteriorly, thus forming a cyclocoel.
also in uncovering cryptic species (morphologically similar and genetically distinct). Delimiting species of digeneans is often difficult owing to their limited morphological characters, and this may have resulted in a gross underestimation of the true number of species. In this sense, DNA-based identification and discovery of helmint species has implications for our understanding of global biodiversity (Poulin and Morand, 2004) and, in this case, it has a direct implication for efforts to establish the species richness of digeneans in Mexican vertebrates (Pérez-Ponce de León, 2001, 2007). Thus, the validity of the new species herein described is strongly supported as it is based on both morphological and molecular evidence.

Cribb (2005) pointed out that the geographic distribution of *Crassicutis* species is restricted to the Atlantic coast of North and South America. Diagnosis of the genus should indicate, from this point on, that species of this genus are mostly restricted to the Atlantic coast. Some records have been made on the Pacific slope, since records of particular species have been established in cichlids along the Pacific coast of Costa Rica in the province of Guanacaste (see Rodríguez-Ortíz et al., 2004), as well as the record we establish in the present study. Likewise, our finding of a new species of *Crassicutis* poses an interesting question regarding the biogeography of cichlids and their helminth parasites. Recently, Pérez-Ponce de León and Choudhury (2005) discussed the biogeographical implications of the helmint fauna of Mexican cichlids. This group of freshwater fishes apparently utilized the developing Isthmus of Panama in the late Tertiary period to colonize the southern lowlands of Mexico. A few species penetrated farther north along the Pacific coastal drainages, resulting in the presence today of *Ci. beani* (Miller and Smith, 1986). Freshwater members of *Crassicutis* are, without doubt, part of the biogeographical core parasite fauna in cichlids and, with the exception of *Cr. cichlasomae*, all the other species are geographically restricted. The new species we describe herein represents the northernmost record of a species of *Crassicutis*; however, in the absence of a robust phylogenetic analysis among species of *Crassicutis*, it is impossible to establish the sister group relationships between the new species and their congeners. Nonetheless, it can be hypothesized that this species evolved as a peripheral isolate of ancestral populations of *Cr. cichlasomae* that were isolated following the colonization and concomitant speciation of their host in Pacific drainages of Mexico.

**ACKNOWLEDGMENTS**

We thank Omar Domínguez, Rodolfo Pérez, Jaqueline Bravo, Elizabeth Martínez, Martín García, and Serapio López for their help during our field work. Stephen Curran kindly loaned specimens of *C. archosargi* he collected in Mississippi, and provided insights to compare the new species with other apocreadiids. Laura Márquez provided technical assistance with the sequencer and Virginia León-Régagnon provided access to the Molecular Systematics Laboratory. We want to thank the following people for their help with collecting fish: Diana Liz Trejo Torre and Roman Chan Chi, Cenote Xlacah, Zona Arqueológica Dzibilchaltun; and Miguel Angel García Estraga, Sociedad Cooperativa El Corchito, Yucatán. Scott Gardner, Curator, and Agustín Jiménez, Collection Manager, kindly provided specimens from the HWML. Luis García-Prieto kindly provided specimens from the CNHE. Leopoldina Garda-Prieto kindly provided specimens from the CHCM (Colección Helminológica del CINVESTAV, Mérida, Yucatán, Mexico); Leticia Magaña provided specimens from the CIUJAT (Colección Helminológica del Sureste de México, Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco, Mexico); and Serapio López provided specimens from his personal collection. U.R.M. wishes to thank CONACYT for his postdoctoral fellowship. R.R.V. and H.M.M. wish to thank DGEP and CONACyT respectively for scholarships for accomplishment of their Ph.D. degrees. This study was supported by grants from PAPIIT-UNAM IN220605 and CONACyT 47233 to G.P.P.

**LITERATURE CITED**


A NEW SPECIES OF OSWALDOCRUZIA (MOLINEIDAE: NEMATODA) IN CHAUNUS MARINUS (AMPHIBIAN: BUFONIDAE) (LINNEAUS, 1758) FROM BRAZIL

Jeannie N. Santos, Elane G. Giese, Arnaldo Maldonado, Jr.*, and Reinalda M. Lanfredi†
Laboratório de Biologia Celular–Centro de Ciências Biológicas, Universidade Federal do Pará, Av. Augusto Correa, s/n CEP. 66075.110 Belém, Pará, Brazil. e-mail: reylanf@biof.ufrj.br

ABSTRACT: Oswaldocruzia belenensis n. sp. (Strongyliida: Molineidae) from the small intestine of Chaunus marinus (L.) from Belém, Pará State, Brazil is described and illustrated by light and scanning electron microscopy. Oswaldocruzia belenensis n. sp. is a neotropical species of this genus, harboring caudal bursa Type II, spicules divided in 3 branches, i.e., a blade, shoe, and fork. The blade is divided in 4 points, of which at least 2 are bifurcated. Cervical alae are absent; there is a simple cephalic vesicle and synlophe with low ridges perpendicular to the body without chitinous supports. The most closely related species are O. bonsi and O. lesurei. Oswaldocruzia belenensis n. sp. differs from O. lesurei and O. bonsi by the number and location of cephalic papillae, rays 2–3 and 5–6 running parallel and slightly separated, ray 6 not overlapping ray 8, and body structure morphology. Oswaldocruzia belenensis n. sp. also differs from O. lesurei by the discontinuity of the longitudinal ridges, the number of subdivisions of the blade, and the absence of extra processes at the bifurcation level of the fork of the spicules. The new species differs from O. bonsi by male and female body dimensions, the symmetry of the caudal bursa, dimension and subdivisions of the spicules without extra processes of the fork, 2 extra processes at the distal division of the blade, and location of ray 7 at the anterior margin of the cloacal aperture. Oswaldocruzia belenensis n. sp. represents the 82nd species assigned to the genus.

Currently 81 species are assigned to Oswaldocruzia Travassos, 1917; these nematodes are typically found in the intestine of amphibians and reptiles (Ben Slimane et al., 1996, Bursey and Goldberg, 2005; Bursey et al., 2006; Durette-Desset et al., 2006), but only 10 species are known to infect Bufonidae in South America. Chaunus marinus (Linnaeus, 1758) is a medium-size toad with a wide distribution across Brazil (Kwet et al., 2006). The purpose of the present paper is to describe a new species of Oswaldocruzia harbored by C. marinus and to add ultrastructural features by scanning electron microscopy, contributing to the taxonomy of this group.

MATERIAL AND METHODS

Five C. marinus were collected in Belém, Pará State, Brazil, between May and December 2006 and examined for helminths. Intestinal nematodes found were fixed in AFA (2% glacial acetic acid and 3% formaldehyde in 95% ethanol), postfixed in 1% OsO4 and 0.8% K2Fe(CN)6, and processed for examination by light and scanning electron microscopy (SEM) following Mafra and Lanfredi (1998). Illustrations were made using a Zeiss microscope with the aid of the camera lucida. Measurements are given in micrometers unless otherwise indicated as the mean and standard deviation with range in parentheses.

RESULTS

All 5 of the toads presented intestinal infection with the new species of nematode. Voucher helminths were deposited in the Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC), Fundação Oswaldo Cruz, Brazil.

DESCRIPTION

Oswaldocruzia belenensis n. sp. (Figs. 1–11)

General: Small, slender, uncoiled nematodes. Obvious sexual dimorphism (Figs. 1–9), males approximately two-thirds length of females.

Excretory pore without circular cuticular disk around it, situated on posterior third of esophagus, deirids at level of excretory pore on esophageal region (Fig. 1). Anterior region presents simple cephalic cuticular vesicle with anterior swelling and transversal annulations (Fig. 1). Junction of cephalic vesicle with rest of body oblique (Fig. 1). No cervical alae. Cuticle with parallel, low longitudinal cuticular ridges without

Oswaldocruzia belenensis n. sp. by scanning electron microscopy. (12) Anterior end lateral view showing cephalic structures, simple cuticular vesicle with transversal annulations, cuticle at distal end smooth, mouth surrounded by dorsal and ventrolateral lips, longitudinal ridges. Bar: 10 μm. (13) Apical view of the anterior end, mouth opening with esophageal teeth, dl with a pair of cephalic papillae and lv lips with one cephalic papillae and one amphid each. Bar: 10 μm. (14) Detail of the mouth showing dorsal and ventrolateral lips and esophageal teeth with a pore on its distal end. Bar: 5 μm. (15) Anterior region ventral view evidencing excretory pore, deirid, and longitudinal ridges emerging and disappearing. Bar: 50 μm. (16) Detail of a deirid. Bar: 5 μm. Abbreviations: lr—longitudinal ridges; dl—dorsal lip; cv—simple cuticular vesicle; vl—ventrolateral lip; a—amphid; pa—papillae; e—esophageal teeth; p—pore of the esophageal teeth; ep—excretory pore; de—deirid.
chitinous support, occasionally one disappears or appears along body, comprising the synloph (Figs. 10–11), ridges beginning obliquely posterior to cephalic vesicle (Fig. 1).

**Male (based 1 holotype and 9 paratypes):** Length: 6.4 mm ± 0.6 (6.0–7.2 mm), width at esophagus-intestinal junction 109.3 μm ± 19.6 (71.4–128.6 μm). Nerve ring, excretory pore and deirids: 218.5 μm ± 26.1 (171.4–247.3 μm), 354.0 μm ± 50.6 (256.2–426.1 μm) and 380.0 μm ± 47.0 (300.0–456.5 μm) from apex, respectively. Claviform oesophagus 491 μm ± 48.0 (400–550 μm) long. Anterior end with simple cuticular vesicle 86.2 μm ± 7.3 (67.2–100.0 μm) in length, with transversal cuticular striations. Cuticle with 29 conspicuous longitudinal ridges in transversal section at posterior region of esophagus (Fig. 10). Caudal bursa trilobed and symmetric, Type II with 2-1-2 pattern (Figs. 2, 3) (see Ben Slimane et al., 1996), rays 2 and 3 running parallel throughout, but slightly separated, reaching edge of bursal membrane, rays 4, 5, and 6 with common origin, ray 4 short turning cephalad, not reaching bursal margin and separated from 5 and 6. Rays 5 and 6 running throughout, but slightly separated, turning caudad and reaching edge of bursal membrane. Rays 6 and 8 with independent origin. Origin of ray 8 on and perpendicular to dorsal trunk, turning caudad near its origin and running parallel, but not overlapping rays 6 and distancing from it in distal third, not reaching bursal margin. Ray 9 arising about...
Oswaldocruzia belenensis n. sp. males by scanning electron microscopy. (24) Lateral view of the posterior region: longitudinal ridges, some disappearing and others appearing near the caudal bursa, rays 2–6 and 8, papillae of rays 4 and 8. Bar: 50 μm. (25) Laterodorsal view of the caudal bursa showing the site were longitudinal ridges end with some still appearing near this point, dorsal lobe of the bursa with rays dorsal and 9 projected on the surface, ray 6 separated from ray 8. Papillae at the distal end of rays 4 and 8. Rays 5 and 6 distal end at the bursa margin. Bar: 10 μm. (26) Ventral view of the caudal bursa showing genital cone, cloacal aperture, dorsal lobe of the bursa with rays dorsal and 9, rays 2, 3, 4, and 8 and papillae of ray 4. Bar: 10 μm. (27) Ventral view of the bursa evidencing the genital cone, ray 7, and zero and dorsal lobe of the bursa with dorsal ray and 3 pairs of small papillae at the distal end of ray 9 and dorsal (thin arrows), cell-like structure flanking dorsal ray. Bar: 10 μm. Abbreviations: ri—ridge; r2, r3, r4, r5, r6, r7, r8, r9, rz—rays; dr—dorsal ray, dl—dorsal lobe; gc—genital cone; ce—cell-like structure.

one-third of distal end of dorsal trunk; ray 9 right arises more anteriorly than left one, before division of dorsal ray that divides in 2 branches, each branch bifurcates; internal ones longer than externals (Fig. 3). On both sides of dorsal ray is a cell-like structure (Fig. 3). Right spicule 161.4 μm ± 3.4 (154.4–183.9 μm) longer than left spicule 160.5 μm ± 6.8 (148.4–187.1 μm), both divided proximally in 3 branches: shoe spoolike, blade divided distally in 4 tips, 2 divided in 2, and fork divided in posterior third (Figs. 4–7).

Female (based 1 allotype and 9 paratypes): Length 9.1 mm ± 1.4 (6.1–10.4 mm), width 123.8 μm ± 27.1 (100–165.2 μm) at the widest region of the esophagus. Simple cephalic cuticular vesicle 89.4 μm ± 9.8 (74.1–104.3 μm) in length with transversal striations. Claviform esophagus 540.3 μm ± 24.7 (478.6–571.4 μm) in length. Nerve ring, excretory pore, and deirids: 238.8 μm ± 28.4 (200–292.9 μm), 401.1 μm ± 35.9 (347.8–478.3 μm), and 431.2 μm ± 52.6 (356.5–513.1 μm) from anterior end, respectively. Cuticular ridges evenly spaced, approximately 29–31 in number at level of the esophagus (Fig. 11). Vulva transverse slit (Fig. 8), anterior lip no salient, 3.2 mm ± 0.5 (2.1–3.7 mm) from posterior end. Vagina short, emerging vestibule dislocated from midpoint, vestibule 355.8 μm ± 57.0 (192.9–443.5 μm) (Fig. 8). Anus 176.8 μm ± 23.6 (143.5–95.7 μm) in length and tail terminating in flexible filament (Fig. 9). Amphidelphic, eggs in double file in uterus at morula stage of development, 70.6 μm ± 3.3 (67.8–75.2 μm) long by 38.3 μm ± 1.5 (36.6–40.3 μm) wide (Fig. 8).

Scanning electron microscopy: Scanning electron microscopy revealed details of surface ultrastructure of O. belenensis, adding new features of taxonomic value to species of Oswaldocruzia. Anterior region of males and females similar, with females slightly larger. Anterior
end dome-shaped with smooth cuticle; triradiated mouth opening surrounded by 3 lips, with dorsal one possessing 2 cephalic papillae and ventrolateral 1 papilla and 1 amphid each (Figs. 12, 13). So-called esophageal tooth (Figs. 12, 13, 14) seems to originate at dorsal internal rays distal ends with small papillae; dorsal one flanked by pair of cell-like structures (Fig. 27). Genital cone well developed; its anterior margin presents ray zero flanked by rays 7 (Figs. 26, 27).

**Taxonomic summary**

*Host:* Chaunus marinus (Linnaeus, 1758).
*Site:* Small intestine.
*Locality:* Belém, Pará State, Brazil, 01°28′03″S, 48°20′18″W.

*Type specimens:* Holotype male, CHIOC 36855; allotype female, CHIOC 36855b; paratypes CHIOC 36855c-d-e-f.

**Etymology:** The new species is named in reference to the city of collection.

**REMARKS**

Among the 18 Neotropical *Oswaldocruzia* with Type II caudal bursa (Ben Slimane and Durette-Desset, 1993; Ben Slimane et al., 1996; Bursey and Goldberg, 2006; Durette-Desset et al., 2006), *O. lescreui* Ben Slimane and Durette Desset, 1996, a parasite of *Hoplobatrachus tigerinus* from French Guiana, *O. bonsi* Ben Slimane and Durette Desset, 1993, a parasite of *Bolitoglossa equatoriana* of rays 7 at the anterior margin of the cloacal aperture.

**ACKNOWLEDGMENTS**

Brazilian financial support came from Conselho Nacional Desenvolvimento Científico e Tecnológico (CNPq), Fundação Estadual de Amparo a Pesquisa Carlos Chagas Filho, (FAPERJ), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PROCAD). We thank Dr. Gerald W. Esch and the anonymous referees for valuable comments and suggestions.

**LITERATURE CITED**


REPLACEMENT OF THE PREOCCUPIED NAME DAVISIA LAIRD 1953 AND DESCRIPTION OF A NEW MYXOZOAN SPECIES (MYXOSPOREA: SINUOLINEIDAE) FROM SEBASTISCUS MARMORATUS (CUVIER, 1829) IN THE EAST CHINA SEA

Yuanjun Zhao*, Yang Zhou, Michael L. Kent†, and Christopher M. Whippst
The Key Laboratory of Animal Biology of Chongqing, Chongqing Normal University, Chongqing 400047, China

ABSTRACT: The myxozoan genus Davisia Laird 1953 is preoccupied by Davisia Del Guercio 1909 (Insecta: Hemiptera). Here, Myxodavisia nomen novum is proposed to replace the preoccupied name, and a new species is described. Myxodavisia sebistisca n. sp. was found in the urinary bladder of Sebastiscus marmoratus, collected from coastal waters off Xiamen in the East China Sea. The parasite is characterized by a disporous trophozoite; spherical to subspherical spore, 13.1 ± 0.3 (12.7–13.6) by 12.3 ± 0.9 (10.9–13.5) μm in size; curved sutural line; 2 shell valves each with a long lateral appendage 119.4–335.2 μm in length; and 2 spherical or subspherical polar capsules, equal in size, 4.6 ± 0.6 (3.2–4.6) μm in diameter. Traditionally, Myxodavisia is distinguished from Ceratomyxa, Sinuolinea, and Sphaerospora spp. by having spores that possess a distinct central chamber and lateral appendages. A review of the literature reveals that the presence or absence of a clear septum between these spore components is open to interpretation. Indeed, in immature spores of M. sebistisca n. sp., there was an indication of a demarcated appendage, but in some mature spores, no clear separation was apparent. Our findings suggest that future revision of this genus is warranted, particularly once DNA sequence data become available.

In China, myxosporean parasites remain a poorly studied group compared with the abundant marine fishes found in Chinese sea waters. Early research focused on myxozoans of fishes in the South China Sea, although these reports are scattered (Xie and Chen, 1988; Wu, 1991; Wu et al., 1993, 1994). The knowledge base of myxozoans from northern Chinese sea waters, i.e., the Yellow Sea, has been greatly expanded by recent works (Zhao and Song, 1999, 2001, 2003a, 2003b; Zhao et al., 2000, 2002). However, little attention has been paid to the myxozoan parasites infecting fishes in the East China Sea. Thus, studies have been aimed to extend information on the geographical distributions and diversity of myxosporeans in that region. During surveys on marine fish parasites from the East China Sea, a myxozoan morphologically consistent with members of the genus Davisia Laird 1953 was detected in a rockfish, Sebastiscus marmoratus (Cuvier, 1829), caught off the coast of Xiamen, China. The parasite is distinct from previously described Davisia species. However, in our examination of the literature, we discovered that the genus name is preoccupied by Davisia Del Guercio, 1909 (Insecta: Hemiptera) (cf. Miller, 1938). Thus a replacement name, Myxodavisia, is provided and we summarize the known members of the genus.

MATERIALS AND METHODS

Host fish caught by native fishers were collected from Xiamen coastal waters between July and August 2005. Urinary bladders were removed and examined immediately after host fishes were killed. Myxosporeans from urinary bladder were collected and observed in their fresh state. A mixture of glycerine–alcohol–formalin (G.A.F) was used to reveal the morphological structure of spores. All specimens were observed and measured at magnification of ×1,250. Illustrations were drawn with Nikon Y-IDT microscope, computer program CorelDRAW 11 and Adobe Photoshop CS2. The description follows the guidelines of Lom and Arthur (1989). Measurements are based on fresh mature spores and are given as the mean, standard deviation, and range (n = 20).

Received 18 February 2007; revised 18 June 2007; accepted 3 July 2008.
* Center for Fish Disease Research, Department of Microbiology, 220 Nash Hall, Oregon State University, Corvallis, Oregon 97331-3404.
† To whom correspondence should be addressed.

DESCRIPTION

Myxodavisia Zhao, Zhou, Kent, and Whipp, nom. nov.
Phylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Schumil, 1959
Suborder Varsiopora Lom et Noble, 1984
Sinuolineidae Schumil, 1959

Diagnosis: The myxozoan genus Davisia was proposed by Laird (1953) to accommodate species possessing spores with a clearly walled off spherical or ovoid central chamber bearing a pair of lateral appendages. Five species, previously classified as Sinuolinea or Ceratomyxa species, were transferred to the new genus. However, Davisia Laird, 1953 is preoccupied by Davisia Del Guercio, 1909 (Insecta: Hemiptera). Thus, Myxodavisia nomen novum is proposed as a replacement name, for the primary junior homonym Davisia Laird, 1953. Parasites, primarily of urinary bladder or kidney tubules, bear spores with long lateral appendages and have a spore body generally greater in length than the width of lateral appendages. Spherical spore body, noted as key characteristic of genus (Shulman, 1966), is absent in many species, most notably, type species Myxodavisia spinosa (Davis, 1917) as designated by Laird (1953). Authors of more recent publications may have been unaware of this designation, having incorrectly listed Myxodavisia diplorhipis (Laird) as the type species (cf. Lom and Dyková, 2006).

Myxodavisia sebistisca n. sp. (Figs. 1–10)

Vegetative stage: Early stage plasmodia found in urinary bladder. Plasmodia in late stage with an irregular or rounded margin, usually possessing 2 developing spores.

Spore: Body of mature spores spherical or subspherical in sputal view, about 13.1 ± 0.3 (12.7–13.6) μm long by 12.3 ± 0.9 (10.9–13.5) μm thick. Suture line slightly curved. Shell valves smooth, bearing 2 long hollow lateral appendages. Average lateral appendage length 188.3 ± 80.9 (119.4–335.2) μm. Each appendage with cavity full of small granules. In some mature spores, wall present between central chamber and lateral appendage. Spore cavity filled with finely granulated sporoplasm. Two polar capsules spherical or subspherical and equal in size, 4.6 ± 0.6 (3.2–4.6) μm in diameter. Polar filament closely coiled, 5–6 turns. Sporoplasm subspherical or irregular in shape, 7–12 by 4.6–6 μm.

Taxonomic summary

Host: Sebastiscus marmoratus (Cuvier, 1829).
Site of infection: Urinary bladder lumen.
FIGURES 1–4. Illustrations of *Davisia sebastisca* sp. n. (1) Early stage of plasmodia with 2 developing spores. (2) Late stage of plasmodia with 2 developing spores. (3) Mature spore from lateral view. (4) Mature spore with especially long lateral appendage. Bar = 20 μm.

**Geographical distribution:** Coastal waters off Xiamen (24°26'N, 118°04'E), China.

**Prevalence:** 5 of 61 (8%).

**Ecological features:** Salinity c. 34%, water temperature 29°C.

**Date of sampling:** August 2005.

**Pathogenicity:** Not known.

**Type material:** Hapantotypes on slide (xm-050813), deposited in the Collection Centre for Type-specimens, the Key Laboratory of Animal Biology of Chongqing, Chongqing Normal University, Chongqing, China.

**Etymology:** The specific name "sebastisca" was derived from the host genus.
Remarks

Among the other morphologically similar species, ours resembles Myxodavisia longibrachia (Kabata, 1962), Myxodavisia amoena (Gayevskaya et al., 1980), and Myxodavisia newfoundlandia, as described by Gayevskaya et al. (1980) (Tables I, II). Myxodavisia newfoundlandia as originally described by Yoshino and Noble (1973a) has much shorter appendages, 4.5–9.0 μm, and is, therefore, a distinct species. Myxodavisia hexagrammi (Zhao et al., 2002) is also found in Chinese waters but can be distinguished from the present species by having spores with much shorter appendages (Table I). Likewise, on average, the appendages of M. sebastisca n. sp. are much longer than M. longibrachia (188 μm vs. 100 μm). Although Laird (1955) suggested that differences in appendage length may be a function of maturity, Kabata (1962) observed the appendages of M. longibrachia to be fully, or almost fully, developed within the sporoblast. Thus, the variation we observed in appendage length for M. sebastisca n. sp. (119.4–335.2 μm) is likely intraspecific variation as opposed to difference in spore age. The appendages of M. newfoundlandia as described by Gayevskaya et al. (1980) overlap this range in length (123–248 μm) but become filamentous more proximally to the spore body than our species. Nonetheless, the spore body of M. sebastisca n. sp. is more spherical than M. newfoundlandia of Gayevskaya et al. (1980). M. amoena most closely resembles our species, but several distinctions can be made. The suture line of M. amoena is sinuous, whereas that of M. sebastisca n. sp. is curved. Appendage lengths also differ, as does the overall range of spore length (see Table I).

DISCUSSION

The classification of the genus Myxodavisia is somewhat confusing. The more one examines its members, the fewer uniting characteristics are apparent among them (Figs. 11–38). Spores are spherical or sub spherical, may or may not have a sinuous suture, and the length of their appendages varies dramatically. Including M. sebastisca n. sp., 28 species of the genus have been described, most of which infect the urinary bladder. Laird (1953) created the genus Davisia to accommodate Sinuolinea species bearing a distinct central chamber that is “clearly walled-off” from a pair of lateral appendages. Since the formation of Davisia, species have been included that possess appendages that are clearly demarcated and others that are continuous with the spore body. These have been referred to as “hollow” and “solid” appendages (Moser and Noble, 1975), although these terms are misleading because the appendages are not physically hollow or solid. Here, the lateral appendages on immature spores of M. sebastisca n. sp. appeared to be delineated from the body of the spore (Fig. 2). However, in approximately 60% of mature spores, the cavity was continuous with the spore body (Fig. 6). This suggests that the wall of the central chamber might dissolve as spores of Myxodavisia age. Moser and Noble (1975) made similar observations for Myxodavisia anoplopoma and Myxodavisia pectoralis but suggested that this variation in attachment was most likely an illusion because of the angle of observation and focal plane or an effect of dehydration or fixation on the sporoplasm. They strengthened their argument by sonicating M. pectoralis spores to release the sporoplasm, after which, no separation between the appendages and spore body was visible. In a subsequent article, Love and Moser (1976) supported those observations, describing Myxodavisia reginae as having lateral appendages continuous with the spore body. Whether this holds for other Myxodavisia species is unknown, but careful consideration to this feature should be given in future descriptions. Detailed ultrastructural studies may also shed light on whether appendage attachment varies between species or maturity or is merely an illusion.

As it stands, members of Myxodavisia may or may not possess a “clearly walled-off” lateral appendage as described by Laird (1953) to distinguish them from Sinuolinea, Ceratomyxa, or Sphaerospora species. Regardless of their mode of attachment, the lateral appendages of most members are much narrower than the central part of the spore, thus distinguishing them from Ceratomyxa. In most cases, lateral bumps and protuberances that might be observed on Sphaerospora spores (Shulman, 1966; Lom and Dyková, 2006) are shorter than the length of the appendages of Myxodavisia spp. Sinuolinea species possess no appendages. It seems clear that Myxodavisia species are distinguished from other myxozoans by possessing spores with lateral appendages that are less broad than the central part of the spore, which may be walled off, and are of sufficient length to give the impression that they are not simply protuberances. There are notable exceptions to this definition. In particular, Myxodavisia nototheniae (Fig. 28) possesses extremely short appendages (Kovaleva et al., 2002) and may be more appropriately considered a Sphaerospora species. Myxodavisia sauridae (Fig. 32) and Myxodavisia murtii (Fig. 36) spores bear a resemblance to those of Ceratomyxa species. Conversely, some Ceratomyxa species appear to possess spores and lateral appendages that would qualify them as Myxodavisia species. In particular, we recognized Ceratomyxa sphairophora (Davis, 1917) and Ceratomyxa lianoides (Aseeva, 2003a), although there may be others. The importance of tissue specificity as it relates to phylogeny among myxozoans has been emphasized in recent publications incorporating DNA sequence analyses (Fiala, 2006). Some of these exceptions are also parasites of tissues other than those of the urinary system, the most common tissue site for Myxodavisia spp.

The placement of Myxodavisia in the Sinuolineidae is also somewhat tenuous. Lom and Dyková (2006) describe the family as having members with spores possessing a sinuous suture, although Shulman (1966) did not include a sinuous suture in the description of the Sinuolineidae. Indeed, several species of Myxodavisia possess a straight suture, and less than half actually have what was described as a sinuous or somewhat sinuous suture (Table II). Jameson (1931) considered the sinuous suture alone insufficient grounds to establish a new genus, i.e., Sinuolinea, in the first place, noting the similarities to Sphaerospora species. Suture morphology may have diagnostic value among other myxozoans, but the character is clearly variable among Myxodavisia species.

Thus, the taxonomy of Myxodavisia is sometimes inconsistent. Nonetheless, we hesitate to make any major taxonomic revisions at this time, primarily because there are so many species that occupy what can best be considered a grey area and are unavailable for examination. Second, as recent studies have shown (Whipp et al., 2004; Fiala, 2006), molecular systematics will likely play an important role in teasing apart the relationships among myxozoan species. Because future revisions are very likely once molecular data are available, we choose not to “muddy the waters” further. For the time being, species bearing spores with distinct lateral appendages that appear to be distinct or give the impression of distinctness should be considered Myxodavisia species, e.g., M. spinosa, M. sebastisca n. sp., M. longibrachia, M. diplocrepis, M. amoena, and others. When encountered, species that possess Ceratomyxa-like spores, e.g., M. murtii, or spores with very small appendages, e.g., M. no-
Table I. Morphological features of the species of *Myxodavisia*. Mean and/or range are provided in micrometers, with range in parenthesis if both were reported. Tissue location listed as UB = urinary bladder, KT = kidney tubules, or GB = gall bladder. Spore length, width, or thickness are listed as assigned in the original descriptions and, therefore, may not correspond to guidelines for spore descriptions as recommended by Lom and Arthur (1989). Suture line morphology was recorded as S = sinuous, SS = slightly sinuous, SC = slight curve, C = curved, or T = straight. Overall polar capsule (PC) morphology was often spherical, therefore, length (PCL) is synonymous with diameter. Polar capsule width (PCW) is provided for other PC morphologies. Appendage length (AL) and, if available, appendage width (AW) are also provided.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host(s)</th>
<th>Tissue</th>
<th>Locality</th>
<th>Length</th>
<th>Width</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. sebastiscus</em> n. sp.</td>
<td><em>Sebastiscus marmoratus</em></td>
<td>UB</td>
<td>East China Sea</td>
<td>13.0 (12.7–13.6)</td>
<td>—</td>
<td>12.3 (10.9–13.5)</td>
</tr>
<tr>
<td><em>M. amoena</em></td>
<td><em>Hippoglossoides platessoides</em></td>
<td>UB, KT</td>
<td>Northwestern Atlantic</td>
<td>10.6–13.9</td>
<td>10.7–13.9</td>
<td>—</td>
</tr>
<tr>
<td><em>M. anoplopoma</em></td>
<td><em>Anoplopoma fimbria</em></td>
<td>UB, KT</td>
<td>U.S.A., central and south</td>
<td>12.75 (12.0–14.0)</td>
<td>15.20 (13.0–17.0)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>California coast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. aurita</em></td>
<td><em>Albatrossia pectoralis</em></td>
<td>UB</td>
<td>Okhotsk Sea</td>
<td>13.3–15.0</td>
<td>13.3–15.2</td>
<td>—</td>
</tr>
<tr>
<td><em>M. bidens</em></td>
<td><em>Porichthys notatus</em> Girard, 1854</td>
<td>UB, KT</td>
<td>U.S.A., California coast</td>
<td>6.5–9*</td>
<td>—</td>
<td>8–11.5*</td>
</tr>
<tr>
<td><em>M. brachiophora</em></td>
<td><em>Paralichthys albigutta</em> Jordan &amp; Gilbert, 1882</td>
<td>UB</td>
<td>U.S.A., North Carolina coast</td>
<td>9–11</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td><em>M. cella</em></td>
<td><em>Porichthys notatus</em> Girard, 1854</td>
<td>UB</td>
<td>U.S.A., California coast</td>
<td>8–10*</td>
<td>—</td>
<td>9–13*</td>
</tr>
<tr>
<td><em>M. cornuta</em></td>
<td><em>Neogobius fluviatilis</em> Pallas, 1814</td>
<td>UB</td>
<td>Sea of Azov</td>
<td>8.8–10.6</td>
<td>—</td>
<td>9.4–11.9</td>
</tr>
<tr>
<td><em>M. coryphaenoidia</em></td>
<td><em>Coryphaenoides acrolepis</em> Bean, 1884</td>
<td>UB</td>
<td>U.S.A., Southern California coast</td>
<td>18.5 (17–23)</td>
<td>11.3 (9–14)</td>
<td>—</td>
</tr>
<tr>
<td><em>M. cynoglossi</em></td>
<td><em>Cynoglossus sp.</em></td>
<td>GB</td>
<td>India, Bay of Bengal</td>
<td>14.24 (12.6–16.8)</td>
<td>12.6 (11.4–15.8)</td>
<td>—</td>
</tr>
<tr>
<td><em>M. diplocrepis</em></td>
<td><em>Diplocrepis puniceus</em> (Richardson, 1846)</td>
<td>UB</td>
<td>New Zealand</td>
<td>10.7 (9.0–12.0)</td>
<td>—</td>
<td>13.0 (12.1–14.0)</td>
</tr>
<tr>
<td><em>M. donecae</em></td>
<td><em>Trachurus capensis</em> Castelnau, 1861</td>
<td>UB</td>
<td>Namib shelf, Atlantic Ocean</td>
<td>10–12</td>
<td>9.6–12</td>
<td>10.6</td>
</tr>
<tr>
<td><em>M. filiformis</em></td>
<td><em>Thryssa setirostris</em> (Broussonet, 1782)</td>
<td>GB</td>
<td>India, Bay of Bengal, Visakhapatnam</td>
<td>12.3 (12–14)</td>
<td>19.73 (16–20)</td>
<td>—</td>
</tr>
<tr>
<td><em>M. galeiforme</em></td>
<td><em>Lycodapus australis</em> Norman, 1937</td>
<td>UB</td>
<td>Falkland-Patagonia, South-west Atlantic</td>
<td>12.0–17.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>M. hexagrammi</em></td>
<td><em>Hexagrammos otakii</em> Jordan and Starks, 1859; <em>Agrammus agrammus</em> (Temminck &amp; Schlegel, 1844)</td>
<td>UB</td>
<td>Yellow sea and Bohai Bay, China</td>
<td>9.9 (9.5–10.5)</td>
<td>—</td>
<td>10.8 (10–12)</td>
</tr>
<tr>
<td><em>M. longibrachia</em></td>
<td><em>Callionymus lyra</em> Linnaeus, 1758; <em>Agonus cataphractus</em> (Linnaeus, 1758); <em>Microstomus kitt</em> (Walbaum, 1792); <em>Hippoglossoides platessoides</em> (Fabricius, 1780)</td>
<td>UB</td>
<td>Northern North Sea</td>
<td>12–15*</td>
<td>—</td>
<td>12*</td>
</tr>
<tr>
<td><em>M. longifilus</em></td>
<td><em>Hippoglossoides dubius</em> Schmidt, 1904</td>
<td>UB</td>
<td>Sea of Japan</td>
<td>10.5–13.5</td>
<td>11.7–13.0</td>
<td>—</td>
</tr>
<tr>
<td><em>M. murtii</em></td>
<td><em>Liza macrolepis</em> (Smith, 1846)</td>
<td>GB</td>
<td>India, Bay of Bengal</td>
<td>7.34 (4.8–11.2)*</td>
<td>—</td>
<td>13.86 (11.2–17.6)*</td>
</tr>
<tr>
<td><em>M. narvi</em></td>
<td><em>Myxocephaulus brandti</em> (Steindachner, 1867)</td>
<td>GB</td>
<td>Japan Sea</td>
<td>10.0–11.5</td>
<td>11.5–14.4</td>
<td>—</td>
</tr>
<tr>
<td><em>M. newfoundlandia</em></td>
<td><em>Macrourus berglax</em> Lacepède, 1801</td>
<td>UB</td>
<td>Canada, eastern Newfoundland</td>
<td>11.63 (10.0–14.0)</td>
<td>9.65 (7.5–11.0)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td><em>Macrourus holotrichys</em> Günther, 1878</td>
<td>UB</td>
<td>Falkland-Patagonia, southwest Atlantic</td>
<td>9.6–12.0</td>
<td>10.7–12.8</td>
<td>6.4</td>
</tr>
<tr>
<td><em>M. newfoundlandia 2</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. nototheniae</em></td>
<td><em>Lepidodontothen larseni</em> Lönningberg, 1905</td>
<td>KT</td>
<td>Antarctic Atlantic Ocean</td>
<td>12–13</td>
<td>12–13.3</td>
<td>—</td>
</tr>
<tr>
<td><em>M. opacita</em></td>
<td><em>Paralichthys albigutta</em> Jordan &amp; Gilbert, 1882</td>
<td>UB</td>
<td>U.S.A., North Carolina coast</td>
<td>12–13</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>M. ophidioni</em></td>
<td><em>Ophidion rochei</em> Müller, 1845</td>
<td>UB</td>
<td>Black Sea, Sevastopol</td>
<td>9.1–11.7</td>
<td>13</td>
<td>—</td>
</tr>
<tr>
<td><em>M. pectoralis</em></td>
<td><em>Albatrossia pectoralis</em> Gilbert, 1892</td>
<td>UB, KT</td>
<td>U.S.A. northern California coast</td>
<td>13.0 (10.0–17.0)</td>
<td>13.05 (11.0–17.0)</td>
<td>9.5 (9.0–10.0)</td>
</tr>
</tbody>
</table>
FIGURES 5–10. Photomicrographs of Davisia sebastisca n. sp. (5) Differential interference contrast micrograph of a fresh mature spore of lateral view in urinary bladder. (6) DIC micrograph of mature spore. Arrow indicates there is no wall between central chamber and lateral appendages. (7) DIC micrograph of a mature spore with especially long appendages (arrow). (8–10) Plasmodium with 2 developing spores in different developing stages (from early stage to late stage). Bar = 10 μm.

totheniae, should be carefully considered for inclusion in other genera, or clearly justified for inclusion in *Myxodavisia*.

A synopsis of *Myxodavisia* species sensu lato is given below, complemented by morphometric data (Tables I, II). To the best of our knowledge, the list is exhaustive.

**SPECIES SYNOPSIS AND NOTES**

*Myxodavisia* Zhao, Zhou, Kent, and Whipps, nom. nov.

*Davisia* Laird, 1953 (junior homonym of *Davisia* Del Guercio, 1909)

*Myxodavisia amoena*

(Gayevskaya, Kovaleva, and Umnova, 1980) n. comb.

Syn. *Davisia amoena* Gayevskaya, Kovaleva, and Umnova, 1980 (Fig. 20)

*Description*: Spores subspherical with tapering appendages
Table II. Morphological features of the species of Myxodavisia. Mean and/or range are provided in micrometers, with range in parenthesis if both were reported. Suture line morphology was recorded as S = sinuous, SS = slightly sinuous, SC = slight curve, C = curved, or T = straight. Overall polar capsule (PC) morphology was often spherical, therefore, length (PCL) is synonymous with diameter. Polar capsule width (PCW) is provided for other PC morphologies. Appendage length (AL) and, if available, appendage width (AW) are also provided.

<table>
<thead>
<tr>
<th>Species</th>
<th>Suture</th>
<th>PC morph</th>
<th>PCL</th>
<th>PCW</th>
<th>AL</th>
<th>AW</th>
<th>Coils</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. sebastiscus n. sp.</td>
<td>SC</td>
<td>spherical</td>
<td>4.6</td>
<td>3.2–4.6</td>
<td>188.3</td>
<td>119.4–335.2</td>
<td>5–6</td>
<td>Present</td>
</tr>
<tr>
<td>M. amoena</td>
<td>S</td>
<td>spherical</td>
<td>3.4</td>
<td>4.0</td>
<td>150–167</td>
<td>5.3–7.4</td>
<td>7</td>
<td>Aseeva (2003)</td>
</tr>
<tr>
<td>M. anoplompa</td>
<td>SS</td>
<td>spherical</td>
<td>4.95</td>
<td>4.0–6.0</td>
<td>34.7</td>
<td>30.0–38.0</td>
<td>5–7</td>
<td>Moser and Noble (1975)</td>
</tr>
<tr>
<td>M. aurita</td>
<td>C</td>
<td>spherical</td>
<td>3.32</td>
<td>3.9–4.2</td>
<td>4.38</td>
<td>3.0–5.5</td>
<td>7</td>
<td>Kovaljova et al. (1986)</td>
</tr>
<tr>
<td>M. bidens</td>
<td>S</td>
<td>spherical</td>
<td></td>
<td></td>
<td>6–10</td>
<td>5.4</td>
<td></td>
<td>Jameson (1931)</td>
</tr>
<tr>
<td>M. brachiophora</td>
<td>SC</td>
<td>spherical</td>
<td>3.5</td>
<td>18–22</td>
<td></td>
<td></td>
<td></td>
<td>Davis (1917)</td>
</tr>
<tr>
<td>M. cella</td>
<td>SC</td>
<td>spherical</td>
<td></td>
<td>25–35</td>
<td></td>
<td></td>
<td></td>
<td>Jameson (1931)</td>
</tr>
<tr>
<td>M. cornuta</td>
<td>SC</td>
<td>pyriform</td>
<td>3.0</td>
<td>2.0–2.5</td>
<td>11.3–12.0</td>
<td>5.0–7.0</td>
<td>5–6</td>
<td>Yurakhno and Naidenova (2000)</td>
</tr>
<tr>
<td>M. coryphaenoidia</td>
<td>C</td>
<td>spherical</td>
<td>3.7</td>
<td>3.5–4</td>
<td>4.8</td>
<td>3.6</td>
<td>7–8</td>
<td>Yoshino and Noble (1973)</td>
</tr>
<tr>
<td>M. cynoglissi</td>
<td>SS</td>
<td>ovoid</td>
<td>3.2</td>
<td>2.8–4.2</td>
<td>20.8–36.4</td>
<td>14.5</td>
<td>5–9</td>
<td>Narasimhamurti et al. (1990)</td>
</tr>
<tr>
<td>M. diplocrepis</td>
<td>T-SC</td>
<td>spherical</td>
<td>3.7</td>
<td>3.2–3.5</td>
<td>12.7</td>
<td>3.2</td>
<td>5–6</td>
<td>Laird (1953)</td>
</tr>
<tr>
<td>M. donecae</td>
<td>SC</td>
<td>spherical</td>
<td>4.2</td>
<td>3.9–4.2</td>
<td>45.2</td>
<td>4.3–4.5</td>
<td>5–6</td>
<td>Gaevskaya and Kovaleva (1979)</td>
</tr>
<tr>
<td>M. filiformis</td>
<td>T</td>
<td>pyriform</td>
<td>8.73</td>
<td>8–10</td>
<td>73.5</td>
<td>60–88</td>
<td></td>
<td>Padma Dorothy et al. (1998)</td>
</tr>
<tr>
<td>M. galeiforme</td>
<td>T</td>
<td>spherical</td>
<td>5.5</td>
<td>6.0</td>
<td>25.3–46.0</td>
<td>5.3–8.0</td>
<td>7</td>
<td>Kovaljova and Rodjuk (1991)</td>
</tr>
<tr>
<td>M. hexagrammi</td>
<td>T-SS</td>
<td>spherical</td>
<td>3.8</td>
<td>3.5–4</td>
<td>52.4</td>
<td>44–59</td>
<td></td>
<td>Zhao et al., 2002</td>
</tr>
<tr>
<td>M. longibrachia</td>
<td>SS</td>
<td>spherical</td>
<td>5</td>
<td>100</td>
<td>6.6</td>
<td>4.2–9.6</td>
<td>4–5</td>
<td>Kabata (1962)</td>
</tr>
<tr>
<td>M. murrii</td>
<td>S</td>
<td>spherical</td>
<td>4.56</td>
<td>3.2–4.8</td>
<td>100</td>
<td></td>
<td>6</td>
<td>Nandi et al. (2004)</td>
</tr>
<tr>
<td>M. narvi</td>
<td>S</td>
<td>spherical</td>
<td>4.0</td>
<td>4.5</td>
<td>20–27</td>
<td>3.5–4.5</td>
<td>6</td>
<td>Aseeva (2002)</td>
</tr>
<tr>
<td>M. newfoundlandia</td>
<td>S</td>
<td>spherical</td>
<td>3.06</td>
<td>2.0–4.0</td>
<td>6.58</td>
<td>4.5–9.0</td>
<td>7–8</td>
<td>Yoshino &amp; Noble (1973)</td>
</tr>
<tr>
<td>M. newfoundlandia 2</td>
<td>SC</td>
<td>spherical</td>
<td>2.7</td>
<td>4.3</td>
<td>123–248</td>
<td>1–2</td>
<td>4–5</td>
<td>Gaevskaya et al. (1980)</td>
</tr>
<tr>
<td>M. nototheniae</td>
<td>SC</td>
<td>spherical</td>
<td>4–4.6</td>
<td>1–2</td>
<td>1–28</td>
<td></td>
<td>6–7</td>
<td>Kovaljova et al. (2002)</td>
</tr>
<tr>
<td>M. opacita</td>
<td>S</td>
<td>spherical</td>
<td>4</td>
<td></td>
<td>6</td>
<td></td>
<td></td>
<td>Davis (1917)</td>
</tr>
<tr>
<td>M. ophidionii</td>
<td>SC</td>
<td>spherical</td>
<td>3.9</td>
<td>5.2</td>
<td>21–29</td>
<td></td>
<td></td>
<td>Zaika (1966)</td>
</tr>
<tr>
<td>M. pectoralis</td>
<td>S</td>
<td>spherical</td>
<td>5</td>
<td>2.7</td>
<td>12.20</td>
<td>4.4–3.6</td>
<td>6–9</td>
<td>Love and Moser (1976)</td>
</tr>
<tr>
<td>M. reginae</td>
<td>SS</td>
<td>pyriform to spherical</td>
<td>3.5</td>
<td>2.5–4.5</td>
<td>40.5</td>
<td>28–85</td>
<td>5.56</td>
<td>8–14</td>
</tr>
<tr>
<td>M. sauridae</td>
<td>S</td>
<td>ovoid</td>
<td>1.8</td>
<td>1.6</td>
<td>3.52</td>
<td>2.6–5.4</td>
<td>2–3</td>
<td>Narasimhamurti et al. (1990)</td>
</tr>
<tr>
<td>M. spectabilis</td>
<td>T</td>
<td>spherical</td>
<td>a) 5.0; b) 3.0</td>
<td>a) 37.0–55.0; b) 33.0–40.0</td>
<td></td>
<td></td>
<td>Shulman (1966), Aseeva (2003)</td>
<td></td>
</tr>
<tr>
<td>M. spinosa</td>
<td>T</td>
<td>spherical</td>
<td>4</td>
<td></td>
<td>70§</td>
<td></td>
<td></td>
<td>Davis (1917)</td>
</tr>
</tbody>
</table>

* In descriptions where measurements were not assigned to a particular dimension (i.e., length, thickness, or width), we made the assignment based on the figures available.
† Width measured at mid appendage.
‡ Appendages defined as unequal, thus dimensions are provided for both the longer and shorter appendages.
§ Estimated from figure or other spore dimensions in original publication.
and sinuous suture (Gaevskaya et al., 1980). Resemblance to *M. longibrachia* striking. Both parasites of American plaice in North Atlantic, with significant overlap in spore dimensions (Table I). Appendages of *M. amoena* lengthier than *M. longibrachia*, and polar capsules smaller; whether these species conspecific necessitates further investigation.

**Myxodavisia anoplopoma**  
(Moser and Noble, 1975) n. comb.

Syn. *Davisia anoplopoma* Moser and Noble, 1975 (Fig. 11)  
*Description:* Spore oval with downward hollow lateral appendages, distinct suture, equal polar capsules (Moser and Noble, 1975).

**Myxodavisia aurita**  
(Kovaleva, Gayevskaya, and Krasin, 1986) n. comb.  

Syn. *Davisia aurita* Kovaleva, Gayevskaya, and Krasin, 1986 (Fig. 14)  
*Description:* Spore subspherical, straight or slightly sinuous sutural line, polar capsules equal, almost no lateral appendages (Kovaleva et al., 1986).

**Myxodavisia bidens**  
(Jameson, 1931) n. comb.  

Syn. *Sinuolinea bidens* Jameson, 1931; *Davisia bidens* Laird, 1953 (Fig. 26)  
*Description:* Oval spore body, short but prominent appendages (Jameson, 1931). Laird (1953) suggested this species as conspecific with *Myxodavisia cella* when both transferred to
Davisia; difference in size of lateral appendages related to spore maturity. Both species with same host and site of infection. Further investigation needed to address possible conspecificity.

**Myxodavisia brachiophora** (Davis, 1917) n. comb.

*Syn. Sinuolinea brachiophora* Davis, 1917; *Davisia brachiophora* Laird, 1953 (Fig. 19)

*Description:* Spore body spherical with long distinct lateral appendages (Davis, 1917; Kudo, 1919; Meglitsch, 1952; Laird, 1953).

**Myxodavisia cella** (Jameson, 1931) n. comb.

*Syn. Sinuolinea cella* Jameson, 1931; *Davisia cella* Laird, 1953 (Fig. 33)

*Description:* Spore body oval with prominent suture, large appendages with pointed or rounded ends, clearly demarcated from central chamber (Jameson, 1931). See comments for *M. bidens*.

**Myxodavisia cornuta** (Yurakhno and Naidenova, 2000) n. comb.

*Syn. Davisia cornuta* Yurakhno and Naidenova, 2000 (Fig. 23)

*Description:* Spherical to subspherical spore body, posterior–lateral appendages hornlike (Yurakhno and Naidenova, 2000).

**Myxodavisia coryphaenoidia** (Yoshino and Noble, 1973) n. comb.

*Syn. Davisia coryphaenoidia* Yoshino and Noble, 1973 (Fig. 17)

*Description:* Spore semicircular to oval, polar capsules equal, sutural ridge distinct, lateral appendages short with broadly rounded ends (Yoshino and Noble, 1973b).

**Myxodavisia cynoglossi** (Narasimhamurti, Kalavati, Anuradha, and Padma Dorothy, 1990) n. comb.

*Syn. Davisia cynoglossi* Narasimhamurti, Kalavati, Anuradha, and Padma Dorothy, 1990 (Fig. 30)

*Description:* Spore body oval, broad lateral appendages with distinct junction (Narasimhamurti et al., 1990).

**Myxodavisia diplocrepis** (Laird, 1953) n. comb.

*Syn. Davisia diplocrepis* Laird, 1953 (Fig. 12)

*Description:* Spore body oval, distinct lateral appendages curve downward. Immature spores possess more of a spherical spore body; appendages shorter than in mature spores, projecting laterally or curving downward in sutural aspect (Laird, 1953). This species mistakenly considered type for genus in recent publications.

**Myxodavisia donecae** (Gayevskaya and Kovaleva, 1979) n. comb.

*Syn. Myxodavisia donecae* Gayevskaya and Kovaleva, 1979 (Fig. 29)

*Description:* Spores rounded, polar capsules equal (Gayevskaya and Kovaleva, 1979).

**Myxodavisia filiformis** (Padma Dorothy, Kalavati, and Vaidchi, 1998) n. comb.

*Syn. Davisia filiformis* Padma Dorothy, Kalavati, and Vaidchi, 1998 (Fig. 27)

*Description:* Spore body oval, lateral appendages clearly demarcated, filamentous, and unequal (Padma Dorothy et al., 1998).

**Myxodavisia galeiforme** (Kovaleva and Rodjuk, 1991) n. comb.

*Syn. Davisia galeiforme* Kovaleva and Rodjuk, 1991 (Fig. 38)

*Description:* Spore body spherical with thin straight suture, broad lateral appendages narrowing to a filamentous end (Kovaleva and Rodjuk, 1991).

**Myxodavisia hexagrammi** (Zhao, Ma, and Song, 2002) n. comb.

*Syn. Davisia hexagrammi* Zhao, Ma, and Song, 2002 (Fig. 15)

*Description:* Spore body spherical or subspherical, suture line straight or slightly sinuus, polar capsules equal, lateral appendages long and tapering with distinct junction to spore body (Zhao et al., 2002).

**Myxodavisia longibrachia** (Kabata, 1962) n. comb.

*Syn. Davisia longibrachia* Kabata, 1962 (Fig. 18)

*Description:* Spores spherical or subspherical, wavy suture, equal polar capsules (Kabata, 1962). Different appendage thicknesses observed for *M. longibrachia* from different hosts. See also comments for *M. amoena*.

**Myxodavisia longifilus** (Aseeva, 2003) n. comb.

*Syn. Davisia longifilus* Aseeva, 2003 (Fig. 25)

*Description:* Spore generally spherical and pointed at poles; 4 equal appendages extend from each valve (Aseeva, 2003b).

**Myxodavisia murtii** (Padma Dorothy and Kalavati, 1994) n. comb.

*Syn. Davisia murtii* Padma Dorothy and Kalavati, 1994 (Fig. 36)

*Description:* Overall spore crescent-shaped, spore body oval, wavy suture, equal lateral appendages with blunt ends (Nandi et al., 2004).

**Myxodavisia narvi** (Aseeva, 2002) n. comb.

*Syn. Davisia narvi* Aseeva, 2002 (Fig. 31)

*Description:* Subspherical spore body with conical posterior pole, thin suture, and broad membrane extensions on valvular surface; lateral appendages equal with filiform ends (Aseeva, 2002).

**Myxodavisia newfoundlandia** (Yoshino and Noble, 1973) n. comb.

*Syn. Davisia newfoundlandia* Yoshino and Noble, 1973 (Figs. 21, 22)
Description: Spore subspherical with tapering appendages, sutural line sinuous; equal polar capsules. Conspicuity of *M. newfoundlandia* as described by Yoshino and Noble (1973a) (Fig. 21) and that described by Gayevskaya et al. (1980) (Fig. 22) highly questionable. Yoshino and Noble (1973a) describe spores with rather short lateral appendages (4.5–9.0 μm), whereas Gayevskaya et al. (1980) describe rather long appendages (123–248 μm). Possible that filamentous appendages observed by Gaevskaya et al. (1980) broke off or were not visible in specimens of Yoshino and Noble (1973a) or that intra-specific variation produced morphological difference. Both parasites found in fish hosts of same genus but in distinct localities (Newfoundland, Canada, vs. the Falkland Islands). Given the morphological differences and potential for geographic isolation, these are likely different species.

**Myxodavisia nototheniae**

(Kovaleva, Rodjuk, and Grudnev, 2002) n. comb.

Syn. *Davisia nototheniae* Kovaleva, Rodjuk, and Grudnev, 2002 (Fig. 28)

Description: Spores rounded, sometimes posteriorly flattened, lateral appendages very short, attached near posterior of spore; immature spores noted as being larger (Kovaleva et al., 2002). Rather small lateral appendages suggestive of *Sphaerospora* spp.

**Myxodavisia opacita** (Davis, 1917) n. comb.

Syn. *Sinuolinea opacita* Davis, 1917; *Davisia opacita* Laird, 1953 (Fig. 34)

Description: Spherical spore body, distinct suture, short and flattened lateral appendages (Davis, 1917; Kudo, 1919; Laird, 1953).

**Myxodavisia ophidioni** (Zaika, 1966) n. comb.

Syn. *Davisia ophidioni* Zaika, 1966 (Fig. 16)

Description: Round spores with weakly curved suture. Equal or unequal, hornlike projections; species sometimes listed with an incorrect subsequent spelling of “*Davisia ophidiin*” (cf. Shulman, 1966). It is also often attributed to Zaika (1965), although Zaika (1966) is the authority for *M. ophidioni*, and *Ophidion rochei* is type host, not *Ophidion barbatum*.

**Myxodavisia pectoralis** (Moser and Noble, 1975) n. comb.

Syn. *Davisia pectoralis* Moser and Noble, 1975 (Fig. 24)

Description: Circular central chamber with hollow, tapering appendages, equal polar capsules (*Moser* and Noble, 1975).

**Myxodavisia reginae** (Love and Moser, 1976) n. comb.

Syn. *Davisia reginae* Love and Moser, 1976 (Fig. 37)

Description: Subspherical spore body with distinct suture, appendages filamentous at extremities with moderately distinct junction to spore body (Love and Moser, 1976).

**Myxodavisia sauridae**

(Narasimhamurti, Kalavati, Anuradha, and Padma Dorothy, 1990) n. comb.

Syn. *Davisia sauridae* Narasimhamurti, Kalavati, Anuradha, and Padma Dorothy, 1990 (Fig. 32)

Description: Spore body rectangular, width twice the length, junction of spore body and appendages marked with distinct furrow (Narasimhamurti et al., 1990).

**Myxodavisia spectabilis** (Dagiel, 1948) n. comb.

Syn. *Davisia spectabilis* Aseeva, 2003 *Ceratomyxa spectabilis* Dagiel, 1948 (Fig. 35)

Description: Spores crescent-shaped overall, spherical to subspherical spore body, lateral appendages constricted at spore body junction with filiformous ends (Shulman, 1966). Aseeva (2003b) listed this as a *Davisia* species; on examination of figures from Shulman (1966), we agree with this generic assignment.

**Myxodavisia sinuolinea** (Davis, 1917) n. comb. (type species)

Syn. *Ceratomyxa sinuolinea* Davis, 1917; *Sinuolinea spinosa* Jameson, 1931; *Davisia spinosa* Laird, 1953 (Fig. 13)

Description: Spore body ovoid with long tapering appendages, polar filament coils indistinct (Davis 1917); *M. spinosa* designated type species of newly described genus *Davisia* by Laird (1953); see also Kudo (1919).

ACKNOWLEDGMENTS

This work was supported by “The National Natural Science Foundation of China” (Project 30370172), and our thanks are also due to Dr. Jingwen Liu, Bioengineering School, Jimei University, Xiamen, China, for supplying a laboratory for collecting the samples. C.M.W. is grateful to the Oregon State University Library interlibrary loan staff for their efforts to obtain several obscure manuscripts and books.

LITERATURE CITED


Kovaleva, A. A., A. V. Gayevskaya, and V. K. Krasin. 1986. New species of Myxosporidians (*Protozoa, Myxosporidia*) from ma-


Likewise, amphotericin B, which binds to cell membranes, and the ketoconazoles that inhibit ergosterol synthesis, interfere in Leishmania: To whom correspondence should be addressed.

The protozoan parasites that cause leishmaniasis are members of the Kinetoplastida, Trypanosomatidae. Leishmania spp. share certain characteristics that suggest a cyanobacterial heritage. Several sequenced enzymes bear signatures of a chloroplast or cyanobacterial nature and their phylogenetic analysis places them on a clade shared by the Kinetoplastidae and euglenids (Hannaert, Bringaud et al., 2003; Hannaert, Saavedra et al., 2003; Martin and Borst, 2003). Furthermore, Leishmania spp. parasites desaturate their fatty acids in a fashion similar to the mechanisms in plants (Korn and Greenblatt, 1963). These relationships are usually explained by the so-called “endosymbiotic hypothesis,” which postulates that in the evolution of the eukaryotic cell, an important event was the fusion of a progenitor eukaryotic cell with a variety of prokaryotes (Rich et al., 1978). The existence of similar metabolic pathways in the Kinetoplastida and cyanobacteria with plants was recently emphasized after sequencing of kinetoplastid genomes (Opperdoros and Szikora, 2006).

The endosymbiotic hypothesis offers new targets for chemotherapy against Leishmania spp. on the basis of the unique parasite characteristics not present in the human host. Of interest in relation to therapeutic intervention are herbicides because of their potential activity on metabolic pathways common to Leishmania spp., fungi, and plants. The toxicity of trifluralin, a herbicide active against Leishmania spp., is due to its inhibition of tubulin synthesis (Chan and Fong, 1990; Chan et al., 1993). Likewise, amphotericin B, which binds to cell membranes, and the ketoconazoles that inhibit ergosterol synthesis, interfere in a metabolic pathway that is essential to plants, fungi, and leishmanial parasites. These compounds are, therefore, effective against both leishmanial and fungal infections. The works of Beach, Holz, Jr., and Anekw (1979), Goad et al. (1984), and Beach, Goad et al. (1989) demonstrated the biosynthetic basis for this inhibition by clarifying the synthesis of ergosterol in both the sandfly and macrophage forms of the parasite. It was noted that imidazole and ketoconazole, active against several leishmanial species, blocked the activity of the C-14 methylation step in fungal synthesis of ergosterol. Fenarimol [α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol, Scheme 1] was introduced into agriculture in the early 1970s. It was suggested that in plant systems fenarimol inhibits ergosterol formation by inhibiting 14α-demethylase, a cytochrome P450-based enzyme (Sisler et al., 1984). Its interference with endocrine activity was also proposed (Andersen et al., 2003). In the present study, we examined the effect of a variety of herbicides, of known structure and mode of action, on leishmanial and other eukaryotic cells. Trifluralin served as a reference herbicide. Fenarimol had a high therapeutic value and, therefore, was chosen for further evaluation. We investigated the specific activity of this compound on lipid metabolism, verified that its site of action is in the membranes of the parasite by using electron microscopy, and demonstrated the docking of the fenarimol molecule in the active site of the enzyme using a model of 14α-lanosterol demethylase.

MATERIALS AND METHODS

In vitro anti-leishmanial activity

Effects on promastigotes: In vitro anti-leishmanial activity was evaluated against Leishmania major Freidlin B4 and Leishmania donovani 160 (isolated from a patient in Sudan) promastigotes. These strains were received from the International Reference Centre of the Kuvin Centre for the Study of Infectious and Tropical Diseases in the Hebrew University of Jerusalem. Serial dilutions of the tested agents were prepared in 20% fetal calf serum (FCS) in RPMI 1640. Wells containing drug-free medium served as a control. The growth inhibition was estimated by the 1H-thymidine incorporation method. Briefly, 96-well plates were seeded with 50,000 promastigotes/200 μl per well in medium, and test solutions were added 3 hr later. After incubation for 24 hr, 0.5 μCi/well 1H-thymidine (in 10% FCS medium) was added, and the cultures were harvested after an additional 24 hr. During the experiment, the plates were incubated at 25 C in air.

Effects on amastigotes: Peritoneal macrophages were obtained from 10- to 12-wk-old BALB/c mice previously stimulated for 4 or 5 days...
with 3% thioglycolate. The macrophages were distributed into 8-well slides (Nunc, Roskilde, Denmark) to 2.5 × 10⁵ cells in 300 µl per well and incubated at 37 C and 5% CO₂ for 3 hr to enable cell adhesion. The supernatant was discarded, and L. major promastigotes (10 × 10⁵ per well) were added. After overnight incubation, fresh medium containing the drugs was added for an additional 48 hr. At the end of this period, the wells were washed, fixed with methanol, and stained with Giemsa. Each treatment was performed in duplicate wells; in each well, 100 macrophages and the number of amastigotes they contained were counted.

In vivo anti-leishmanial effect

Groups of 7 female BALB/c mice (Harlan Laboratories, Jerusalem, Israel), 8 to 10 wk old, were injected subcutaneously (s.c.) at the base of the tail with 5 × 10⁶ stationary-phase promastigotes. Drug treatment was initiated 30 days after parasite injection and was administered intraperitoneally (i.p.), followed by 2 successive days of drug administration, after which 2 additional injections were given on 2 successive days. Lesion size was measured once a week. The diameter was estimated by using the average between the longest distance across the lesion and the length of the line bisecting this distance at a 90° angle. Mice were kept in a university sterile pathogen-free animal facility throughout the experiments.

In vitro toxicity to mammalian cells

The cytotoxicity was evaluated in (human) Hela cells. Cultures were maintained in flat-bottomed flasks at 37 C. Before each experiment, the cells were washed, removed from the flask bottom by trypsin treatment, and an appropriate volume centrifuged, resuspended, and diluted in growth medium to 1.5 × 10⁶/ml. The growth medium consisted of RPMI 1640 and 10% FCS. A 200-µl cell suspension was added to the wells of a microtiter plate. After incubation overnight, the appropriate drug concentration, in triplicate, was added to test wells. Drug-free medium was used as control. Then, 0.5 µCi of [³H]-thymidine in 20 µl of medium was added the next day and the plate was harvested, and read by liquid scintillation counter (LKB, Turku, Finland) after an additional 24 hr. The percentage growth inhibition of the cells by the drug was calculated as (100 − [count with drug/control count] × 100). The effective 50% dose (ED₅₀) of the drugs, defined as the concentration that inhibits 50% thymidine incorporation, was determined graphically from curves of incorporation inhibition.

Lipid metabolism

Lipid extraction: Freeze-dried samples of 10⁶ L. major organisms were extracted by the method of Bligh and Dyer (1959). Before extraction, cells were treated with hot isopropanol (70 C) for 30 min to prevent degradation by lipid catalytic enzymes.

Lipid separation: Total lipid extract was separated into neutral and polar lipids by 1-dimensional thin-layer chromatography (TLC) (silica gel 60, 10 × 10 cm plates, 0.25-mm thickness; MERK, Darmstadt, Germany), using a solvent system of chloroform: methanol:water:acetic acid (60:40:20:1, v/v/v/v). Neutral lipids (NL) were resolved in a system of petroleum ether:diethyl ether:acetic acid (70:30:1, v/v/v). A double development system was utilized to resolve total lipids. After running the gel for two-thirds of its length with chloroform:methanol:water:acetic acid solvent, the plate was dried, then combed through the second solvent, petroleum ether:diethyl ether:acetic acid, to resolve the NL.

Lipids were visualized either by brief exposure to iodine vapors or by spraying with primuline solution (5% in acetone:ddw, 8:2, v/v), followed by exposure to ultraviolet light to view lipid spots. Lipids were identified by comparison of R₁ values with commercial lipid standards. (Sigma, St. Louis, Missouri).

Acetate labeling: Leishmania major promastigotes (cell concentration 2 × 10⁷/ml) were incubated in 100-ml flasks containing 100 ml of RPMI medium with 20% FCS. Cells were incubated with fenarimol (100 µM) for 1 hr before addition of 25 µCi of (1-¹⁴C)acetate (Amersham, London, U.K.). Cultures were then incubated for 6 and 24 hr at 25 C to follow changes in acyl lipid metabolism. At the end of the incubation period, cells were pelleted by centrifugation and washed 3 times with sterile phosphate-buffered saline (PBS, pH 7) to remove excess acetate. Lipids were extracted as described above. Radioactivity was measured in cell and extract samples. The lipids were separated by TLC to depict the profile of the total and neutral lipids. The distribution of radioactivity in individual lipids was estimated by phosphoimaging (Fujix BAS 1000). Alternatively, spots were scrapped from the TLC plates, transferred to scintillation vials, and counted by a radioactivity counter (Packard liquid scintillation analyzer 1600 TR) using universal liquid scintillation cocktail (Ultima Gold, Packard Bioscience, Meriden, Connecticut).

Electron microscopy (EM)

Leishmania major promastigotes in the logarithmic phase of growth were cultured with or without 100 µM fenarimol. Six and 24 hr later, the samples were fixed in 2.5% 0.1 M cacodylate at pH 7.4. After 2 more hours, the fixatives were replaced with 2% osmium tetroxide. One hour later, the samples were washed with increasing concentrations of ethanol, and introduced to propylene oxide/araldite (2/1L). Two hours later, samples were washed in araldite and kept overnight in 4 C, after which they were polymerized at 60 C for 48 hr. Sixty-nanometer sections were stained with 4% uranyl acetate in lead acetate and 50% ethanol. The sections were evaluated by transmission EM (Philips CM 12, Eindhoven, The Netherlands) at 100 kV.

Model of L. major cytochrome P450 14α-lanosterol demethylase

The model was built using homology modeling with the structure of cytochrome P450 14α-lanosterol demethylase from Mycobacterium tuberculosi, complexed with azole inhibitors (Protein Data Bank ID code 1EAI, 27) serving as a template. This structure was chosen as the best template for model building of the L. major cytochrome P450 14α-lanosterol demethylase by the threading server 3D-PSSM (Kelley et al., 2000). The 2 sequences share 30% identity and 50% similarity. The model, including the heme group and the inhibitor fluconazole, was built using the program Modeller (Accelrys Software, San Diego, California), with the alignment given by the threading server 3D-PSSM.

The fenarimol ligand was built by the program DS Modeling 1.1 (Accelrys Software) and was energetically minimized using CHARMM (Accelrys Software).

Docking of the fenarimol ligand to the binding site was simulated by superimposing the pyrimidine ring of the fenarimol molecule with the PH-1,2,3,-triazole of the fluconazole while keeping the interaction between the nitrogen atom of the pyrimidine ring and the heme iron. After the superimposition the fluconazole was removed and the complex was minimized using CHARMM.

In Table I the anti-leishmanial effects of selected herbicides, promastigotes were exposed for 48 hr to the drugs. [³H]-Thymidine was added 24 hr after onset of the experiment and the cultures were harvested after an additional 24 hr. The incorporation of the marker served as a parameter for parasite development.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>ED₅₀ in L. donovani</th>
<th>ED₅₀ in L. major</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>333 µM</td>
<td>334 µM</td>
</tr>
<tr>
<td>Atrazine</td>
<td>ND*</td>
<td>131 µM</td>
</tr>
<tr>
<td>Trifluralin</td>
<td>57 µM</td>
<td>21 µM</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>2 µM</td>
<td>17 µM</td>
</tr>
</tbody>
</table>

* ND, not done.

In Table II the effects of trifluralin and fenarimol on L. major amastigotes. Infected mouse peritoneal macrophages were exposed to the drugs during 48 hr, after which the cultures were fixed and stained, and the number of parasites determined by microscopic observation.

<table>
<thead>
<tr>
<th></th>
<th>Trifluralin</th>
<th>Fenarimol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED₅₀</td>
<td>21 µM</td>
<td>58 µM</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>17 µM</td>
<td>55 µM</td>
</tr>
</tbody>
</table>
TABLE III. The effect of fenarimol on acetate uptake. Leishmania major promastigotes were incubated for 1 hr, after which radioactive acetate was added. Six and 24 hr later the amount of incorporated radioactive marker into the cells and the lipids was estimated.

<table>
<thead>
<tr>
<th>Duration of treatment (hr)</th>
<th>Cells</th>
<th>Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>78*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>82</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>6</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>83</td>
</tr>
</tbody>
</table>

* Percentage incorporation of added acetate.

RESULTS

Replication of experiments

Experiments were repeated at least twice. Results from representative experiments are presented. Experiments were performed in triplicate (Table I) or duplicate (Tables II, III). The range of results in each individual experiment (Tables I–III) did not exceed 10% of the average.

In vitro anti-leishmanial effects

Effects on promastigotes: A series of herbicides was examined for biological activity against leishmanial parasites. These included the SAN series, 6706, 9774, 133-410, and 9785, and pebulate, SHAM, fenarimol, and atrazine. These compounds act on the photosynthetic system. Trifluraline served as a reference because of its known anti-leishmanial effect (Chan and Fong, 1994). Most compounds were not active at concentrations of up to 400 μM. SHAM had an EDso (the concentration that inhibits 50% thymidine incorporation by control PBS-treated cells) of 330 μM against the 2 examined leishmanial strains; trifluralin had an EDso of 21 μM and 57 μM against L. major and L. donovani promastigotes, respectively. Fenarimol was found to be the most active compound, with an EDso of 17 μM and 2 μM against L. major and L. donovani, respectively (Table I). Consequently, fenarimol was used throughout this study.

Effects on amastigotes: The anti-leishmanial effects of fenarimol and trifluralin were further investigated against amastigotes, the pathogenic leishmanial stage within the macrophage host cell. The drugs reduced both the number of infected macrophages and the number of amastigotes in the infected macrophages (Table II). The data indicate that the concentrations that are needed to reduce by half both the number of infected macrophages (70% of the macrophages were infected in the untreated control) and the average amastigote number in the infected macrophages (5/macrophage in the control). Thus, 20 μM and 55 μM of trifluralin and fenarimol, respectively, were needed to reduce the total number of parasites in the culture by 75% (100 – 100/2).

In vitro effects on Hela cells

The cytotoxicity of both drugs was also examined in Hela cell cultures. Trifluralin was less toxic, with an EDso above 370 μM. The EDso of fenarimol was 125 μM and its therapeutic index (TI), according to the effects on amastigotes and Hela cells, was about 5. The TI for trifluraline was at least 30.
Effects on leishmanial lipid metabolism

General effect: Changes in the lipid metabolism of leishmanial promastigotes were examined by acetate labeling. The parasites were treated with 100 μM fenarimol; radioactive acetate was added and samples were examined 6 and 24 hr after the treatment. Most of the radioactive acetate was incorporated into the cells within the first 6 hr. This incorporation was not affected by the treatment. Seven and 27% of the acetate were incorporated into the lipids of the control cells within 6 and 24 hr, respectively. The treatment reduced these values to 5 and 11%, respectively (Table III).

Specific changes: TLC demonstrates that within 6 hr of treatment, there was a reduction in sterols, sterol esters, and phosphatidyl ethanolamine. There was a slight increase in triacylglycerol and no change in the free fatty acids (Fig. 1). Another TLC that examines the specific effects on neutral sterols emphasizes the accumulation of intermediate products and the inhibition of the various sterols (Fig. 2). These changes reflect the inhibition of sterol biosynthesis. The results correlate with impairment of 14-sterol demethylase, a specific enzyme in the sterol biosynthetic pathway.

Electron microscopy

Ultrastructural observation of L. major promastigotes treated with fenarimol revealed a “rounding-up” of the cells and a general loss of endoplasmic reticulum. At 6 hr after the initiation of treatment, a number of dark, small, osmophilic vacuoles appeared, which increased and became a dominant feature at 24 hr. At this time, multivesicular bodies could be seen (Fig. 3).

Docking of fenarimol in the catalytic site of 14α-sterol demethylase

The model of 14α-sterol demethylase was built on the basis of the crystallographic structure of the enzyme of M. tubercu-
A. The catalytic binding site of the model.

B. The residues that generate the hydrophobic pocket of the catalytic site.

**FIGURE 4.** The model of *L. major* cytochrome P450 14α- lanosterol demethylase. (A) The catalytic binding site of the model. It consists of 4 conserved regions, Y102-F109, M122-L126, F289-T294, and L355-L358, depicted in a ribbon presentation, and colored in red, cyan, blue, and magenta respectively. These regions generate a hydrophobic pocket that with the heme group embed the fenarimol molecule. The fenarimol molecule and the heme group are depicted in stick presentation and colored in gray, blue, red, and green for carbon, nitrogen, oxygen, and chlorine atoms, respectively. The iron atom of the heme group is depicted in yellow space-filling presentation.

**DISCUSSION**

In this work, we have demonstrated that the herbicide fenarimol inhibits both promastigote and amastigote development. Electron microscopic images revealed parasite membrane disruption. The TI of fenarimol, according to the effects on amastigotes and Hela cells, was about 5. This is not a high TI. However, fenarimol may be derivatized to produce a more effective compound analogously to trifluralin (Callahan et al., 1996). Moreover, the in vivo potential of fenarimol was reflected in significant anti-leishmanial activity in a mouse model. Labeling studies indicated an inhibition of lipid synthesis after 6 hr. There was a slight increase in triacylglycerol and no change in the free fatty acids. Examination of the neutral sterols emphasizes the accumulation of intermediates and the loss of various sterols. These results correlate with impairment of 14α-sterol demethylase. Modelling 14α-sterol demethylase, the suspected enzymatic site of inhibition with fenarimol, indicates the possible way for docking the inhibitor.

The anti-leishmanial activity of amphotericin B and ketoconazole also point to ergosterol synthesis as a susceptible metabolic pathway. Beach et al. (1988) demonstrated that parasite synthesis of ergosterol was an essential function. They argued that there were both “bulk” and essential sterol requirements for membranes, and that ergosterol fell into the latter category. The bulk requirements can be met by utilization of exogenous cholesterol found in the host serum or host cell, while ergosterol, a 28-sterol, must be synthesized de novo. Beach et al.
(1989) proposed that drugs inhibiting the lanosterol-14α demethylation produce a destabilization of membrane structure. They postulated that accumulation of 14α-methyl sterols and cholesterol in azole-treated organisms disordered the membranes by the protrusion of the “methyl group from the sterol α-face, compromising sterol interactions with the paraffin chains of the fatty acyl group of lipid bilayer phospholipids.” Electron microscope images revealed the gradual accumulation of electron-dense large vesicles and multilamellar osmophilic granules, which resemble those appearing after treatment with azasterols (Lorente et al., 2004). These have been also observed in *Leishmania* spp. exposed to elevated temperatures, where interruption is observed in desaturation of long-chain fatty acids (Greenblatt and Wetzel, 1966; Beach et al., 1982). Furthermore, they also occur in plant cells treated with anionic surfactants (Healey et al., 1971). These activities may possibly reflect membrane alterations related to dysfunction in lipid metabolism.

In eukaryotic, rather than prokaryotic, parasites, there are many metabolic systems shared with the host. Finding unshared metabolic targets is an advantage in the design of certain therapeutical agents. Chan and Fong (1990) showed that trifluralin, a herbicide, could distinguish between host and parasite tubulins. Although trifluralin reacted with leishmanial tubulins and inhibited their morphological changes that are dependent on tubulin, it did not bind to rat brain tubulin. The inhibitory effects of fenarimol on *Leishmania* spp. parasites support the supposition that the ergosterol pathway is a susceptible parasite target, and its inhibition is associated with disrupted cellular structure.

A model of 14α-lanosterol demethylase of *L. major* was built, on the basis of the crystallographic structure of the enzyme of *M. tuberculosis*, using a homology modeling method. Docking of the fenarimol into the catalytic binding site showed that it has a geometrical fit. Fenarimol is stabilized via hydrophobic interactions with the residues that surround it and interaction with the heme ring. The modeling results are an additional support to the hypothesis that fenarimol inhibits sterol biosynthesis by interfering with the enzyme 14α-lanosterol demethylase.

Molecular signatures of plantlike characteristics have remained functional in *Leishmania* spp. in a number of metabolic areas, including ergosterol synthesis, fatty acid desaturation, and certain glycosomal functions (Korn and Greenblatt, 1963; Hannaert, Bringaud et al., 2003; Hannaert, Saavedra et al., 2003; Opperdoes and Szikora, 2006). An in silico investigation of 110 of these *L. major* glycosomal enzymes revealed that 18 were homologous to bacterial ones (Opperdoes and Szikora, 2006). It has been suggested that having lost the chloroplast, these enzymes found a new home in the unique eukaryotic organelle, the glycosome (Martin and Borst, 2003). Unfortunately, in this eukaryotic group, no clear remains of a plastid or a residual ancestral organism exist as in the Apicomplexa to convince us of an actual endosymbiotic event (Gleeson, 2000). Recently the concept of the endosymbiotic event has been challenged on the basis of a growing body of information suggesting multiple events of lateral gene transfer, which may better explain these relic pathways (Katz, 2002; Simpson et al., 2006). For the scientist seeking susceptible parasite targets, their actual evolutionary origin is of less importance than being aware of their existence, and designing relatively nontoxic agents that successfully target them.

**ACKNOWLEDGMENTS**

We thank Professor Zvi Cohen from the Blaustein Institute for Desert Research, Ben Gurion University of the Negev for the gift of herbicides and radioactive acetate. We thank Dr. Ezra Rahamim from the Department of Electron Microscopy, The Hebrew University—Hadassah School of Medicine, Jerusalem for the electron microscopy. This work was supported by The Centre for the Study of Emerging Diseases and The Gretel B. Bloch Trust. We thank Makhteshim-Agan Industries, Beer-Sheva, Israel, for the gift of fenarimol.

**LITERATURE CITED**


CHAN, M., AND D. FONG. 1990. Inhibition of leishmanias but not host macrophages by the antitubulin herbicide trifluralin. Science 249: 924–926.


ZEIMAN ET AL.—FENARIMOL AND LEISHMANIA 285


Observations on the Sporozoite Transmission of *Plasmodium vivax* to Monkeys

William E. Collins, JoAnn S. Sullivan, Douglas Nace, Tyrone Williams,* Allison Williams,† and John W. Barnwell, Division of Parasitic Diseases, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30341; *Atlanta Research and Education Foundation, Atlanta, Georgia 30033; †Animal Resources Branch, Division of Scientific Resources, CDC, Atlanta, GA 30341. e-mail: wec1@cdc.gov

ABSTRACT: *Saimiri boliviensis* monkeys were infected via sporozoites with the Salvador I strain of *Plasmodium vivax* that had been stored frozen for periods ranging from 12 to 5,312 days. Prepatent periods ranged from 16 to 53 days.

Previously, we reported on the development of models for the testing of antimalarial vaccines using spleenectomized *Saimiri boliviensis* monkeys with varying numbers of sporozoites of the Salvador I strain of *Plasmodium vivax* (Collins et al., 1988). In total, 20 monkeys were used, and the results indicated that when 100,000 sporozoites were injected intravenously into these animals, the prepatent periods averaged 16.6 days versus prepatent periods of 19.4 days after injections of 10,000 sporozoites. When 5 monkeys were injected with 1,000 sporozoites, only 4 developed detectable parasitemia, and the prepatent periods in the 4 infected animals ranged from 24 to 35 days. It seemed that the threshold for infection was between 1,000 and 10,000 sporozoites, and the prepatent period decreased with an increase in challenge dosage.

Subsequently, additional studies were conducted with many more animals and different numbers of sporozoites (Sullivan et al., 1996). Prepatent periods ranged from 10 to 63 days. By 1996, 193 *S. boliviensis* monkeys in total had been infected, 4 via bites, and the rest by the intravenous injection of 10,000 or more sporozoites of the Salvador I strain of *P. vivax*. The requirement for such large numbers of sporozoites and the resultant long prepatent periods contrasted markedly with the results of studies with *Plasmodium knowlesi* in the same animal model (Collins et al., 2004). As we reported, when *S. boliviensis* monkeys were given intravenous injection of 50 sporozoites of the H strain of *P. knowlesi* that had been stored frozen, the prepatent periods ranged from 11 to 16 days. In addition, sporozoite numbers ranging from 450 to 45,000 that had been frozen for over 11 years induced infections with prepatent periods of 10 to 13 days.

The objective of this current study was to determine whether sporozoites of the Salvador I strain of *P. vivax* that had been stored frozen for varying periods could be used to induce infections in spleenectomized *S. boliviensis* monkeys for challenge in vaccine trials.

The *S. boliviensis* were colony-born animals. On arrival at the facility, all animals were quarantined for a 2-mo conditioning period, weighed, and tested for tuberculosis. Parasitologic and serologic examination indicated that the animals were free of infection with malarial parasites before primary inoculation. The animals had previously been spleenectomized, infected with *Plasmodium spp.*, and cured of their infections before participation in this study. All animals were fed a diet that has been proven to provide adequate nutrition and calories to captive *S. boliviensis* used in malaria-related research. Meals were free of contaminants and freshly prepared. Daily observations of the animals’ behavior, appetite, stool, and condition were recorded. An attending veterinarian treated all animals as medical conditions arose.

*Anopheles stephensi* (originally from India) were laboratory reared and maintained at the CDC/DDP insectaries. Mosquito infection was obtained by allowing the caged anophelines to feed through a membrane on blood obtained from an infected chimpanzee. Mosquitoes were held in an incubator at 25 C until sporozoites were present in the salivary glands. Glands were dissected into sterile 50% fetal bovine serum/50% phosphate-buffered saline from groups of 10 mosquitoes, triturated, and an aliquot was counted in a Neubauer cell-counting chamber. Dilutions were made, and the sporozoites were distributed into sterile vials in volumes of 0.5 ml. They were directly stored in the vapor-phase of a liquid nitrogen freezer. For challenge, a vial was removed from storage, thawed quickly, drawn into a syringe, and injected intravenously into the monkey.

Beginning 14 days after sporozoite injection, thick (Earle and Perez, 1932) and thin blood films were made daily and stained with Giemsa. Parasite counts were recorded per microliter of blood. At the end of the study, all animals were administered 30 mg of chloroquine (base) by oral intubation over a period of 3 days and 2.5 mg of primaquine daily for 7 days.

Fifteen of the 21 animals that were injected with sporozoites that had been stored frozen became infected (Table I). The prepatent periods that ranged from 16 to 53 days (median of 32 days) were, in most instances, at the high end of the reported period that we had seen in previous studies (Collins et al., 1988; Sullivan et al., 1996). In our report on studies with frozen sporozoites of *P. knowlesi*, we concluded that the survival of frozen sporozoites may be less that 10% using the current freezing and storage procedures. With a parasite model system that requires between 1,000 and 10,000 sporozoites to predictably ensure infection, such as the *S. boliviensis*/P. vivax combination, the number of sporozoites available was apparently insufficient and thus we obtained only approximately 70% infection. The extended prepatent periods and the 6 failed infections are indications that improvement in the freezing procedure is needed.

Nonetheless, it was demonstrated that sporozoites of *P. vivax* could be stored frozen for greater than 14 yr and be made available for challenge studies withFrozen refrigerated procedures. With a parasite model system that results in patent periods ranging from 16 to 53 days.

**Table I. Endohelmiths recollected of 7 fish species from La Mintzita Reservoir, Michoacan, Mexico.**

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>Previous infections with <em>Plasmodium spp.</em> †</th>
<th>Number of sporozoites (×10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Period Frozen (days)</th>
<th>Prepatent Period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI-0260</td>
<td>None</td>
<td>25,000</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>SI-063</td>
<td>None</td>
<td>25,000</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>SI-0277</td>
<td>None</td>
<td>25,000</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>SI-0266</td>
<td>None</td>
<td>25,000</td>
<td>12</td>
<td>47</td>
</tr>
<tr>
<td>SI-0207</td>
<td>None</td>
<td>30,000</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>SI-0080</td>
<td>None</td>
<td>30,000</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>SI-0091</td>
<td>None</td>
<td>30,000</td>
<td>15</td>
<td>NI*</td>
</tr>
<tr>
<td>SI-0192</td>
<td>None</td>
<td>30,000</td>
<td>15</td>
<td>NI</td>
</tr>
<tr>
<td>SI-0967</td>
<td><em>P. falciparum</em>, <em>P. falc.</em></td>
<td>90,000</td>
<td>296</td>
<td>39</td>
</tr>
<tr>
<td>SI-0966</td>
<td><em>P. falciparum</em>, <em>P. falc.</em></td>
<td>90,000</td>
<td>296</td>
<td>43</td>
</tr>
<tr>
<td>SI-0975</td>
<td><em>P. falciparum</em>, <em>P. falc.</em></td>
<td>90,000</td>
<td>296</td>
<td>45</td>
</tr>
<tr>
<td>SI-0964</td>
<td><em>P. falciparum</em>, <em>P. falc.</em></td>
<td>90,000</td>
<td>296</td>
<td>NI</td>
</tr>
<tr>
<td>SI-0276</td>
<td>None</td>
<td>130,000</td>
<td>405</td>
<td>53</td>
</tr>
<tr>
<td>SI-0258</td>
<td>None</td>
<td>130,000</td>
<td>405</td>
<td>NI</td>
</tr>
<tr>
<td>SI-2045</td>
<td><em>P. vivax</em>, <em>P. knowlesi</em>, <em>P. sim.</em></td>
<td>75,000</td>
<td>5,312</td>
<td>23</td>
</tr>
<tr>
<td>SI-2047</td>
<td><em>P. vivax</em>, <em>P. sim.</em></td>
<td>75,000</td>
<td>5,312</td>
<td>32</td>
</tr>
<tr>
<td>SI-2028</td>
<td><em>P. falciparum</em>, <em>P. knowlesi</em>, <em>P. sim.</em></td>
<td>75,000</td>
<td>5,312</td>
<td>NI</td>
</tr>
</tbody>
</table>

† *P. falc* = *Plasmodium falciparum*; *P. falc* = *P. falciparum*; *P. vivax*; *P. knowlesi* = *P. knowlesi*; *P. sim* = *P. simium*.

* NI = No infection.
leng of *S. boliviensis* monkeys either for vaccine or other biologic studies.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. This study was supported in part by an Interagency Agreement 936-3100-AA6-0-P0-0006-07 between the United States Agency for International Development and the Centers for Disease Control and Prevention.

**LITERATURE CITED**


**Estimation of the Endohelminth Parasite Species Richness in Freshwater Fishes From La Mintzita Reservoir, Michoacán, Mexico**

María de la Luz Romero-Tejeda, Luis García-Prieto, Lorena Garrido-Olvera, and Gerardo Pérez-Ponce de León, Laboratorio de Helmintología, Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Coyoacán, C.P. 04510, Apartado Postal 70-153, México, D.F., México. e-mail: gprieto@biologia.unam.mx

**ABSTRACT:** In total, 9 endohelminth species were found to parasitize 7 fish species (2 cyprinids, 4 goodeids, and 7 poeciliids) from La Mintzita Reservoir, Michoacán, in central Mexico; 5 were larvae, including 3 allogenic species (*Clinostomum complanatum*, *Tylodelphys* sp., *Posthodiplostomum minimum*) and 2 autogenic species (*Serpinema trispinosum*, *Spirooxys* sp.). Four were enteric autogenic adults, i.e., *Margotremia bravae*, *Bothrioccephalus aechilognathi*, *Proteocephalus longicollis*, and *Rhabdochona lichtenfelsi*. The metacercariae of *P. minimum* reached the highest levels of prevalence and mean abundance among host species. Our results confirm the depauperate nature of the helminth communities of freshwater fishes from central Mexico. On the basis of this data set, we estimated the total endohelminth species richness for each component community by using 7 nonparametric estimators whose performance was evaluated with the unscaled measures of bias, precision, and accuracy. We found that Chaol and Bootstrap are the most precise and least biased methods for the 7 component communities; however, species richness was consistently underestimated. The underestimation was an unavoidable consequence of the patchy distribution of helminth species among different component communities, particularly at the small sample size used in our study.

Central Mexico is a region where surveys of freshwater fish parasites have been conducted extensively over the last decades. Most of these studies are focused on taxonomic records of a particular host species (Osorio-Sarabia et al., 1986), a host group in 1 locality (Peres barbosa et al., 2000); 1 species, frequently the metacercariae of the digenean *Posthodiplostomum minimum*, a species that matures in piscivorous birds (Espinosa-Huerta et al., 1996; Rojas et al., 1997; Pérez-Ponce de León et al., 2000; Sánchez-Nava et al., 2004). Even though a great amount of information has been produced, the total helminth species richness of only 3 host species has been estimated using empirical data and the same methodological approaches (Martínez-Aquino et al., 2004, 2007). The objectives of the present work are (1) to describe the endohelminth parasites of 7 fish species sampled concurrently in La Mintzita, Michoacán, a natural water reservoir located in central Mexico; (2) on the basis of the number of endohelminth species recorded in these 7 fish species (observed richness), to estimate the total species richness for each component community (observed species richness + number of missing species remaining to be found in each component community) using 7 nonparametric richness estimators; and (3) to compare the usefulness of these estimators in predicting the species richness in each component community by evaluating their performance with the unscaled measures of bias, precision, and accuracy (see Walther and Moore, 2005).

In total, 166 adult fishes representing 7 of the 13 fish species inhabiting La Mintzita Reservoir were collected in September 2003: *Cyprinidae*: *Notropis caliensis* (12), *Yuritia alta* (30); *Goodeidae*: *Alloophorus robustus* (27), *Skiffia iberma* (30), *Xenotoca varia* (7), and *Zoogeneticus quitzeoensis* (30); *Poeciliidae*: *Xiphophorus helleri* (30). La Mintzita Reservoir is located in the northern portion of the Michoacán State, 7 km SW from Morelia City (19°38′40″N, 111°16′28.7″W). The total area of the reservoir is 7.65 km², with a mean depth of 1.5 m (Medina-Nava et al., 2003).

Fishes were captured using seine nets and examined within 4 hr of capture using standard procedures. Voucher specimens of all helminth taxa were deposited at the Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Mexico City, Mexico. Prevalence and mean abundance of infections were calculated following Bush et al. (1997).

By computing 7 nonparametric species richness estimators, we evaluated the total endohelminth species richness in the 7 component communities of fish species from La Mintzita Reservoir; the program EstimateS 7.5 was used (Colwell, 2005). For comparative purposes, we chose ACE, ICE, Chao1, Chao2, Jackknife1, Jackknife2, and Bootstrap estimators, which were assessed by Walther and Morand (1998) on parasite component communities. Each host was considered a sample and the entire component community was considered the data set. For each component community, the sample order was randomized 100 times without replacement; for each new random combination of samples, all estimators were used to calculate the total species richness. The mean values and standard deviation of the 100 resulting estimates at each level of sampling effort were used to calculate the bias, precision, and accuracy of each estimator. The difference between the mean value of the estimates and the total species richness represents the bias of each estimator at each level of sampling effort. Accuracy is the square of these differences; the standard deviation of the estimates yields the precision of each estimator at each level of sampling effort. The performance measures are presented as the average of each performance measure over all levels of sampling effort (Walther and Moore, 2005).

In total, 9 endohelminth species (observed species richness) were...
| Host species         | Parameters | Cyprinidae | | Goodeidae | | Poeciliidae |
|---------------------|------------|------------|------------|------------|-------------|
|                     |            | Notropis calientis | Yuriria alta | Allophorus robustus | Skiffia lermae | Xenotoca variata | Zoogoneticus quitzeoensis | Xiphophorus helleri |
| Length (mm)         | 2.5–3.7    | 5–15.6     | 3.8–10.3   | 3.9–7      | 2.9–4      | 2.9–5.7   | 3.4–7.3 |
| Digenea             | %, Ab ± SD | %, Ab ± SD | %, Ab ± SD | %, Ab ± SD | %, Ab ± SD | %, Ab ± SD | %, Ab ± SD |
| M. bravae           |            | C. complanatum* |            |            |            |            |            |
| Intestine           | 5038–39    | 5044–46    | 7.4, 0.07 ± 0.27 | 3.33, 0.03 ± 0.18 | 14.29, 0.14 ± 0.38 | 10, 0.1 ± 0.31 | 3.33, 0.03 ± 0.18 |
| Body cavity         |            |            |            |            |            |            |            |
| Tylodelphys sp.*    | 5041–43    |            |            |            |            |            |            |
| Eyes                |            |            |            |            |            |            |            |
| P. minimum*         |            |            |            |            |            |            |            |
| Body cavity, brain, liver | 5051–53 | 91.67, 8.58 ± 8.33 | 100, 473.8 ± 749.54 | 30, 1.27 ± 3 | 14.29, 0.14 ± 0.38 |            |            |
| Cestoda             |            |            |            |            |            |            |            |
| B. acheilognathi    |            |            |            |            |            |            | 16.67, 0.43 ± 1.36 |
| Intestine           | 5054–57    | 58.33, 0.83 ± 0.94 | 10, 0.13 ± 0.43 | 7.4, 0.19 ± 0.79 |            |            |            |
| P. longicollis      | 5040       |            |            |            | 16.67, 0.2 ± 0.48 |            |            |
| Nematoda            |            |            |            |            |            |            |            |
| S. trispinosum*     |            |            |            |            |            |            |            |
| Intestine           | 5061       |            |            |            | 3.3, 0.03 ± 0.18 |            |            |
| Spiroxys sp.*       |            |            |            |            |            |            |            |
| Body cavity, intestine, mesentery | 5062–63 |            | 3.7, 0.07 ± 0.38 |            | 3.33, 0.3 ± 1.64 |            |            |
| R. lichtenfelsi     | Intestine  | 5058–60    | 3.7, 0.04 ± 0.19 | 96.67, 11.33 ± 7.93 | 6.67, 0.8 ± 4.2 |            |            |

CNHE = Accession number to Colección Nacional de Helmintos; % = Prevalence; Ab = Mean abundance; SD = Standard deviation. * = larvae.
TABLE II. Total endohelminth species richness for 7 fish species from La Mintzita Reservoir, Michoacán, Mexico, obtained with 7 estimators and performance measures of them.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sobs</th>
<th>ACE</th>
<th>ICE</th>
<th>Chao1</th>
<th>Chao2</th>
<th>Jack1</th>
<th>Jack2</th>
<th>Bootstrap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notropis calientis</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Bias</td>
<td>-1.94</td>
<td>-0.03</td>
<td>-0.15</td>
<td>-0.007</td>
<td>-0.04</td>
<td>-0.05</td>
<td>-0.09</td>
<td>-0.03</td>
</tr>
<tr>
<td>Precision</td>
<td>0.03</td>
<td>0.16</td>
<td>0.29</td>
<td>0.029</td>
<td>0.15</td>
<td>0.06</td>
<td>0.2</td>
<td>0.15</td>
</tr>
<tr>
<td>Accuracy</td>
<td>3.77</td>
<td>0.01</td>
<td>0.07</td>
<td>0.0003</td>
<td>0.019</td>
<td>0.01</td>
<td>0.2</td>
<td>0.0027</td>
</tr>
<tr>
<td>Yuriria alta</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2.04</td>
</tr>
<tr>
<td>Bias</td>
<td>-1.78</td>
<td>-0.0003</td>
<td>-0.13</td>
<td>-0.0003</td>
<td>-0.004</td>
<td>-0.97</td>
<td>-0.10</td>
<td>-0.12</td>
</tr>
<tr>
<td>Precision</td>
<td>0.16</td>
<td>0.26</td>
<td>0.42</td>
<td>0.04</td>
<td>0.009</td>
<td>0.97</td>
<td>0.90</td>
<td>0.35</td>
</tr>
<tr>
<td>Accuracy</td>
<td>3.15</td>
<td>0.0004</td>
<td>0.06</td>
<td>0.0004</td>
<td>0.0008</td>
<td>0.06</td>
<td>0.14</td>
<td>0.018</td>
</tr>
<tr>
<td>Alloophorus robustus</td>
<td>4</td>
<td>4.65</td>
<td>6</td>
<td>4</td>
<td>4.32</td>
<td>5.93</td>
<td>6</td>
<td>4.97</td>
</tr>
<tr>
<td>Bias</td>
<td>-2.42</td>
<td>-1.3</td>
<td>-2.3</td>
<td>-0.4</td>
<td>-1.2</td>
<td>-1.9</td>
<td>-2.8</td>
<td>-0.9</td>
</tr>
<tr>
<td>Precision</td>
<td>2.60</td>
<td>1.8</td>
<td>2.19</td>
<td>0.60</td>
<td>1.61</td>
<td>1.13</td>
<td>2.12</td>
<td>0.94</td>
</tr>
<tr>
<td>Accuracy</td>
<td>5.86</td>
<td>2.07</td>
<td>6.61</td>
<td>0.21</td>
<td>1.65</td>
<td>3.91</td>
<td>8.9</td>
<td>0.91</td>
</tr>
<tr>
<td>Skiffia lermae</td>
<td>5</td>
<td>9.05</td>
<td>7.24</td>
<td>6*</td>
<td>5.97*</td>
<td>6.93</td>
<td>8.8</td>
<td>5.73</td>
</tr>
<tr>
<td>Bias</td>
<td>-3.82</td>
<td>-1.96</td>
<td>-1.81</td>
<td>-0.59</td>
<td>-0.74</td>
<td>-1.29</td>
<td>-2.17</td>
<td>-0.57</td>
</tr>
<tr>
<td>Precision</td>
<td>0.89</td>
<td>1.83</td>
<td>2.05</td>
<td>1.25</td>
<td>1.5</td>
<td>0.92</td>
<td>1.67</td>
<td>0.80</td>
</tr>
<tr>
<td>Accuracy</td>
<td>14.6</td>
<td>5.21</td>
<td>3.39</td>
<td>0.42</td>
<td>0.56</td>
<td>1.85</td>
<td>5.95</td>
<td>0.35</td>
</tr>
<tr>
<td>Xenotoca variata</td>
<td>3</td>
<td>6</td>
<td>5.57</td>
<td>6</td>
<td>5.57*</td>
<td>5.57</td>
<td>7.71</td>
<td>4.02</td>
</tr>
<tr>
<td>Bias</td>
<td>-1.71</td>
<td>-1.19</td>
<td>-1.02</td>
<td>-1.10</td>
<td>-1.02</td>
<td>-1.33</td>
<td>-2.19</td>
<td>-0.56</td>
</tr>
<tr>
<td>Precision</td>
<td>0.67</td>
<td>1.054</td>
<td>0.97</td>
<td>1.78</td>
<td>1.59</td>
<td>0.70</td>
<td>0.92</td>
<td>0.74</td>
</tr>
<tr>
<td>Accuracy</td>
<td>2.94</td>
<td>2.51</td>
<td>1.85</td>
<td>2.38</td>
<td>1.85</td>
<td>2.53</td>
<td>7.85</td>
<td>0.44</td>
</tr>
<tr>
<td>Zoogoneticus quitzeoensis</td>
<td>4</td>
<td>4</td>
<td>4.57</td>
<td>4</td>
<td>4</td>
<td>4.97</td>
<td>4.1</td>
<td>4.66</td>
</tr>
<tr>
<td>Bias</td>
<td>-2.67</td>
<td>-0.6</td>
<td>-1.59</td>
<td>-0.15</td>
<td>-0.61</td>
<td>-1.36</td>
<td>-1.68</td>
<td>-0.68</td>
</tr>
<tr>
<td>Precision</td>
<td>2.72</td>
<td>1.2</td>
<td>1.9</td>
<td>0.32</td>
<td>1.04</td>
<td>0.94</td>
<td>2.11</td>
<td>0.84</td>
</tr>
<tr>
<td>Accuracy</td>
<td>7.13</td>
<td>0.52</td>
<td>3.31</td>
<td>0.04</td>
<td>0.52</td>
<td>2.06</td>
<td>3.69</td>
<td>0.52</td>
</tr>
<tr>
<td>Xiphophorus helleri</td>
<td>2</td>
<td>2</td>
<td>3.12</td>
<td>2</td>
<td>2*</td>
<td>2.97</td>
<td>3.9</td>
<td>2.37</td>
</tr>
<tr>
<td>Bias</td>
<td>-1.38</td>
<td>-0.31</td>
<td>-0.60</td>
<td>-0.02</td>
<td>-0.12</td>
<td>-0.68</td>
<td>-1.11</td>
<td>-0.31</td>
</tr>
<tr>
<td>Precision</td>
<td>0.24</td>
<td>0.77</td>
<td>0.82</td>
<td>0.21</td>
<td>0.35</td>
<td>0.62</td>
<td>1.21</td>
<td>0.61</td>
</tr>
<tr>
<td>Accuracy</td>
<td>1.89</td>
<td>0.13</td>
<td>0.50</td>
<td>0.001</td>
<td>0.021</td>
<td>0.51</td>
<td>1.49</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Sobs = Observed richness; * Computed using the classic formula.

Six of the 9 helminth species were transmitted via trophic dynamics, whereas 3 metacercariae, i.e., P. minimum, C. complanatum, and Tylocephalus sp., penetrated their hosts through the skin. The goodeid, S. lermae, harbored the richest component community (5 species), whereas the remaining fish species were parasitized by 2–4 helminth species.

On the basis of the values of the 7 estimators used in this study, the minimum number of missing species remaining to be found in the 7 host species studied from La Mintzita Reservoir varies from 1 to 5 (Table II). However, considering performance measures obtained for Chao1 and Bootstrap (the least biased and most precise estimators; see Table II), the total number of species is known for 5 fish species, i.e., A. robustus, N. calientis, Y. alta, X. helleri, and Z. quitzeoensis, whereas in S. lermae and X. variata, 1 more species would be expected for each species.

With the exception of the nematode Serpinema trispinosus, all other helminth species have been recorded previously in La Mintzita Reservoir (Mejía-Madrid et al., 2005; Garrido-Olvera et al., 2006; Salgado-Maldonado, 2006). Moreover, the larva of S. trispinosus had not previously been recorded in fishes from central Mexico; however, adults of this nematode species have been found to parasitize garter snakes (Thamnophis spp.) and mud turtles (Kinosternon spp.) from 4 lakes located within the same geographical region (see Pérez-Ponce de León et al., 2001).

The helminth fauna of the 7 fish species in La Mintzita Reservoir included only 2 specialist species (Margotrema bravae and Rhabdorchon hochenfeldi), which were considered to be part of the biogeographical core helminth fauna of the Goodeidae in accordance with Mejía-Madrid et al. (2005). Additionally, a greater number of generalist species (C. complanatum, P. minimum, Tylocephalus sp., B. aichelognathi, Proteocephalus longicollis, S. trispinosus, and Spiroxys sp.) was recorded. The 3 metacercariae, B. aichelognathi, and the nematode Spiroxys sp. are the most commonly found parasite species in freshwater.
fishes from Mexico; each infects more than 20 freshwater fish species (Pérez-Ponce de León et al., 1996; Salgado-Maldonado, 2006). Likewise, Tylodelphys species are widely distributed in southeast Mexico, where they mainly infect cichlid fishes (Vidal-Martínez et al., 2001). Finally, we considered P. longicollis as a generalist species because this cestode has been reported from several salmonoid fishes in North America (Hoffman, 1999), even though in central Mexico it infects exclusively goodeid fishes.

The taxonomic composition and the low abundances of the helminths in each host species, as well as the number of uninfected hosts in La Mintzita Reservoir, correspond with the pattern described for 12 fish species (3 atherinopsids, 1 centrarchid, 1 cichlid, 2 cyprinids, and 5 goodeids) studied in other water bodies from central Mexico (Espinosa-Huerta et al., 1996; Rojas et al., 1997, Pérez-Ponce de León et al., 2000; Salgado-Maldonado, 2001; Martínez-Aquino et al., 2004; Sánchez-Nava et al., 2004). Our results confirm the depauperate nature of the helminth communities of freshwater fishes from central Mexico (Pérez-Ponce de León et al., 2000). These communities have been described as species poor, open to the invasion by allogetic generalist species (particularly avian-transported larval stages such as P. minimum and Tylodelphys sp.), and with a reduced number of specialist species that are not typically abundant. A further component of these helminth faunas is represented by helminth species anthropogenically introduced, i.e., B. acheilognathi (Martínez-Aquino et al., 2004).

Some historical and ecological factors have been identified as determinants of the depauperate character of these component communities: (1) the young geological age of several fish families from central Mexico; (2) the trophic status of the lake; (3) host feeding and behavioral habits; and (4) availability of parasite species and their colonization capabilities (Espinosa-Huerta et al., 1996; Pérez-Ponce de León et al., 2000; Pineda-López et al., 2005). Additionally, in La Mintzita Reservoir, anthropogenic factors such as pollution, introduction of exotic fish species, and extraction of water for domestic use (Medina-Nava et al., 2003) have modified the ecological conditions and the stability of this ecosystem. These conditions would preclude the colonization of new helminth species or would cause the extinction of others from this water body.

The extrapolations obtained using nonparametric estimators are common for many kinds of ecological studies; however, relatively little attention has been focused on their applicability in parasitological investigations. Previous studies have assessed the performance of nonparametric estimators for simulated data of parasite communities, pointing out that Chao1, Chao2, Bootstrap, and Jacknife1 are the most robust estimators (see Poulin, 1998; Walther and Morand, 1998; Zelmer and Esch, 1999). When real data sets of helminth species are used, Chao2 and Jacknife1 were consistently the most precise and least biased methods (see Walther and Morand, 1998). However, for different data sets, different estimators are expected to perform better (Colwell and Coddington, 1994).

When we applied the 7 nonparametric estimators to our data set, we found that Chao1 and Bootstrap are the most precise and least biased methods (Table II). The performance of Chao 1 was better in helminth communities with a high proportion of rare species (N. calientis, Y. alta, A. robustus, Z. quitsoeensis, and X. helleri); likewise, Bootstrap performed better in the component community with a high proportion of quadrants (individual hosts) containing 1 helminth species, i.e., in S. lermae, where R. lichtenfelsi had a prevalence of 96.67%, as well as in the component community where helminth species infected a similar proportion of hosts, i.e., in X. variata, where the digeneans C. complanatum, Tylodelphys sp., and P. minimum had the same prevalence (Table I).

However, the rates of species accumulation of Chao 1 and Bootstrap estimators increased slowly and did not reach a stable value of richness even though all sampling levels were considered. This condition determined that species richness was consistently underestimated, as it is indicated by the negative bias values obtained for both estimators (Table II). The underestimation was an unavoidable consequence of the patchy distribution of helminth species among the component communities, particularly at the small sample sizes used in our study. Therefore, the sample size should increase to account fully for all of the rare species and then, to predict accurately the number of “unfound” species (see Chazdon et al., 1998). In general, the patchy distribution of parasites is due to individual host differences in diet, susceptibility, behavior, and immune response, as well as the viability, dispersion, and behavior of individual parasites (Kennedy, 1975, 1977).

Recent helminthological surveys in the La Mintzita Reservoir included some of the host species analyzed in our study, i.e., A. robustus, N. calientis, S. lermae, X. variata, and Z. quitsoeensis (Mejía-Madríd et al., 2005; Garrido-Olvera et al., 2006; Salgado-Maldonado, 2006). Considering these studies, helminth species richness for each component community increased from 1 (in N. calientis) to 5 (in X. variata) species; this would confirm that the estimators yielded underestimated values of species richness for all component communities analyzed (Table II). However, only 2 endohelminth species reported in these studies are different from those recorded in our sampling efforts, i.e., the cystacanth of Polymorphus brevis in X. variata and the larval nematode Eustrongylides sp. in S. lermae (Salgado-Maldonado, 2006).

With the exception of M. bravoae, R. lichtenfelsi, and probably S. trispinosum, the helminth taxa recorded in our study seem to constitute a species pool for this particular area, parasitizing indistinctly all component communities, i.e., host sharing at this level is independent of host phylogeny, being determined primarily by niche sympathy and by the generenalist character of these helminth species (see Nelson and Dick, 2002). Eustrongylides sp. and P. brevis (also generalist species) could not be detected with the reduced sample size that we used in this study because of the low levels of infection present in host populations. On the basis of previous and present information regarding the estimation of helminth species richness in real and simulated communities, it is evident that there is not any estimator that is especially suitable for this particular parasite group. Therefore, further studies are necessary to establish if one of them can be recommended.

We thank María Antonieta Arizmendi for field assistance, Omar Domínguez and his students from the Universidad Michoacana de San Nicolás de Hidalgo, who helped us with the sampling and identification of hosts, and 2 anonymous referees whose suggestions improved this work. Hosts were captured under the collecting permit number FAUT 0057 issued by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT), Mexico. This study was funded by grants from PAPIIT-UNAM IN-220605 and CONACyT 47233, to G.P.P.L. L.M.R.T. thanks the program PAPIIT-UNAM (IN-220605) for providing a scholarship to conduct her undergraduate research project.

LITERATURE CITED


RESERCH NOTES 291
Parasite-Induced Changes in Nitrogen Isotope Signatures of Host Tissues

Hideyuki Doi†, Natalia I. Yurlova†, Svetlana N. Vodyanitskaya†, Eisuke Kikuchi‡, Shuichi Shikano‡, Elena I. Yadenkina§, and Elena I. Zuykova†

†Graduate School of Life Sciences, Tohoku University, 2-1-1, Katahira, Aoba-ku, Sendai 980-8577, Japan; ‡Present address: School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, Washington 98195; §Institute of Animal Systematics and Ecology of Animals Siberian Branch of Russian Academy Sciences, Frunze St., 11, Novosibirsk, 630091, Russia; §Center for Northeast Asian Studies, Tohoku University, Kawauchi, Aoba-ku, Sendai 980-8576, Japan. e-mail: hdoi@u.washington.edu

ABSTRACT: To estimate isotopic changes caused by trematode parasites within a host, we investigated changes in the carbon and nitrogen isotope ratios of the freshwater snail Lymnaea stagnalis infected by trematode larvae. We measured carbon and nitrogen stable isotopes within the foot, gonad, and hepatopancreas of both infected and uninfected snails. There was no significant difference in the δ13C and δ15N values of foot and gonad between infected and uninfected snails; thus, trematode parasite infections may not cause changes in snail diets. However, in the hepatopancreas, δ15N values were significantly higher in infected than in uninfected snails. The 15N enrichment in the hepatopancreas of infected snails is caused by the higher 15N ratio in parasite tissues. Using an isotope-mixing model, we roughly estimated that the parasites in the hepatopancreas represented from 0.8 to 3.4% of the total snail biomass, including the shell.

Parasitic infections introduce additional demands on host resources, either through direct competition for energy (Coop and Holmes, 1996; Sorensen and Minchella, 1998) or by stimulating the host’s immune system (Moret and Schmidt-Hempel, 2000). Recently, parasites have been shown to modify the feeding patterns of their intermediate hosts (e.g., Thompson, 1990; Miura et al., 2006). Hosts may attempt to compensate for the increased nutritional demands caused by parasites by increasing their foraging effort (Thompson, 1990).

Stable isotope ratios of carbon and nitrogen are increasingly being used to analyze the food sources of macroinvertebrates in various ecosystems (e.g., Vander Zanden and Vadeboncoeur, 2002; Doi et al., 2004, 2006). Stable isotope techniques can provide continuous measures of trophic positions that integrate the assimilation of energy or mass flow through all the various trophic pathways leading to an organism (McCutchan et al., 2003).

The freshwater snail Lymnaea stagnalis is an intermediate host for many species of trematode parasites (Yurlova, 2003). Lymnaea stagnalis is the first intermediate host for at least 15 trematode species that parasitize the hepatopancreas and the second intermediate host for 18 trematode parasite communities of Characodon audax and C. lateralis (Pisces: Goodeidae) endemic freshwater fishes from Durango, Mexico. Southwestern Naturalist 52: 125–130.


We investigated changes in the isotope ratios of infected and uninfected \textit{L. stagnalis} to determine whether parasites can affect the nature and concentration of isotopes. Changes in food sources of hosts because of infection have been reported for marine gastropods (Miura et al., 2006); the phenomenon was observed using stable isotopes. We hypothesized that (1) an infected snail will change food sources because of changes in feeding behavior and habitat, and (2) the isotope ratios in the tissues of infected snails will differ from those of uninfected snails. The confirmation of these hypotheses will provide key information for understanding host–parasite interactions because changes in host food sources and feeding habits because of parasites could affect survival and reproductive rates within a host population.

We studied the common freshwater snail \textit{L. stagnalis} inhabiting the riverine portion of Lake Chany, Siberia, Russia. \textit{Lymnaea stagnalis} is a dominant gastropod snail in western Siberia (Yurlova and Vodyanitskaya, 2005). Lake Chany is located in the Barabinskaya lowland of West Siberia, Russia (54°30'-55°00'N, 76°48'-78°12'E). The lake is located in a forest–steppe region at an altitude of 106 m above sea level. It is a shallow, inland, saline system (average depth, 2.2 m; maximum depth, 8.5 m; Aladin and Plotnikov, 1993; Doi et al., 2004). The study was conducted in the inflow part of the Kargat River (54°37.76'N, 78°13.07'E) of Lake Chany. The substrata at the site varied from detritus to sediment. Information regarding larval trematodes and their effects on the behavior, growth, fecundity, and population dynamics of their snail hosts in West Siberia has been reported previously (Yurlova, 1987, 2003, 2006; Yurlova et al., 2000, 2006).

Specimens of \textit{L. stagnalis} and their parasites were sampled in August 2004. Snails were collected by hand at a depth of 0.1–0.5 m. We collected 20 \textit{L. stagnalis} (10 infected and 10 uninfected). Under laboratory conditions, the snails were measured using a slide caliper (length of shell from apex to aperture), then dissected, and examined for the presence of trematode parasites using a microscope. We saved the foot, gonad, and hepatopancreas for isotope ratio analyses (n = 20). The tissues were first dried at 60°C and then stored at ~20°C. Before analysis, the dry weight of each tissue was measured. We calculated the relative contribution of each tissue to the dry weight of the body. We used t-tests to examine the effect of infection on tissue dry weight.

Before isotope measurement, the lipids in all tissues were removed using a chloroform:methanol mixture (2:1 by weight) because of the high lipid content in muscle tissue (Kling, 1992). The isotopic ratios of carbon and nitrogen in the samples were measured with a mass spectrometer (Integra CN, SerCon Co., Cheshire, U.K.). The results are presented using common delta notation, calculated as: $\delta^{13}C$ or $\delta^{15}N = \left( \frac{R_{sample}}{R_{standard}} - 1 \right) \times 1,000$ ($\%$), where \( R \) is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio for $\delta^{13}C$ or $\delta^{15}N$, respectively. See Dee Belelmine and atmospheric nitrogen were used as international standards for $\delta^{13}C$ and $\delta^{15}N$, respectively. The results during the overall analyses were within $\pm0.2\%$ for $\delta^{13}C$ and $\delta^{15}N$. The effects of infection on the carbon and nitrogen isotope values in the different tissues were determined using t-tests.

Four of 10 infected \textit{L. stagnalis} had sporocysts of Plagiorchis mutations; 6 of 10 snails were infected by sporocysts of \textit{Plagiorchis} sp. larvae. We determined the total dry weight of each snail by calculating the dry weight and the percentage of total weight represented by each tissue (foot, gonad, and hepatopancreas) from infected and uninfected \textit{L. stagnalis} (Table I). The shell lengths of uninfected and infected snails were 41.9 ± 0.43 and 42.5 ± 0.42 mm, respectively, and were not significantly different ($P = 0.90$). The dry weight and percentage of total weight of infected hepatopancreas were significantly higher than those of uninfected hepatopancreases ($P < 0.01$; Table I). However, the dry weight and percentage of the total weight of infected gonads were significantly lower than those of uninfected snails’ gonads ($P < 0.001$; Table I). The dry weight and percentage of total weight of foot tissue did not differ between infected and uninfected snails (Table I).

The $\delta^{15}N$ values from infected hepatopancreas were significantly higher than those of uninfected hepatopancreases ($P = 0.03$, Table II). However, there were no significant differences in $\delta^{15}N$ between infected and uninfected foot or gonad tissues, or in $\delta^{13}C$ values for foot, gonad, or hepatopancreas tissues ($P > 0.09$; Table II). Except for the $\delta^{15}N$ of the hepatopancreas, there were no significant effects of infection on the $\delta^{13}C$ and $\delta^{15}N$ values of the different tissues (Fig. 1). There were no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of tissues between snails infected with \textit{Plagiorchis} sp. and \textit{P. mutations} ($P = 0.3$). It is possible that our small sample sizes limited our ability to detect differences between snails infected by different trematode species and between infected and uninfected snails. We tested 2 hypotheses: (1) the food sources of host snails are altered by trematode infection, and (2) the isotopic ratios of infected tissues differ from uninfected tissues because of parasites. We did not observe the migration of cercariae through the tissues. Parasites can become significantly $^{15}N$-enriched from the host because parasites feed mainly on host tissues (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O’Grady and Dearing, 2006). Changes in host food sources because of infection have been reported for marine gastropods (Miura et al., 2006). Thus, parasites can modify the feeding patterns of snails that serve as their intermediate hosts (Levri, 1999; Levri and Fisher, 2000). However, the $\delta^{13}C$ and $\delta^{15}N$ values of foot and gonad tissues did not differ significantly between the infected and uninfected snails that we examined. Moreover, we did not find differences in the habitat use or feeding behavior of infected or uninfected snails in August. Also, we collected all snails (infected and uninfected) at the same sites and observed that they were moving in many directions. Thus, trematode infection may not have a strong effect on snail diet and may not affect the $\delta^{13}C$ and $\delta^{15}N$ values in snail tissues, except within the hepatopancreas. An additional explanation for the observed foot and gonad isotope values is that significant isotope changes were not detected because the snails fed on various food sources.

### Table I. The dry weight of the total body, foot, gonad, and hepatopancreas from infected and uninfected \textit{Lymnaea stagnalis} and the percentage of each tissue in the total dry weight of the snails (mean ± 1 SD, n = 10 infected and 10 uninfected snails). The \( t \)- and \( P \)-values are for \( t \)-tests comparing infected and uninfected snails.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dry weight (μg)</th>
<th>( t )</th>
<th>( P )</th>
<th>% of Total weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total body of snail with shell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>794 ± 116</td>
<td>-0.8</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
<td>Infected</td>
<td>838 ± 118</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Foot</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>104 ± 26</td>
<td>0.20</td>
<td>0.67</td>
<td>10.0 ± 1.9</td>
</tr>
<tr>
<td>Infected</td>
<td>88 ± 27</td>
<td>-1.3</td>
<td>0.28</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td><strong>Gonad</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>59 ± 17</td>
<td>-4.2</td>
<td>&lt;0.001</td>
<td>5.6 ± 1.5</td>
</tr>
<tr>
<td>Infected</td>
<td>33 ± 10</td>
<td>&lt;0.001</td>
<td>3.3 ± 0.8</td>
<td>-4.2</td>
</tr>
<tr>
<td><strong>Hepatopancreas</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>41 ± 11</td>
<td>0.01</td>
<td>0.99</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>Infected</td>
<td>58 ± 15</td>
<td>4.0</td>
<td>&lt;0.001</td>
<td>6.0 ± 1.6</td>
</tr>
</tbody>
</table>
TABLE II. The δ¹³C and δ¹⁵N values of the foot, gonad, and hepatopancreas of infected and uninfected *Lymnaea stagnalis* (mean ± 1 SD, n = 10 infected and 10 uninfected snails). The *t* and *P*-values are for *t*-tests comparing infected and uninfected snails.

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>Infected</th>
<th><em>t</em></th>
<th><em>P</em></th>
<th>Uninfected</th>
<th>Infected</th>
<th><em>t</em></th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Foot</td>
<td>−32.3 ± 1.6</td>
<td>−31.1 ± 0.9</td>
<td>−1.77</td>
<td>0.10</td>
<td>3.4 ± 1.5</td>
<td>4.3 ± 1.7</td>
<td>−1.24</td>
<td>0.23</td>
</tr>
<tr>
<td>Gonad</td>
<td>−32.2 ± 1.2</td>
<td>−31.5 ± 1.2</td>
<td>−1.36</td>
<td>0.19</td>
<td>3.9 ± 1.5</td>
<td>3.9 ± 2.0</td>
<td>0.11</td>
<td>0.91</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>−31.6 ± 1.5</td>
<td>−32.3 ± 1.0</td>
<td>0.87</td>
<td>0.39</td>
<td>3.1 ± 1.5</td>
<td>4.5 ± 1.1</td>
<td>−2.23</td>
<td>0.03</td>
</tr>
</tbody>
</table>

with various isotope ratios, such as sediment organic matter, benthic microalgae, and macroalgae.

In addressing our second hypothesis, the δ¹⁵N values of hepatopancreases from infected snails were significantly higher than those of uninfected hepatopancreases. Moreover, the dry weight of infected hepatopancreases was significantly greater than that of uninfected hepatopancreases. Many generations of trematode asexual larval stages, i.e., sporocysts and/or rediae, develop and reproduce in the hepatopancreas of an infected snail. In most snail–trematode systems, the growth and reproduction of trematode larval sporocysts and rediae occur in resource-rich host tissues, such as the digestive gland and gonads (Kube et al., 2006). Thus, a possible explanation for the greater weight of infected hepatopancreases is the altered composition of tissues such as the increased storage of calcium granules. Also, the dry weight of infected gonads was significantly lower than that of uninfected gonads. Some trematode species possess larval stages that castrate their snail hosts, either chemically or directly (mechanical castration), when the larvae are located in the gonads of the host and cause complete destruction of the gonad (Wilson and Denison, 1980; Sluiter et al., 1984; De Jong-Brink, 1990; Probst and Kube, 1999). In infected snails, the hepatopancreas contains trematode parasite tissues.

We conclude that the trematode parasites caused the δ¹⁵N enrichment and increased the weight of infected hepatopancreases, even though Cheng et al. (1983) indicated that the soft tissues of infected and uninfected snails do not differ after trematode parasites are removed. Parasites such as cestodes and nematodes become significantly δ¹⁵N-enriched from their hosts because the parasites feed mainly on host tissues (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O’Grady and Dearing 2006). Parasite δ¹⁵N-enrichment likely causes the δ¹⁵N values of infected and uninfected hepatopancreases to differ, although we did not directly measure the δ¹⁵N values of the trematode parasites.

We calculated the contribution of parasites to the isotope values of infected hepatopancreases using the mass–balance isotope-mixing model of Phillips (2001) with various isotope fractions of parasites as follows:

\[
\delta^{15}N_{\text{infected hepatopancreas}} = a(\delta^{15}N_{\text{uninfected hepatopancreas}} + \Delta^{15}N) + (1 - a)\delta^{15}N_{\text{infected hepatopancreas}}
\]

where Δ¹⁵N is the isotope fractionation of the cestode and nematode parasites, ranging from +1.0 to +4.0‰ (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O’Grady and Dearing, 2006). Based on the isotope-mixing model with various isotope fractions, the contribution of parasites to the isotope values of infected hepatopancreases ranged from 12.5 to 50.0%. Thus, the dry weight of the parasites was calculated using the assumed isotopic contribution from the parasites and the dry weight of the hepatopancreas. In this way, we roughly estimated that the dry weight of parasites in an infected hepatopancreas ranged from 7.3 to 29.0 µg. Parasites in the hepatopancreas thus accounted for between 0.8 and 3.4% of the total snail biomass. The high proportion of parasite biomass within the host probably causes remarkable changes in host reproduction and tissue weight. However, we acknowledge several caveats with this approach that affect its scope. Thus, the species composition of trematode parasites varies among individual snails, and the isotope estimation includes the contributions of many trematode species. In future studies, we plan to estimate and compare parasite biomass in various hosts and parasite species using stable isotopes.

We tested 2 hypotheses using stable isotopes of carbon and nitrogen in snail tissues. We showed that the food sources of snails are not changed by trematode infection, but the nitrogen isotope ratio differs between infected and uninfected hepatopancreases, probably because of the direct effect of parasite biomass. Using an isotope mixing model, we estimated the biomass of parasites in the snails. Our results provide an initial step for estimating the presence and biomass of parasites in host tissues using stable isotope techniques.

We thank Dr. K. Itoh, Department of Agriculture, Tohoku University, for her permission to use the stable isotope analytical facilities in her laboratory. We thank to Dr. A. Yurlov, Institute of Animal Systematics and Ecology, Siberian Branch of Russian Academy of Sciences (SB
RAS), for his help in the field survey. This study was partly supported by Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science (13570004, 16405005), Russian Found Basic Research (RFBR; 07-04-01416), and Integration Interdisciplinary Project Institute of Systematics and Ecology of Animals Siberian Branch of Russian Academy of Sciences (19).

LITERATURE CITED


A Tick From a Prehistoric Arizona Coprolite

Keith L. Johnson, Karl J. Reinhard†, Luciana Sianto‡, Adauto Araújo†, Scott L. Gardner§, and John Janovy, Jr.‡, Department of Anthropology, Butte 311, California State University, Chico, California 95929-0400; †719 Hardin Hall, School of Natural Resource Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68583-0887; ‡To whom correspondence should be addressed. e-mail: kreinhard1@unl.edu; †Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz, 1480 Rua Leopoldo Bulhões, Rio de Janeiro, Brazil; §CAS Nebraska Hall, School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588-0514, †424 Manter Hall, School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588-0118

ABSTRACT: Ticks have never been reported in archaeological analyses. Here, we present the discovery of a tick from a coprolite excavated from Antelope Cave in extreme northwest Arizona. Dietary analysis indicates that the coprolite has a human origin. This archaeological occupation is associated with the Ancestral Pueblo culture (Anasazi). This discovery supports previous hypotheses that ticks were a potential source of disease and that ectoparasites were eaten by ancient people.

Nearly 1,000 coprolites from the desert west of the United States have been analyzed for parasite remains (Reinhard, 1990, 1992). Although arthropod parasites are rare, they are occasionally found in coprolites. The discovery of lice in human coprolites led Fry (1977) to conclude that arthropods were consumed to control infestation. In 2005, we commenced the dietary and parasitological analysis of coprolites from Antelope Cave in the northwestern corner of Arizona. At this site, we discovered a tick from a coprolite. This discovery has health, behavioral, and ecological implications for the Puebloan people that once occupied the cave.

Antelope Cave is a large limestone cavern sunk into the gently rolling terrain of the Unkar Plateau some 40 km southeast of St. George, Utah. Prehistoric Native Americans occupied it, probably intermittently, for at least 3,000 yr (2028 B.C. to A.D. 1100). The most intense habitation of the cave is attributed to Ancestral Puebloan peoples (Anasazi) who lived there 1,300 to 1,000 yr ago. Antelope Cave lies within the Virgin River Branch of prehistoric western Anasazi territory, and the great majority of artifacts (for example, woven fiber sandals, plaited basketry, Virgin series pottery) in the cave reflects Puebloan (Kayenta) affiliation. There is scant evidence of Fremont cultural influence from the north in Utah. Cultural debris left in the cave by its prehistoric inhabitants forms a 1.52-m-thick layer and contains mostly perishable artifacts, including wooden arrow shafts, basketry, string, netting, sandals, needles and thread, etc., as well as painted pottery and various lithic tools. The Pueblo people used the cave for shelter, and in the surrounding area, they grew corn and beans, gathered wild plant foods, and hunted game, mostly rabbits.

Professional archaeologists have conducted excavations in the cave, off and on, since 1954 (Janetski and Hall, 1983; Janetski and Wilde, 1989). The most extensive excavations were undertaken by the University of California–Los Angeles (UCLA) in 1959–1960 (Johnson and Pendergast, 1960). The coprolite specimen discussed here was recovered by UCLA and came from 1,000-yr-old Puebloan deposits at the rear of the cave. The date is based on cross-dated Anasazi artifacts pending C14 assay.

In 1959, archaeologists from UCLA excavated five 2 × 2 m pits into the midden deposit of Antelope Cave. The excavation units were designated AC59-1 through AC59-5. The coprolite of concern here was recovered from the 60–76-cm level below the surface in pit AC59-2. Along with the coprolite, this level yielded a wide variety of cultural debris, including fragments of Pueblo pointed-toe sandals, sandal ties, a net bag, fiber cordage, feather cordage, and pottery. No features, such as fire hearths, storage pits, latrines, etc., were exposed in this or any other level.

Eight Antelope Cave coprolites have been analyzed to date. Five are consistent with humans and 3 are consistent with canids, probably dogs. Laboratory sample 2 is the focus of this report. Its field context is AC 1516, pit AC 59-2, 60–76 cm below surface. After contextual information was recorded, the coprolite was cleaned of extraneous dirt, photographed, and weighed. Its weight, 2.67 g was then recorded. Observations relative to biological origin were made. The coprolite was then rehydrated in 0.5% trisodium phosphate for 48 hr. It was placed in a 300-ml beaker, and rehydration solution was added until the coprolite was completely immersed. Parafilm was used to cover the beaker to prevent potential modern airborne pollen contamination. Observations were made after 24 hr of rehydration. Rehydration fluid color is sometimes useful in verifying human origin (Reinhard and Bryant, 1992). Human coprolites tend to turn the rehydration solution dark brown or black, although this is not always the case. In addition, the rehydrating coprolite was examined for a mucilage coat, which sometimes forms on dog coprolites after rehydration (Reinhard et al., 1988).

After 48 hr of rehydration, 3 Lycopodium sp. spore tablets were added to the coprolite to facilitate quantification (Warnock and Reinhard, 1994; Sianto et al., 2005). For this analysis, Lycopodium sp. spore batch 212761 was used. Previous analysis has shown that approximately 12,500 spores are present in each tablet (values presented from different analyses of tablets are 12,432, 12,489, and 12,542). The tablets were dissolved in a few drops of hydrochloric acid and added to the rehydrated coprolite.

The coprolite was then disaggregated. It was transferred to a 600-ml beaker along with the rehydration solution and dissolved Lycopodium sp. tablets. A magnetic stir rod was added to the beaker, which was placed on a stir plate. The coprolite in the solution was then stirred for 45 min until it was completely disaggregated.

Microscopic remains were separated from macroscopic remains by pouring the disaggregated coprolite through a 300-µm mesh screen. A stream of distilled water under pressure was used to thoroughly wash the microscopic remains through the screen and into a 600-ml beaker. The macroscopic remains on top of the screen were dried on cotton filter paper. The microscopic remains were sedimented by centrifugation in 50-ml tubes.

The microscopic remains were then analyzed for parasite eggs and microscopic dietary evidence such as plant cells, phytoliths, and starch grains. Nine microscope preparations were made for helminth eggs or protozoan cysts. The dietary residues were categorized and counted. Next, using the following formula, the numbers of each category of dietary residue per gram of coprolite were calculated: concentration = ([d] × n)/w, where i is items counted, n is marker Lycopodium spores counted, n is marker Lycopodium spores added, and w is weight of coprolite in grams.

Subsequent to this analysis, about 10 ml of sediment were processed in hydrofluoric acid and acetolysis solution for pollen following the methods of Reinhard et al. (2006). Pollen grains were counted, and the numbers of pollen grains per gram were calculated using the above formula. When the analysis of microscopic remains was completed, the dried macroscopic remains were examined for food residues and arthropods.

One fragment of a tick was found. Images of the tick were made with a Synecrscope Auto-Montage digital microscope system at the University of Nebraska State Museum Biodiversity Synthesis Laboratory, Lincoln, Nebraska. This system eliminates depth of field limitation problems by automatically capturing in the- focus regions from a range of focal planes and combining them into a single, fully focused, high-resolution image. Adobe Photoshop was used to reconstruct the complete view of the dorsal posterior of the tick so that festoons could be more accurately accounted.

The specimen was not cleared or mounted. We are saving the specimen for future study, including molecular analysis. Therefore, we felt that it was best to preserve the specimen with no further chemical treat-
The tick was fractured away and was not discovered in extensive examination of the coprolite residues. The incomplete nature of the specimen leaves some doubt as to the identification of species and developmental stage. We believe the tick is an adult or nymph of *D. andersoni* (Stiles). It is 3.4 mm long and 2.0 mm wide. This is in the range for adults and nymphs. The reconstruction of the tick (Fig. 3) shows that it originally had 11 festoons. The ventral surface is fractured posterior to the first left coxa and second right coxa. The outline of a broken-off third coxa is visible, but it is impossible to ascertain if there was a fourth. Three coxa would be consistent with a larva, and 4 would be consistent with a nymph or adult. There are no visible spiracles. If this was an adult, the spiral plate had to have been present just posterior the fourth coxa. Larvae have no spiracles. We believe that spiracles have been simply fractured away from the specimen since the region of the fourth coxa is missing. To support our identification, there is a distinct spur on the first coxa that is characteristic for larvae and nymphs of *D. variabilis* and *D. andersoni*. Relative to the three-legged larval stage, the general morphology of the scutum is consistent with an adult or nymphal male. The larval scutum is not as elongate as adults and nymphs. We cannot determine whether *D. variabilis* or *D. andersoni* is represented by this specimen. The key anatomical elements, i.e., spiracular plate goblets, are not present. *Dermacentor variabilis* is not present in the Rocky Mountain region and is not endemic to Utah due to climate (Longstreth and Wiseman, 1989). However, Antelope Cave is within the southern-most range of *D. andersoni*. Therefore, in all likelihood, the tick discovered at Antelope Cave is an adult or nymph *D. andersoni*.

From the perspective of the function of a tick as a disease vector, specific identification matters. Tickborne diseases have been suggested as potential health threats for Ancestral Puebloans (Stodder and Martin, 1992, p. 62). The discovery of *Dermacentor sp.* at Antelope Cave is the first empirical evidence that ticks and humans were in contact. Which diseases were potential threats for the Ancestral Puebloans at Antelope Cave? The fact that we found a *Dermacentor sp.* tick limits the number of disease possibilities (Roberts and Janovy, 2003; Bowman, 2003; PAHO, 2003). Lyme disease is transmitted by *Ixodes* spp. and *Amblyomma* spp. ticks. *Ehrlichiosis* is transmitted by ticks of 2 genera, i.e., *Ixodes* and *Amblyomma*. Tickborne relapsing fever is transmitted by *Ixodes* spp. and *Ornithodoros* spp. ticks. These diseases probably were not potentialities for Antelope Cave.
Rocky Mountain spotted fever is spread to humans by *Dermacentor* sp. ticks. Tularemia is caused by the bacterium *Francisella tularensis*, which is transmitted by species of *Amblyomma* and *Dermacentor*. Therefore, these 2 infectious diseases could have been transferred by ticks at Antelope Cave. Tick paralysis is a condition that results from neurotoxins secreted by ticks during their feeding process, so tick paralysis or serious skin reactions such as dermatosis, inflammation, swelling, ulceration, and itching are possible.

The question might be raised as to whether this tick was accidentally consumed inadvertently in grain or some other food product. The maize and sunflower found in the coprolite was thoroughly ground. Had the tick been a contaminant of the grain, it would have been ground into powder. It is not likely that ticks would have been in the cave, since *D. andersoni* feeds on small animals in brushy areas. The human coprolites from Antelope Cave contain bones of *Sylvilagus* sp. Rabbits, and the trash deposits contained bones from a variety of small vertebrates. Thus, humans came into contact with the ticks when they hunted small mammals. The fact that this *Dermacentor* sp. was found in an apparent human coprolite and the finding that it was partially crushed indicate that the tick was pinched between the teeth and swallowed. This action reflects a response on the part of one ancient person who chose to remove the tick, bite it, and swallow it, thus suggesting a prehistoric behavior pattern of eliminating arthropod pests by eating them.

We are currently examining more human and dog coprolites from Antelope Cave and anticipate reconstructing the parasite ecology of this site.

This research was supported, in part, by National Science Foundation (NSF) grant DBI-0506767 and by CNPq (Brazilian Research Council).

**LITERATURE CITED**


**J. Parasitol.,** 94(1), 2008, pp. 298-300

© American Society of Parasitologists 2008

**Observations on Cryptosporidium Life Cycle Stages During Excystation**

Panagiotis Karanis, Akio Kimura*, Hideyuki Nagasawa†, Ikuo Igarashit, and Naoyoshi Suzuki†, Medical and Molecular Parasitology Laboratory, Medical School, Center of Anatomy, Institute II, University of Cologne, 50937 Cologne, Germany; *Osaka Prefectural Institute of Public Health, Department of Virology, Osaka 537-0025, Japan; †National Research Center for Protozoan Diseases, Obihiro University for Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan. e-mail: karanis@obihiro.ac.jp

**ABSTRACT:** *Cryptosporidium parvum* (HJN-1 strain, genotype 2) mer­ozoa­ites were released from oocysts directly during an incubation and excystation procedure without bleach treatment. They were polymor­phic, mostly spindled-shaped; others were bean shaped, actively motile, and underwent division. Merozoites survived for short time–period in an in vitro culture system, but could not be established in a subsequent cultivation effort in RPMI medium.

Many vertebrates, including humans, are hosts to the intestinal protozan parasite, *Cryptosporidium parvum*. Species in this genus have a worldwide distribution and in the last 3 decades have become an increasingly important public health problem. *Cryptosporidium parvum* has been reported as the causative agent of a number of waterborne outbreaks of diarrheal disease and is considered one of the most important contaminants of drinking water (Karanis et al., 2007). The general life cycle and biology of *C. parvum* are comprised of an exogenous stage (oocysts with 4 sporozoites) and an endogenous phase (trophozoites, merozoites, and sexual stages) as extensively described in Fayer et al. (1997). Oocysts of *Cryptosporidium* spp. can be exposed to different media to produce sporozoite excystation. In vitro tech­niques for sporozoite excystation from oocysts have been reported by sev­eral investigators (Upton, 1997). Exposure to an acid pH during in vitro excystation protocols for *Cryptosporidium* spp. mimic host-derived signals, but some of these host-derived triggers seem to be unessential. There is still an insufficient understanding of the hierarchy or synergism of specific
after incubation in excystation solution with all of the mentioned forms: (a) unexcysted oocyst, and 2 empty oocysts with a residual body inside; (b) oocyst with sporozoites inside; (c) oocyst with a sporozoites outside attached the outer part of the oocyst wall; (d) oocyst wall with residual 0.50 % extraction were genetically analyzed, identified by nested PCR-RFLP containing antibiotics (penicillin and streptomycin). Observations during the present study were made using confocal laser scanning microscopy. Preparations, filter-sterilized medium composed of acidic H2O and the authors concluded that the in vitro propagation of Cryptosporidium spp. is not a universal phenomenon.

The aim of the present study is to report observations on C. parvum life cycle stages, which could be of interest for the in vitro developmental biology of this protozoon. Oocysts of C. parvum (HNJ-1 strain, genotype 2) are potentially infective for humans and other animals such as cattle (Satoh et al., 2005). This isolate was purified from an adult woman in Japan, and has been largely maintained in SCID mice and used in various laboratories in Germany and in Japan. During our observations, oocyst aliquots from the investigated stocks used for DNA extraction were not pretreated with bleach for decontamination. During incubation, 1 × 10^6, 1-mo-old oocysts were excysted in a freshly prepared, filter-sterilized medium composed of acidic H2O containing 0.50% trypsin and 0.75% taurocholate, using a 0.20-μm filter and incubated at 37 C for 40 min. Trials have been performed in Eppendorf tubes in a 0.5 ml suspension. After 20 min incubation, a small drop (2 μl) was resuspended on cover slips and examined microscopically. The suspension was spun in an Eppendorf centrifuge for 5 min at room temperature. A small drop taken from the bottom of the tube containing the parasites was re-suspended on cover slips and examined microscopically. To promote further development in vitro, equal portions of the remaining suspension was added to previously prepared culture flasks containing 40 ml of maintenance RPMI medium according to Hijjawi et al. (2004). Using light microscopy, we observed excysted and unexcysted oocysts (Fig. 1a) with banana-shaped sporozoites inside (Fig. 1b) of the oocysts, as well as a considerable number of banana-shaped sporozoites outside of the oocysts (Fig. 1c, d, e). Merozoites, observed at the same time, were plentiful, active, and pleomorphic (Fig. 1e–j); they variously measured (Table I) 3.12 μm × 1.47 μm (bean-shaped), 5.07 μm × 1.63 μm (long spindle-shaped), and 3.04 μm × 1.05 μm (short spindle-shaped). In addition, a few spherical stages, approximately 3.5–4 μm in diameter, were likewise seen; these forms were round and smaller than oocysts (not shown). The appearance of these stages was mostly similar to macrogamonts. In vitro propagation in monophasic and biphasic RPMI 1640 maintenance medium according to Hijjawi et al. (2004) was only possible for a limited duration of approximately 48 hr; efforts failed to establish a long-term axenic in vitro culture system using 50-ml culture flasks. Using oocysts pretreated with bleach may kill the very sensitive merozoites, which we report here. In accompanying work (not reported here), we have not observed the same stages when oocysts were pretreated with bleach. Most investigators of Cryptosporidium HNJ-1 developmental stages described.

<table>
<thead>
<tr>
<th>Form and properties</th>
<th>Oocysts of HNJ-1 (one month olds)</th>
<th>Merozoites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average sizes* (length × width) in μm</td>
<td>5.87–6.58 × 0.88–1.20</td>
<td>(a) 2.56–3.85 × 1.27–1.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) 2.71–3.71 × 0.95–1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) 4.25–5.98 × 1.20–1.98</td>
</tr>
</tbody>
</table>

* For the mean sizes at least 10 single measures were plotted.

**FIGURE 1.** Cryptosporidium HNJ-1 developmental stages and observations during excystation observed by CLSM. Images of different stages after incubation in excystation solution with all of the mentioned forms: (a) 1 unexcysted oocyst, and 2 empty oocysts with a residual body inside; (b) oocyst with sporozoites inside; (c) oocyst with a sporozoites outside attached the outer part of the oocyst wall; (d) oocyst wall with residual body and banana-shaped sporozoite; (e) sporozoite and merozoite. Merozoite images: different merozoite images; long spindle shaped (f); short spindle shaped (g) and bean shaped (h, i, j); a prominent nucleus is visible in all stages.
tosporidium spp. biology during in vitro studies treat purified oocysts with sodium hypochlorite in order to prevent microbial contamination. Bleach treatment triggers excreta and may enhance overall excreta rates and provide additional sanitization of the oocyst sample. However, Arrowood (2002) did not find bleach necessary when performing disinfection or survival studies. Moreover, we cannot exclude the possibility that this sort of in vitro emergence and development of Cryptosporidium sp. life cycle stages is strain-dependent. In addition, this organism may need several media manipulations to become adapted to axenic culture conditions, e.g., RPMI 1640 medium, mono- or biphasic growth media, or other media. Media manipulation may ultimately enable the development, propagation, and establishment of additional Cryptosporidium species in axenic in vitro cultures. Despite the impressive increases in the understanding of this parasite, much more remains to be explored on Cryptosporidium spp. evolution, life cycle, and development. We expect that the new findings will contribute to a better understanding of the life cycle of Cryptosporidium spp. and of various aspects of its developmental biology.

Some authors have suggested that classification of Cryptosporidium species needs to be reconsidered. The uncertain taxonomic position of Cryptosporidium species is based on the recent description of new developmental stages in the life cycle, which have characteristics similar to those of gregarines (Carreno et al., 1999; Zhu et al., 2000; Rosales et al., 2005). Our findings indicate a natural pressure on Cryptosporidium spp. for a development outside of its host’s cells. Perhaps the endogenous development in the host intestine is not obligate for this parasite to complete its life cycle. Sequence polymorphism for the HNJ-1 strain reflects the extent of subpopulation diversity within the genotype (Sato et al., 2005). Whether or not the genetic polymorphism of this strain also reflects some biochemical and/or physical properties, and if these findings can be extended to other strains remains to be demonstrated. The possibility of the exogenous in vitro development will offer more possibilities for Cryptosporidium spp. research. Data regarding the Cryptosporidium spp. genome and on nucleotide biosynthesis will provide significant insight on genetics, physiology, and metabolism for the group (Striepen et al., 2004; Xu et al., 2004). We suggest that future research using in vitro models and the broad array of molecular details should proceed in parallel with detailed work on morphology of the parasite to provide additional insight to the developmental biology of Cryptosporidium species. The success of axenic in vitro culture for mass production of the parasite will make the use of experimental animal models unnecessary. It should also be adaptable to drug sensitivity assays and vaccination studies.

We thank Dr. Jerry Ongerth for his review of the manuscript. The C. parvum HNJ-1 strain used in the present study was originally supplied by Dr. Motohiro Iseki of Kanazawa University, Japan.

LITERATURE CITED


Chinese Liver Flukes in Latrine Sediments From Wong Nim’s Property, San Bernardino, California: Archaeoparasitology of the Caltrans District Headquarters

Karl J. Reinhard, Adauto Araújo,* Luciana Sianto,* Julia G. Costello,† and Karen Swope‡, 719 Hardin Hall, School of Natural Resource Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68583-0987. e-mail: reinhard1@unl.edu; *Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz, 1480 Rua Leopoldo Bulhões, Rio de Janeiro, Brazil; †P.O. Box 288, 9686 Sport Hill Road, Mokelumne Hill, California 95245; ‡Caltrans San Bernardino District 8, 247 W. 3rd Street, San Bernardino, California 92402

ABSTRACT: Parasitological analysis of 5 sediment samples from San Bernardino, California latrine deposits spanning the time period from about 1880 to the 1930s are presented. Two sediment samples are from a latrine used by Chinese-Americans. Two European-Americans. The Chinese latrines are from latrines used by Chinese-Americans on the property of Wong Nim, an important member of the Chinese community. Two of the Chinese latrines were positive for human parasites. The human parasites encountered include the human whipworm (Trichuris trichiura), the giant intestinal roundworm (Ascaris lubricoides, c.f.), and the Chinese liver fluke (Clonorchis sinensis). Evidence of the liver fluke is especially important. This parasite cannot complete its life cycle outside of its end-host range in Asia because suitable intermediate hosts are not present in the American continents. Its presence signals that at least some of the Chinese-Americans who used the latrines were immigrants who were infected in Asia and then sustained infections while in the Americas.
Parasitological analysis of archaeological sediments can provide insights into human transhumance (Ferreira et al., 1984; Araújo et al., 1984; Costello and Hallaran, 2004; Costello et al., 2006). By 1880, the countywide Chinese population was about 150. Initially, they lived in various places throughout the town of San Bernardino. They farmed, operated laundries, worked in restaurants and hotels, and were employed as domestic servants or farm laborers. In 1878, the city prohibited laundries within the town limits, and, subsequently, a Chinese quarter was established. By the turn of the twentieth century, as many as 600 Chinese lived in Chinatown. Initially, Chinatown was virtually all male. It was composed of shops, boarding houses, gambling parlors, a temple, labor contractors, and other establishments. By 1893, Chinatown had electricity, and it had piped water by 1900. Human waste disposal was managed by construction of backyard latrines.

Three artifact-filled latrines were discovered during excavations. They were located on property purchased by California-born Wong Nim in 1900. Wong Nim was born in Alameda County, California, and moved to San Bernardino about 1875. He was successful. He first worked as a laundryman but eventually opened a mercantile shop and acted as a labor contractor. He also opened a temple. Wong remained on the corner of Third and B Streets until his death at age 89 in 1941. At that time, he had earned the honorary title of “Mayor of Chinatown.” When the State of California purchased Wong Nim’s property in 1943, all of the remaining buildings on his property were demolished. At least 1 latrine (number 1035) was filled at this time with debris from the abandoned buildings.

The privies were used by people who built residences and businesses on Wong’s original property. However, Wong’s house, store, and temple were located a half block away from the latrines and associated houses. It is possible that the latrines were communally used by several Chinese households and businesses.

Processing of the latrine sediment samples was done in 2001, following the methods of Reinhard et al. (1986), Warnock and Reinhard (1992), and Siano et al. (2005). Sediment was removed from each sample bag. The sediment was freed of large fragments of detritus. From the loose sediments, 30 ml were removed. Next, 3 Lycopodium sp. spore tablets were added to each 30-ml sample (about 1,250 Lycopodium sp. spores were added to each ml of sediment). For this analysis, Lycopodium sp. spore batch 212761 was used. Previous analysis has shown that ~12,500 spores are present in each tablet (values presented from different analyses of tablets are 12,432, 12,489, and 12,542). The tablets were dissolved in a few drops of hydrochloric acid in 300-ml beakers. Next, the 30-ml aliquots of sediment were added to the beakers with 50 ml of distilled water. Subsequently, 20 ml of 10% hydrochloric acid in distilled water was added to dissolve calcium carbonates in the sediment. More water was added until the reaction between the acid and the carbonates in the sediment stopped.

Once the calcium carbonates were dissolved, the samples were treated with the swirl technique. The contents of the beaker were swirled until all particles were in suspension. The beaker was placed on a flat surface for 30 sec. After 30 sec, the fluid was poured through a 300-μm mesh. This was repeated twice. The macrofossils on the mesh were examined for parasites, especially the presence of Rubus sp. seeds. Next, the screened fluid was concentrated by centrifugation in 50-ml centrifuge tubes. The sediments were washed 3 times in distilled water. Preliminary microscopic examinations were made of the samples to determine if further chemical processing was necessary. It was found that the high content of fine silicates required further processing, so 20 ml of 40% hydrochloric acid were added to each tube, and the sediments were thoroughly mixed in the acid. The samples were left in the hydrochloric acid for 24 hr and were stirred occasionally during this period. Next, the sediments were concentrated by centrifugation. The acid was replaced by water, and the sediments were reexamined. The vast majority of silicates were dissolved, and microscopic examination was deemed to be possible. The sediments in the tubes were then washed 3 times in distilled water.

Drops of the sediments were transferred to glass microscope slides with Pasteur pipettes. The sediment drops were mixed with glycerin and cover-slipped. For each sample, a total of 25 Lycopodium sp. spores was counted along with all parasite eggs found in the process of counting the spores. A count of 25 spores represents 0.02 ml of the sediment sample. After counting, at least 3 more microscope preparations were counted to assess the presence of trace amounts of parasite eggs.

We quantified the parasite eggs for 0.02 ml of processed sediment for each sample in order to standardize the results of each analysis in terms of parasite eggs per ml of sediment. For latrine contexts, we found that 0.02 ml is sufficient to identify parasite egg quantities as low as 50 eggs per ml. We then scanned an additional 0.06 to 0.08 ml of processed sediment to identify trace amounts of parasite eggs.

The concentrations of eggs of each species were calculated using the following formula: concentration = ([pml] × a/v), where p is parasite eggs counted, m is marker Lycopodium sp. spores counted, a is marker Lycopodium sp. spores added, and v is volume of sediment.

Identification of the species of the parasite eggs was done by morphological analysis. In the case of trichurid eggs, the dimensions of the eggs were taken and compared to those of trichurid species from a variety of hosts, including humans, domestic animals, and rodents that commonly infest habitations, outbuildings, and yards. Operculated eggs were compared to the morphology of a variety of cestode and trematode genera. These included species of Clonorchis, Paragonimus, Fasciola, Diphyllobothrium, and Dicrocoelium.

Based on many years of experimentation (Reinhard et al., 1986; Warnock and Reinhard, 1992; Siano et al., 2005), we have found this method to be superior to all clinical methods for recovery of parasite eggs from latrine sediments (soil derived from feces). This is because parasite eggs in latrine soils do not respond to flotation in the same way as modern eggs. Parasite eggs are trapped in calcium carbonate deposits and must be freed by chemical means. The calcium carbonate deposits are a special problem in latrines because people added lime to the latrines when they were in use.

We reanalyzed the processed sediments in 2003 to verify the diagnoses based on observations of more eggs. A third analysis was done in 2005 to photograph the eggs.

Samples from the Euro-American latrines were negative for parasite eggs. Two of three samples from Chinese-American latrines were positive for parasite eggs (Fig. 1). Latrine 1056 was the earliest latrine and was used from the 1880s to about 1900. Latrine 1058 was built in 1900 and filled in 1910; number 1035 was the final latrine and was used from 1910 to 1944.
Latrine 1035 yielded 1,062 ascarid eggs and 710 whipworm eggs per ml of sediment, while latrine 1056 contained 3,374 ascarid eggs and 358 whipworm eggs per ml of sediment. These faecal-borne whipworm eggs are nearly ubiquitous in historical town sites. These numbers are not high for latrine sediments and are relatively normal for historical sediments. At low or moderate infections, these parasites rarely cause severe disease. No eggs were found in latrine 1058. The whipworm and ascarid eggs were morphologically identical to eggs of Trichuris trichiura and Ascaris lumbricoides, respectively. We acknowledge that A. lumbricoides is morphologically identical to A. suum. We believe that the eggs are from A. lumbricoides because pigs did not live at the site and the latrines were used for human waste. There has been a debate concerning the value of egg measurements for diagnosis of T. trichiura and Trichuris suis. Horne and Tuck (1996) argued that this diagnosis is not possible with archaeological remains. In contrast, Fernandes et al. (2005) presented the majority view that whipworm egg dimensions can be obtained from archaeological sediments for diagnosis.

The most interesting discovery in both of these privies was the delicate eggs of Clonorchis sinensis, the Chinese liver fluke. The discovery of these eggs shows that the Chinese immigrants in California brought with them at least 1 species of parasite from Asia. Latrine 1035 contained 710 C. sinensis eggs per ml, and latrine 1056 contained 533 eggs per ml. The 3 latrines were used at different times by the same Chinese community. It is noteworthy that the earliest latrine deposits (1880–1900) and the latest latrine deposit (1910–1941) were positive for parasite eggs, but the 1900–1910 latrine sediment contained no eggs. The absence of evidence of parasitism in the middle period is unexplained.

The Chinese liver fluke, like most trematodes, has a multistage life cycle, which includes fishes and snails. These intermediate hosts have important roles in the life cycle of the parasite. The parasite goes through asexual reproduction in the snails. Thus, the number of parasites produced by a single egg is amplified by the snail stage of the life cycle. The fish is important in conveying the parasites to their definitive hosts, a fish-eating mammal. The definitive host is the animal that harbors the sexually active stages of the parasite. If the parasite survives the culinary preparation of the fish, it will eventually migrate to the liver of the definitive host and live there for many years, mating and laying eggs. The eggs pass through the bile duct into the digestive tract and are passed with feces.

However, the introduction of this parasite to California was a dead end. The intermediate snail hosts to which it is adapted in Asia are not high for latrine sediments and are relatively normal for historical sediments. The finding of Diphyllobothrium in human coprolites (4,100–1,950 B.C.) from northern Chile. Memórias do Instituto Oswaldo Cruz 79: 175–180.


SIANTO, L., K. J. REINHARD, M. L. C. GONÇALVES, and A. ARAÚJO. 2005. The finding of Echinostoma (Trematoda: Digenea) and hookworm
Seroprevalence of *Neospora caninum* Antibodies in Dogs in India

S. Sharma, M. S. Bal, Meenakshi, K. Kaur, K. S. Sandhu, and J. P. Dubey*, Department of Epidemiology and Preventive Veterinary Medicine, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, India; *To whom correspondence should be addressed. United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, Maryland 20705-2350. e-mail: jitender.dubey@ars.usda.gov

**ABSTRACT:** *Neospora caninum* is one of the most important causes of abortion in cattle worldwide, and dogs are an important risk factor for *N. caninum* infection in cattle. Antibodies to *N. caninum* were determined in 184 (126 rural, 58 urban) dogs from the Punjab State, India, using commercial monoclonal antibody-based competitive ELISA and found in 16.8% of the animals. The prevalence of *N. caninum* antibodies was significantly higher in rural dogs (21.4%, 27 of 126) than city dogs (6.9%, 4 of 58). To our knowledge this is the first report of *N. caninum* infection in canines from India.

The domestic dog is a definitive host for *N. caninum*, and the oocysts shed in canine feces are considered essential in the epidemiology of this parasite (McAllister et al., 1998; Dubey et al., 2007). *Neospora caninum* is one of the most important causes of abortion in cattle worldwide, and dogs are an important risk factor for *N. caninum* infection in cattle. Therefore, there have been many surveys for *N. caninum* infections in dogs worldwide, and these were recently summarized (Dubey et al., 2007). Here we report the prevalence of *N. caninum* antibodies in dogs from India for the first time.

For the present study, 4 villages from the Punjab State were selected using simple random sampling, without replacement, using the ‘Random Village’ program of Survey toolbox (Cameron, 1999). Punjab has approximately 500,000 dogs; of these, 378,000 are pets and 119,000 strays (www.husbandrypunjab.org).

Serum samples from all 126 dogs in the 4 villages were collected. Additionally, serum samples were collected from 58 urban dogs brought to the Small Animal Veterinary Clinics of the College of Veterinary Science, Ludhiana, Punjab. In total, 184 serum samples were collected from June to December 2005. Blood (3–5 ml) was collected from a recurrent tarsal vein of dogs, centrifuged, and sera stored at −20 °C until assayed. A standardized questionnaire for animals sampled was completed at the time of blood collection.

Sera were tested for presence of *N. caninum*–specific antibodies using a monoclonal antibody–based competitive ELISA (VMRD, Inc., Pullman, Washington) as described by Baszler et al. (1996, 2001) and recently used by us to detect antibodies to *N. caninum* in cattle and buffaloes in Punjab, India (Meenakshi et al., 2007). The ELISA kits were imported from VMRD, and the test was performed according to instructions supplied by the manufacturer. Positive and negative controls were run in duplicate according to manufacturer’s instructions. The cutoff value considered positive was 30% inhibition (Meenakshi et al., 2007).

The data were analyzed using SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, Illinois) for Windows version 11.0.1. The associations were evaluated between binary outcome variable and a variety of risk factors (age, breed, sex, and spatial distribution) of dogs. Antibodies to *N. caninum* were found in 31 (16.8%) of 184 dogs. Most dogs had a high SP ratio in the ELISA; percentage inhibition of 31 seropositive dogs was 30–40% (2 dogs), 41–50% (3 dogs), 51–60% (5 dogs), 62–70% (6 dogs), 71–80% (7 dogs), and 81% or more (8 dogs). Seroprevalence was higher in older dogs; 7.3% (3 of 41) of <12-mo-old were seropositive compared with 13.6% (3 of 22) prevalence in 13- to 24-mo-old, and 20.7% (25 of 121) seropositive in >24-mo-old dogs, but there was an insufficient number in each age group to establish an age-related relationship. Seroprevalence in males (18.4%, 6 of 48) was higher than in females (12.5%, 25 of 136), but statistically there was no differences between sexes. The seroprevalence was not significantly higher ($\chi^2 = 3.45; P = 0.063$) in mongrel dogs (21.1%, 23 of 109) compared with that in defined breeds (10.7%, 8 of 75).

The prevalence of *N. caninum* antibodies in the present study was significantly higher ($\chi^2 = 5.99; P < 0.05$) in rural dogs (21.4%, 27 of 126) when compared with that from city dogs (6.9%, 4 of 58); the risk of seroprevalence was 3.5 times higher in rural dogs in comparison to city dogs. These findings are in agreement with reports by others (Sawada et al., 1998; Wouda et al., 1999; for review see Dubey et al., 2007). In India, domestic and stray dogs on farms have easy access to placentas and dead animals, and the ingestion of infected tissues by dogs can lead to shedding of *N. caninum* oocysts (McAllister et al., 1998; Dijkstra et al., 2001). Canids (dogs, coyotes, and possibly red foxes) are the only hosts that can shed *N. caninum* oocysts (McAllister et al., 1998; Gondim et al., 2004; Wapenaar et al., 2006). There are no coyotes in India, and foxes are rarely seen on cattle farms in India. Thus, dogs appear to be the main reservoir of *N. caninum* in India.

**LITERATURE CITED**


CONTENTS
THE JOURNAL OF PARASITOLOGY
VOL. 94, NO. 1 February 2008

DEVELOPMENT
Evagination and Infectivity of Taenia crassiceps Cysticerci in Experimental Animals. RIMMA ZURABIAN, LAURA AGUILAR, JOSÉ A. JIMÉNEZ, LILIA ROBERT, and KAETHE WILLMS ................................................................. 1

ECOLOGY-EPIDEMIOLOGY-BEHAVIOR
The Role of Phylogeny and Ecology in Experimental Host Specificity: Insights From a Eugregarine-Host System. J. DETWILER and J. JANOY, JR. ...................................................... 7
Short-Term Seasonal Changes in Parasite Community Structure in Northern Leopard Frogs (Rana pipiens) Inhabiting Agricultural Wetlands. KAYLA C. KING, ANDRÉE D. GENDRON, J. DANIEL McMCLAUGHLIN, ISABELLE GIROUX, PAULINE BROUSSEAU, DANIEL CYR, SYLVIA M. RUBY, RICHARD FOURNIER, and DAVID J. MARCOGLIESE ....... 13

ECTOPARASITOLOGY
New Species of Rhabdosynochus Mizelle and Blatz 1941 (Monogenoidea: Diaplectanidae) From the Gills of Centropomid Fishes (Teleostei) Off the Pacific Coast of Mexico. EDGAR F. MENDOZA-FRANCO, JUAN VIOLANTE-GONZÁLEZ, and VÍCTOR M. VIDAL-MARTÍNEZ ..................................................... 28

GENETICS-EVOLUTION
Isolation and Genetic Characterization of Toxoplasma gondii From Raccoons (Procyon lotor), Cats (Felis domesticus), Striped Skunk (Mephitis mephitis), Black Bear (Ursus americanus), and Cougar (Puma concolor) From Canada. J. P. DUBEY, T. QUIRK, J. A. PITT, N. SUNDAR, G. V. VELMUERGAN, O. C. H. KWOK, D. LECLAIR, R. HILL, and C. SU ....... 42
Morphology and 18S rDNA of Hennegunya gutlei (Myxosporea) From Ameinarius nebulosus (Siluriformes) in North Carolina. LUKE R. IWANOWICZ, DEBORAH D. IWANOWICZ, LINDA M. POTE, VICKI S. BLAZER, and WILLIAM B. SCHILL ...................................................... 46
Sequence Analysis of Ribosomal and Mitochondrial Genes of the Giant Liver Fluke Fascioloides magna (Trematoda: Fasciolidae): Intraspecific Variation and Differentiation From Fasciola hepatica. IVICA KRÁLOVÁ-HROMADOVÁ, MARTA ŠPAKULOVÁ, EVA HORÁČKOVÁ, LUDMILA TURČEKOVÁ, ADAM NOVOBILSKÝ, REJÁ BECK, BRÉTISLAV KOUDELA, ALBERT MARINCULIĆ, DUŠAN RAJSKÝ, and MÁRGO PYBUS ........... 58

IMMUNOLOGY
Primary Culture of Skeletal Muscle Cells as a Model for Studies of Toxoplasma gondii Cystogenesis. ERICK VAS GUI-MARÁEZ, LAIS RE CARVALHO, and HELENE SANTOS BARBOSA .......... 72
Transcriptional Inhibition of Interleukin-12 Promoter Activity in Leishmania spp.-Infected Macrophages. ASHA JAYA-KUMAR, ROBYN WIDENMAIER, XIAOJING MA, and MARY ANN MCDOWELL ................. 84
Detection of Cryptosporidium parvum Oocysts by Dot-Blotting Using Monoclonal Antibodies to Cryptosporidium parvum Virus 40-kDa Capsid Protein. MARK C. JENKINS, CELIA N. O'BRIEN, and JAMES M. TROUT .... 94
Inhibition of Interleukin-12 Production by Trypanosoma brucei in Rat Macrophages. KAZUHIKO NISHIMURA, SHINSUKE SAKAKIBARA, KOUSUKE MITANI, JYOJI YAMATE, YOSHIHIRO OHNISHI, and SHINJI YAMASAKI ... 99
Cytokine and Nitric Oxide Production of Trypanosoma brucei Infection in Rats Fed Polymine-Deficient Chow. KAZUHIKO NISHIMURA, MICHIKO YAGI, YOSHIHIRO OHNISHI, and SHINJI YAMASAKI .......... 107
Antibodies in Cold Stressed Mice Recognize a Surface Protein in Toxoplasma gondii Tachyzoites. ERIC G. THOMPSON, HERNAN O. AVILES, and FERNANDO P. MONROY ................. 114
Heat Shock Response of Babesia gibsoni Heat Shock Protein 70. MASÁHIRO YAMASAKI, MOTOSHI TAJIMA, OSAMU YAMATO, SHIANG-YI HWANG, HIROSHI OHTA, and YOSHIMITSU MAEDA ............. 119

INVERTEBRATE-PARASITE RELATIONSHIPS
Numerical Quantification of Perkinsus marinus in the American Oyster Crassostrea virginica (Gmelin, 1791) (Mollusca: Bivalvia) by Modern Stereology. ANTONIO REMACHA-TRIVISO, DORANNE BORSAY-HOROWITZ, CHRISTOPHER DUNGAN, XIMO GUAL-ARNAU, JAVIER GÓMEZ-LEON, LUISA VILLAMIL, and MARTA GÓMEZ-CHIARRI .................. 125

LIFE CYCLES-SURVEY
Ultrastuctural Developmental Cycle of Haplosporidium montforti (Phylum Haplosporidia) in Its Farmed Abalone Host, Haliotis tuberculata (Gastropoda). CARLOS AVEDEO, GRAÇA CASAL, and JAIME MONTES ........... 137
Examination of Naturally Exposed Bottlenose Dolphins (Tursiops truncatus) for Microsporidia, Cryptosporidium, and Giardia. R. FAYER, P. A. FAIR, G. D. BOSSART, and M. SANTÍN .......... 143
First Record of Trypanosoma challengeri in Brazil and Occurrence of Other Trypanosoma Species in Brazilian Frogs (Anura, Leptodactyliidae). M. LEMOS, D. H. MORAIS, V. T. CARVALHO, and M. D'AGOSTO .......... 148
Effect of Age of the Intermediate Host Tribolium confusum (Coleoptera) on Infection by Hymenolepis diminuta (Cestoda). ALLEN W. SHOSTAK ..................... 152

MOLECULAR-CELL BIOLOGY
GpMyoF, a WD40 Repeat-Containing Myosin Associated with the Myonemes of Gregarina polymorpha. MATTHEW B. HEINTZELMAN and MARCUS J. MATER ................. 158

(Continued on inside back cover)
PATHOLOGY
Kinetics of Encephalitozoon Spp. Infection of Human Macrophages. JEFFREY FISCHER, DIANA TRAN, RICHARD JU-NEAU, and HOLLIE HALE-DONZE .......................................................... 169

SYSTEMATICS-PHYLOGENETICS
A New Species of Allocreadium (Trematoda: Allocreadiidae) From Freshwater Fishes in the Danjiangkou Reservoir in China. DIAN GAO, GUI TANG WANG, BING WEN XI, WEI JIAN YAO, and PIN NIE .......................................................... 176

New and Already Known Acanthocephalans From Amphibians and Reptiles in Vietnam, with Keys to Species of Pseudoco-acanthocephalus Petrochenko, 1956 (Echinorhynchidae) and Sphaerocinuroidea Johnston and Deland, 1929 (Plagiorhynchidae). OMAR M. AMIN, NGUYEN VAN HA, and RICHARD A. HECKMANN .......................................................... 181


New and Already Known Acanthocephalans Mostly From Mammals in Vietnam, with Descriptions of Two New Genera and Species in Archiacanthocephala. OMAR M. AMIN, NGUYEN VAN HA, and RICHARD A. HECKMANN .......................................................... 194

Sandonella sandoni (Lynsdale, 1960), an Enigmatic and Morphologically Unique Cestode Parasite in the Osteoglossiform Fish Heterotis niloticus in Africa. ALAIN DE CHAMBRIER, JEAN MARIAUX, AMINATA SÈNE, ZUHEIR N. MAHMOUD, and TOMÁŠ SCHOLZ .......................................................... 202


A New Species of Myxospore (Myxosporea) From the Brain and Spinal Cord of Rainbow Trout (Oncorhynchus mykiss) From Idaho. CARLA I. HOGGE, MATTHEW R. CAMPBELL, and KEITH A. JOHNSON .......................................................... 218

A New Dicyemid From Sepiella japonica (Mollusca: Cephalopoda: Decapoda). HIDETAKA ITÅ .......................................................... 223

An Exploration of Diversity Among the Osotergiinae (Nematoda: Trichostrongyloidea) in Ungulates From Sub-Saharan Africa with a Proposal for a New Genus. ERIC P. HOBERG, ARTHUR ABRAMS, and VANESSA O. EZENWA .......................................................... 230

Detection and Species Identification of Cryptosporidium From Taiwan Feeding Animals. BING-MU HSU, HAU-YANG WUN, and CHIH-LI LILIAN HSU .......................................................... 252

Description of a New Species of Grassclites Manter, 1936, Parasite of Cirrhosoma beani Jordan (Osteichthyes: Ichthyidae) in Mexico, Based on Morphology and Sequences of the ITS1 and 28S Ribosomal RNA Genes. GERARDO PÉREZ-PONCE DE LEÓN, ULISES M. AMIN, and HUGO MEJÍA-MADRID .......................................................... 257

A New Species of Oswalducruzie (Molineidae: Nematoda) in Chaunus marinus (Amphibian: Bufonidae) (Linnaeus, 1758) From Brazil. JEANNIE N. SANTOS, ELANE C. GIESE, ARNALDO MALDONADO, JR., and REINALDA M. LAFREDI .......................................................... 264

Replacement of the Preoccupied Name Davisia Laird 1953 and Description of a New Myxozoan Species (Myxosporea: Sinuolineidae) From Sebastiscus marmoratus (Cuvier, 1829) in the East China Sea. YUANJUN ZHAO, YANG ZHOU, MICHAEL L. KENT, and CHRISTOPHER M. WHIPPS .......................................................... 269

THERAPEUTICS-DIAGNOSTICS
Mode of Action of Fenarimol Against Leishmania Spp. EINAT ZEIMAN, CHARLES L. GREENBLATT, SHARONA EL-GAVISH, INA KHOZIN-GOLDBERG, and JACOB GOLENSER .......................................................... 280

RESEARCH NOTES
Observations on the Sporozoite Transmission of Plasmodium vivax to Monkeys. WILLIAM E. COLLINS, JOANN S. SUL-LIVAN, DOUGLAS NACE, TYRONE WILLIAMS, ALLISON WILLIAMS, and JOHN W. BARNWELL .......................................................... 287

Estimation of the Endophelminth Parasite Species Richness in Freshwater Fishes From La Mintzita Reservoir, Michoacán, Mexico. MARÍA DE LA LUZ ROMERO-TEJEDA, LUIS GARCÍA-PRIETO, LORENA GARRIDO-OLVERA, and GERARDO PÉREZ-PONCE DE LEÓN .......................................................... 288

Parasite-Induced Changes in Nitrogen Isotope Signatures of Host Tissues. HIDEYUKI KIMURA, NALATIA I. YURLOVA, SVET-LANA N. VODYANYTSKAYA, EISUKE KIKUCHI, SHUICHI SHIKANO, ELENA N. YADRENKINA, and ELENA I. ZUYKOVÀ .......................................................... 296

A Tick From a Prehistoric: Arizona Coprolite. KEITH R. JOHNSTON, KARL J. REINHARD, LUCIANA SIANTO, ADAUTO ARAÚJO, SCOTT L. GARDNER, and JOHN JANOVY, JR .......................................................... 299

Observations on Cryptosporidium Life Cycle Stages During Excystation. PANAGIOTIS KARANIS, AKIO KIMURA, HIDEYUKI NAGASAWA, IKUO IGARASHI, and NAQOYOSHI SUZUKI .......................................................... 298

Chinese Liver Flukes in Latrine Sediments From Wong Nim’s Property, San Bernardino, California: ArchaeaOpal spheres of the Caltrans District Headquarters. KARL J. REINHARD, ADAUTO ARAÚJO, LUCIANA SIANTO, JULIA G. COSTELLO, and KAREN SWOPE .......................................................... 300

Seroprevalence of Neospora caninum Antibodies in Dogs in India. S. SHARMA, M. S. BAI, MEENAKSHI, K. KAUR, K. S. SANDHU, and J. P. DUBEY .......................................................... 303

ERRATUM .......................................................... 190

COVER CAPTION: Light (LM) and transmission electron microscopy (TEM) aspects of different life-cycles stages of Haplo­sporidium montforti parasite of the abalone Haliotis tuberculata (scale bars µm). Spore excystment showing the endo­sporangium with a nucleus (N) and several haplosporosomes (HS). Externally, the spore wall (Wa), some filament sections (F), and part of the operculum (Op) are present. Figure 4 from Azevedo et al. 94: 137–142.