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A PATHOGENIC NEW SPECIES OF *EIMERIA* FROM THE PYGMY RABBIT, *BRACHYLAGUS IDAHOENSIS*, IN WASHINGTON AND OREGON, WITH DESCRIPTION OF THE SPORULATED OOCYST AND INTESTINAL ENDOGENOUS STAGES

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ABSTRACT: In January 2003, fecal samples from 13 live pygmy rabbits, *Brachylagus idahoensis* (Merriam, 1891), were collected at the Oregon Zoo in Portland, Oregon, and sent to the University of New Mexico (UNM), Albuquerque, New Mexico, to be examined for coccidia. In July 2004, 14 more fecal samples were collected and sent to UNM, 6 from some of the same rabbits and 8 from 16 other rabbits (4 were pooled samples from siblings). In addition, tissue sections from 3 dead rabbits (2 from the Oregon Zoo, 1 from Washington State University) also were examined. Two of 4 (50%) pooled fecal samples and 8 of 17 (47%) 1-rabbit samples were positive for a single species of *Eimeria*, which we describe here as a new species. Sporulated oocysts were subspheroidal, 25.6×23.8 ($22\text{--}28 \times 21\text{--}27$) μm , with a length:width (L:W) ratio of 1.1 (1.0–1.2). A micropyle ($\sim 2 \mu\text{m}$ wide) and 0–1 polar granules were present, but an oocyst residuum was absent. Sporocysts were ellipsoidal, 13.4×8.1 ($11\text{--}16.5 \times 7.5\text{--}9$) μm , with a L:W ratio of 1.7 (1.3–2.2), and they had a Stieda body and sporocyst residuum. Tissue sections showed a heavy infection of the villous epithelial cells of the proximal and mid-small intestine with coccidial endogenous stages, but no stages were found in liver hepatocytes. Meronts with approximately 46 (26–70) merozoites per infected cell appeared to be fully developed and were subspheroidal, 14.8×13.9 ($13\text{--}18 \times 10.5\text{--}16.5$) μm . Developing macro- and microgamonts were indistinguishable from each other and were spheroidal to subspheroidal, 10.4×9.5 ($9\text{--}11 \times 7.5\text{--}10.5$) μm . Mature macrogamonts were spheroidal to subspheroidal, 14.2×13.7 ($12\text{--}17 \times 11\text{--}16$) μm , and mature microgamonts were smaller and subspheroidal, 11.9×10.8 ($10.5\text{--}13 \times 9\text{--}12$) μm . This eimerian seems to be extremely pathogenic to young pygmy rabbits, and given the precarious nature of this unique genetic population, it appears to be an emerging pathogen that deserves immediate further study.

Relatively little is known about the biology of the pygmy rabbit, *Brachylagus idahoensis* (Merriam, 1891). Weighing approximately 400 g as adults, they are the only species in *Brachylagus*, are restricted to the Great Basin of the western United States, and are found only in isolated populations in northeastern California, southern Idaho, southwestern Montana, northern Nevada, eastern Oregon, western Utah, western Wyoming, and southeastern Washington (Green and Flinders, 1980; Campbell et al., 1982; Nowak, 1991). The population in Washington State is confined to the Columbia Basin and thought to have been geographically isolated from other populations of the species for thousands of years (Lyman, 1991, 2004; K. Warheit, pers. comm.).

Pygmy rabbits display several traits that set them apart from species of cottontails (*Sylvilagus*), jackrabbits (*Lepus*), and Old World rabbits (*Oryctolagus*). They are the only leporids in the continental United States that dig their own extensive and intercommunicating burrows. They give alarm calls and other vocalizations. They move by a low, scampering gait (not leaping). They are almost totally dependent on sagebrush (*Artemisia tridentata*) for their diet, especially during winter months, when it may comprise 98–99% of their food intake (Nowak, 1991).

During the last decade, populations have been declining in Washington, Oregon, and California, where sagebrush habitat has been burned, converted to agriculture, or cleared from large areas and replaced with bunch grasses to improve livestock forage. Studies by the Washington Department of Fish and Wildlife (WDFW) in 2001 determined that the Columbia Basin population of pygmy rabbits (Douglas County, Washington) is genetically distinct and has been isolated from the Idaho and Oregon populations for at least 7,000 yr. The population has declined precipitously from approximately 150 pygmy rabbits

in 1995 (WDFW, 1995) to less than 30 in 2001 (Hays, 2001). Because no one had bred this species in captivity successfully, the WDFW initiated a captive-breeding program in cooperation with Washington State University and the Oregon Zoo. It started in December 2000, when 4 pygmy rabbits were brought from the Lemhi Valley, Idaho, to the Oregon Zoo to help develop pygmy rabbit husbandry protocols. Later, 16 Columbia Basin pygmy rabbits were captured as an initial source for captive-breeding efforts, and in 2002, both facilities began breeding the endangered Columbia Basin rabbits for eventual reintroduction to a protected habitat in central Washington. The U.S. Fish and Wildlife Service listed the Columbia Basin pygmy rabbit population under emergency provisions of the Endangered Species Act on 30 November 2001 (USFWS, 2001) and provided the population with full listing status on 5 March 2003 (USFWS, 2003).

As of spring 2004, 22 Columbia Basin pygmy rabbits were in captive-breeding programs in Washington and Oregon, but the program did not produce many offspring during the first 2 yr. In addition, in May 2002, 4 captive-bred young rabbits died in 5 days, apparently because of gastrointestinal coccidiosis. Here, we describe the species responsible for these deaths as a new and potentially pathogenic species of *Eimeria*, and we document the structure of the sporulated oocyst and some of the endogenous stages and histopathology.

MATERIALS AND METHODS

Fecal samples from 13 live pygmy rabbits were collected at the Oregon Zoo in Portland, Oregon, in January 2003 and sent to the University of New Mexico (UNM), Albuquerque, New Mexico. These rabbits were all of Washington origin, and they currently are captive-housed at the Oregon Zoo. In July 2004, 14 additional fecal samples (10 from individual rabbits and 4 pooled samples from siblings) also were collected and sent to UNM for analysis. Fecal material was placed in vials containing 2% (w/v) aqueous potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) solution, mixed thoroughly, and refrigerated for 2–3 days before being shipped to UNM for examination. On receipt, the samples were stored at ambient temperature for approximately 1–2 mo, until they could be processed and screened for coccidia as detailed by Duszynski and Wil-

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TABLE I. Fecal samples from 29 captive-born (C) and wild-caught (W) pygmy rabbits, *Brachylagus idahoensis*, examined for coccidia and those found to be infected with *Eimeria brachylagia*. All samples were collected when animals were at the Oregon Zoo.

Rabbit no.	Sex	C or W	Birth date	Death date	<i>E. brachylagia</i> (+/–) and fecal sample date(s)		Housed*	Subspecies†
					01/03/04	07/13/04		
0	M	W	?	A (?)‡	—		?	ID
10125	M	C	05/22/01	04/03/03	—		OZ	WA
10171	M	W	?	A	+	+	OZ	WA
20076	F	C	05/01/02	A	—	—	OZ	WA
20078	F	C	05/03/02	04/23/04	+		OZ	WA
20079	F	C	05/03/02	A	+	+	OZ	WA
20185	M	W	?	03/08/03	—		OZ	WA
20186	F	C	05/22/01	07/13/04	+	+	OZ	WA
20187	F	C	05/08/02	A	—	—	OZ	WA
20188	F	C	05/05/02	A	+	—	OZ	WA
30015	M	W	?	A	+		OZ/WSU	ID
30016	M	W	?	A	+		OZ/WSU	ID
30017	F	C	05/05/02	01/02/04	—		OZ	WA
30064	F	C	05/09/03	A		—	OZ	IC
30080	F	C	05/22/03	A		—	OZ	IC
40065–66	2F	C	04/15/04	A		+	OZ	IC
40067	F	C	04/15/04	A		+	OZ	IC
40068–71	3F, 1M	C	04/18/04	A		—	OZ	IC
40075–76	2F	C	05/02/04	A		+	OZ	IC
40077	M	C	05/02/04	A		—	OZ	IC
40082–85	3F, 1M	C	05/03/04	A		—	OZ	WA

* Pygmy rabbits were housed at the Oregon Zoo (OZ), but some are now at Washington State University (WSU).

† WA, Washington (Columbia Basin); ID, Idaho (Great Basin); IC, Intercross (WA × ID hybrids of varying proportions).

‡ A, alive. Feces were collected once from Ozzy, but his current whereabouts are not known.

ber (1997). Oocysts were measured and photographed using both bright-field and Nomarski differential interference contrast (DIC) microscopy. Standardized abbreviations for oocyst and sporocyst structures are those used by Wilber et al. (1998) with 1 modification in the abbreviation for sporozoite: *oocyst characters*: length (L) and width (W) with their ranges and ratio (L:W), micropyle (M), residuum (OR), and polar granule (PG); *sporocyst characters*: L and W ranges and L:W ratio, Stieda body (SB), substieda body (SSB), parastieda body (PSB), residuum (SR), sporozoites (SZ), refractile body (RB), and nucleus (N) in SZ. All measurements are in micrometers (μm), with size ranges in parentheses after the means.

Tissue sections also were examined from 3 additional pygmy rabbits. Two of these were 3-wk-old male juveniles, which had died of peracute severe coccidiosis, and the third was an adult female, which had died of other causes (it was killed because of hemangiosarcoma) but also had coccidial endogenous stages in her intestine at necropsy. All tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with standard H&E methods. Endogenous developmental stages were measured with an ocular micrometer in a Zeiss Universal photomicroscope equipped with a neofluar ×100 achromat oil objective lens (NA = 1.3). Only stages within an intact parasitophorous vacuole around the developing parasite were measured to achieve the best op-

FIGURES 1–3. Photomicrographs of sporulated oocysts of *Eimeria brachylagia* from the pygmy rabbit, *Brachylagus idahoensis*. RB, refractile body of sporozoite; SB, Stieda body on pointed end of sporocyst; M, micropyle in oocyst wall. Bar = 10 μm.

FIGURE 4. Line drawing of sporulated oocyst of *Eimeria brachylagia*. Bar = 10 μ m.

tical cross-section possible. Some photomicrographs (Figs. 5–10) of endogenous stages in gut tissue sections were taken on a Zeiss Axioskop 2 photomicroscope with a Plan Apochromat DIC $\times 63$ oil objective lens (NA = 1.4), whereas others (Figs. 11–15) were made using Afga Pan-X 35-mm film in a Zeiss Universal photomicroscope equipped with a $\times 100$ neofluar objective (NA = 1.3).

RESULTS

Feces from 17 individual pygmy rabbits and 4 pools of sibling rabbits (representing 12 rabbits) were examined for coccidia, and 8 of 17 (47%) individual pygmy rabbits and 2 of 4 (50%) pooled samples had eimerian oocysts in them. Six of the 17 individuals were examined twice (Table I). Also, tissue samples from 3 of 3 (100%) rabbits that were necropsied after death also were positive for coccidia. Three of the 5 wild-caught and 7 of the 16 samples from 24 captive-born rabbits (Table I) had oocysts in their feces, and all oocysts seen in these infected rabbits appeared to represent a single species, which we describe here as new.

DESCRIPTION

Eimeria brachylagia

(Figs. 1–15)

Description of sporulated oocyst (Figs. 1–4): Oocyst shape: subspheroidal; number of walls: 2, outer $\sim 3/4$ of total thickness; wall thickness: 0.8–1.1 (1.0); wall characteristics: smooth; L $\times$ W (n = 50): 25.6 $\times$ 23.8 (22–28.5 $\times$ 21–27); L:W: 1.1 (1.0–

1.2); M: present as a thin, membranous area at one end of the oocyst, with the wall ending bluntly on each side of M; OR: absent; PG: 0–1. Distinctive features of oocyst: presence of a M and lack of an OR.

Description of sporocyst and SZ: Sporocyst shape: ellipsoidal; L $\times$ W: 13.4 $\times$ 8.1 (11–16.5 $\times$ 7.5–9); L:W: 1.7 (1.3–2.2); SB: present as a slightly rounded extension of the sporocyst wall; SSB and PSB: absent; SR: present as many fine granules evenly dispersed between SZ, usually in the middle of the sporocyst; SZ: lie head to tail and have a large, posterior RB. Distinctive features of sporocysts: large posterior RB in each SZ.

Taxonomic summary

Type host: *Brachylagus idahoensis* (Merriam, 1891), pygmy rabbit.

Other hosts: None to date.

Type locality: North America, Washington State, Douglas County (47°45'N, 119°42'W).

Geographic distribution: North America; Washington, Oregon, and Idaho.

Prevalence: 11 of 20 (55%); 8 of 17 in feces, and 3 of 3 in tissue sections of intestinal epithelium.

Site of infection: Epithelial cells of proximal and mid-small intestine (Fig. 5). No coccidial endogenous stages found while scanning sections of liver parenchyma for more than 20 min.

Endogenous development (Figs. 6–15): Merogony: Several different merogonous stages seen. These may represent a progression in development of 1 stage (Figs. 11–15), or they may represent 2 or more different merogonous generations. If the former, early merozoites seem to develop and bud from a central globule in the meront (Figs. 11–13) and then enlarge until they become what appear to be fully developed meronts (Figs. 6, 14, 15); these (n = 11) were subspheroidal, 14.8 $\times$ 13.9 (13–18 $\times$ 10.5–16.5), with approximately 46 (26–70) merozoites. Gamogony: Early developing macro- and microgamonts are indistinguishable from each other (Fig. 7). They (n = 20) were spheroidal to subspheroidal, 10.4 $\times$ 9.5 (9–11 $\times$ 7.5–10.5), and stained light purple, with the nucleus staining somewhat darker. Cytoplasm varied from homogeneous to having a slight frothy appearance. The nucleus (~ 2.0) usually was centric, and only rarely was a small, noncentric nucleolus visible. Mature macrogamonts had clearly defined wall-forming bodies that had not yet coalesced at the margins (Fig. 8) and were spheroidal to subspheroidal and 14.2 $\times$ 13.7 (12–17 $\times$ 11–16). Mature microgamonts (Figs. 9, 10) had a central nucleus surrounded by homogeneous cytoplasm and many scattered, small nuclei that eventually end up at periphery and were subspheroidal and 11.9 $\times$ 10.8 (10.5–13 $\times$ 9–12).

Pathology: Case history 1: A 3-wk-old, male, captive-bred rabbit was ill for less than 1 day and found dead. No gross abnormalities were noted other than the absence of pelleted stool in the distal colon. Massive numbers of developing gamonts and a lesser number of meronts were seen in villous en-

FIGURES 5–10. Photomicrographs of endogenous stages of *Eimeria brachylagia* in villous epithelial cells of the small intestine from a pygmy rabbit that died of coccidiosis. 5. Section through 3 villi; note the large number of cells with developing meronts and/or gamonts in them. Bar = 50 μ m.

6. Presumably mature meront (Me) with approximately 46 merozoites in cross-section. Bar = 10 μ m. **7.** Young developing gametocyte (Gcy); note that it is not possible at this stage to distinguish between macro- and microgametocytes. Bar = 10 μ m. **8.** Macrogametocyte (Ma) Bar = with unfused wall forming bodies near the periphery of the cell. Bar = 10 μ m. **9.** Young microgametocyte (Mi) with a few nuclei beginning to accumulate at the periphery of the cell. Bar = 10 μ m. **10.** Two microgametocytes (Mi) in the same cell. Bar = 10 μ m.

FIGURES 11–15. Various stages in a proposed developmental sequence of merogony. **11–12.** Young meronts with approximately 10–12 merozoites (cross-section) budding from a central mass. **13.** Young meront with approximately 12 merozoites (long-section) budding from a central mass. **14–15.** Nearly mature (**14**) and presumably mature (**15**) meronts with approximately 35 and 50+ merozoites, respectively.

terocytes of the mid-small intestine associated with apical necrosis and mild chronic inflammation in lamina propria. Bacterial overgrowth was noted in the central lumen of this section of intestine. Animal was emaciated, with marked atrophy of adipose stores, and severe intestinal coccidiosis likely contributed to this condition. Endogenous stages were succinctly segmental in 1 convoluted section of small intestine, whereas the cecum, colon, and distal part of the small intestine near the ileum were unaffected.

Case history 2: A 26-day-old, male, captive-bred pygmy rabbit found in moribund condition, with moderate, seizure-like muscle movements. The rabbit died within 25 min despite treatment with oxygen, warmth, fluid, dextrose, doxapram, and epinephrine. Necropsy revealed reddening of calvarium and ingesta throughout the gut. Formed fecal pellets in the distal colon also were reddened, but it is not known whether this was fresh blood or a postmortem artifact. The rabbit had pneumonia; its lungs were consolidated with clostridial overgrowth. The proximal and mid-small intestine had large numbers of endogenous developmental stages (mostly gamonts) in villous enterocytes that seemed to be associated with mild elongation of villi (Fig. 5). The presence of lymphocytes and plasma cells in lamina propria is normal, but in infected villi, a mild to moderate increase was found in the number of these infiltrates. Large numbers of unsporulated oocysts were present in central lumen. Adipose stores were markedly atrophic.

Case history 3: A wild-caught, adult, female pygmy rabbit presented with a mass in the neck region that was diagnosed as a hemangiosarcoma. The animal was humanely killed; microscopic examination of the small intestine noted many intra-epithelial coccidial meronts and gamonts. Increased numbers of lymphocytes, plasma cells, macrophages, and hemosiderin-laden macrophages in lamina propria, beyond background infiltrates, were found.

Sporulation: Exogenous.

Prepatent and patent periods: Unknown.

Material deposited: Photosyntypes of sporulated oocysts, no. 095050.00, document 429, and tissue sections of endogenous stages, no. 095051.00, voucher 167A-9, are deposited in the U.S. National Parasite Collection, Beltsville, Maryland.

Remarks

Historically, the monotypic *Brachylagus* was included as a subgenus of *Sylvilagus*, but as more data accumulated from pa-

leontology, morphology, serology, ecology, and behavior, it has become accepted as a distinct genus by most mammalogists (Green and Flinders, 1980; Nowak, 1991). Thus, it is most logical to compare sporulated oocysts of *E. brachylagia* with those found in *Sylvilagus* spp., which is still the closest phylogenetic relative and probably the most similar.

Of the dozen *Eimeria* species known to infect *Sylvilagus* spp., sporulated oocysts of *E. brachylagia* are similar in size only to those of *E. environ*, as described by Honess (1939), from the mountain cottontail, *Sylvilagus nuttallii* (Bachman) (26×24 [$22\text{--}28.5 \times 21\text{--}27$] vs. 27×21 [$22\text{--}30 \times 16\text{--}23$]), but their L:W ratios are different (1.1 vs. 1.3), making *E. environ* more elongate and not subspheroidal. Oocysts of the 2 species also are similar by having a M and by lacking an OR, but the M of *E. environ* often has “a cap which slightly protrudes beyond the curvature of the oocystic wall” (Honess, 1939), which *E. brachylagia* lacks. The size of the sporocysts of *E. brachylagia* also are similar (13×8 [$11\text{--}16.5 \times 7.5\text{--}9$]) to those of *E. environ* (16×8 [$13\text{--}18 \times 7\text{--}10$]), and both species possess a SB. However, the sporocyst L:W ratios are, again, different (1.7 vs. 2.0), with those from *E. brachylagia* being more subspheroid and those from *E. environ* more ellipsoid. Finally, sporocysts of *E. brachylagia* possess a diffuse SR, which those of *E. environ* lack.

DISCUSSION

Lagomorphs include 2 extant families, Ochotonidae and Leporidae; the latter, which includes *Brachylagus*, contains 11 genera with 54 distinct species (Hoffmann, 1995). To date, only 3 of 11 (27%) leporid genera (*Lepus*, *Oryctolagus*, and *Sylvilagus*) and 16 of 54 (30%) leporid species have had coccidia species (all *Eimeria*) described from them (Duszynski et al., 1998). Before the present study, coccidia had not been described from *B. idahoensis*.

It was surprising to see the pathogenic nature of this coccidium. In natural populations of other leporids with which we are familiar, especially *Lepus* and *Sylvilagus* spp., it is not uncommon for an individual to be infected with up to 6 *Eimeria* spp. at a single time yet not exhibit demonstrable pathology (pers. obs.). Yet, when young pygmy rabbits become infected with sporulated oocysts of *E. brachylagia*, it appears that virtually every epithelial cell on an infected villus harbors 1 or more developmental stages of the parasite (Figs. 5–10). This suggests

to us that several successive generations of meronts, each producing many merozoites, eventually may lead to the massive concentration of gamogonous stages we saw infecting epithelial cells. Whether the merogony we saw represents 1 (Figs. 11–15) or possibly 2 different generations (Figs. 11–13, a smaller one; Figs. 6, 14, 15, a larger one) can only be a matter of speculation because of the temporal nature of endogenous development and the time at which the animals we examined either died or had been killed. One question that arises is whether it is the nature of *E. brachylagia* to be pathogenic (as is *E. bovis* in cattle, *E. tenella* in chickens, and *E. stiedai* in other leporids) or whether the pathology we observed was exacerbated by the stress of captivity.

Other questions on the biology of *E. brachylagia* need to be answered soon if, indeed, this coccidium is a contributing factor to the decline of pygmy rabbits in the wild. Does the burrowing nature of these rabbits contribute to the concentration of oocysts in their natural habitat, such that animals are more likely to encounter massive infections early in life? Are there other *Eimeria* spp. that infect *B. idahoensis* and may contribute to the pathology seen? To our knowledge, none of the pygmy rabbit populations of the northwestern United States have been surveyed for coccidia or any other parasites. What are the mechanisms of pathogenicity? Is it simple destruction of epithelium in the mid-small intestine caused by large numbers of merogonous stages, or is some yet unknown mechanism in operation?

Given the severe lesions associated with the presence of *E. brachylagia* documented here and the precarious nature of this unique genetic population of rabbits, it appears that *E. brachylagia* is an emerging pathogen that deserves immediate further study.

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