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Isolation and genetic characterization of *Toxoplasma gondii* from mute swan (*Cygnus olor*) from the USA

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ABSTRACT

Little is known of the genetic diversity of *Toxoplasma gondii* circulating in wildlife. In the present study, antibodies to *T. gondii* were determined in serum samples from 632 mute swans (*Cygnus olor*) collected from different areas of the USA. Sera were tested by *T. gondii* modified agglutination test; 54 (8.5%) of 632 samples were seropositive with titers of 25 in 28 sera, 50 in 22 sera, 100 in three samples, and 200 or higher in one swan. Hearts from 14 seropositive swans were bioassayed in mice and viable *T. gondii* (designated TgSwanUs1–3) were isolated from the hearts of three. These three *T. gondii* isolates were further propagated in cell culture, and DNA isolated from culture-derived tachyzoites was characterized using 11 PCR-RFLP markers (SAG1, 5′- and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico). Results of genotyping revealed that two strains (TgSwanUs1, TgSwanUs2) were Type III (ToxoDB PCR-RFLP genotype #2), and TgSwanUs3 was a new genotype designated here as ToxoDB PCR-RFLP genotype #216. Pathogenicity of oocysts derived from these three strains was determined in Swiss Webster (SW) outbred mice. All mice infected with oocysts and tachyzoites of the atypical isolate (TgSwanUs3) died of acute toxoplasmosis, irrespective of the dose. Oocysts of the remaining two isolates were less pathogenic but differed from each other; 10 oocysts of the TgSwanUs1 killed all inoculated mice whereas 1 million oocysts of the TgSwanUs2 were needed to kill all infected SW mice. Isolation of *T. gondii* from mute swan indicates that the local waters were contaminated by *T. gondii* oocysts, and that mouse *T. gondii* virulent strains are circulating in wildlife. Mute swan is a new host record for *T. gondii*.

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1. Introduction

The protozoan parasite *Toxoplasma gondii* infects virtually all warm-blooded animals, including birds, humans, livestock, and marine mammals (Dubey, 2010). In the USA, various surveys have found that 10–50% of the adult human population has been exposed to this parasite (reviewed in Dubey and Jones, 2008). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, or by consuming food or drink contaminated with oocysts. However, only a small percentage of exposed adult humans or other animals develop clinical signs of disease. It is unknown whether the severity of toxoplasmosis in immunocompetent hosts is due to the parasite strain, host

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Table 1
Occurence of *T. gondii* in mute swan in the USA.

State	Number of swans			
	Total tested	% seropositive	Bioassayed	Bioassay positive
Indiana	7	1	1	0
Michigan	291	12	9	2
New Jersey	187	15	2	0
New York	11	1	0	0
Rhode Island	127	25	2	1
Wisconsin	9	0	0	0
Total	632	54 (8.5%)	14	3

variability, or to other factors. Recently, attention has been focused on the genetic variability among *T. gondii* isolates from apparently healthy and sick hosts (Grigg and Sundar, 2009). Severe cases of toxoplasmosis have been reported in immunocompetent patients in association with atypical *T. gondii* genotypes in certain countries (Ajzenberg et al., 2004; Demar et al., 2007; Elbez-Rubinstein et al., 2009; Grigg and Sundar, 2009; Vaudaux et al., 2010). A variant of Type II (NE-II) was recently found associated with prematurity and severe disease at birth in congenitally infected children in the USA (McLeod et al., 2012). Little is known of the association of genotype and clinical disease in animals (Dubey, 2010). Type II strains are the most prevalent in Europe and the USA (Su et al., 2012).

Historically, *T. gondii* was considered to be clonal with low genetic diversity and grouped into three types I, II, III (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002a,b, 2004; Lehmann et al., 2006; Aubert et al., 2010). However, recent studies have revealed a greater genetic diversity of *T. gondii*, particularly isolates from domestic animals in Brazil and wildlife in the USA (Dubey et al., 2010, 2011a,b; Khan et al., 2011; Dubey et al., 2012; Su et al., 2012). Most intriguing are findings that some genotypes such as Type 12 predominantly found in wildlife in the USA (Dubey et al., 2011a,b; Khan et al., 2011). Though Type 12 has also been identified from pigs and sheep in the USA, the frequency is low, and the dominant genotype in these domestic animals is the Type II (Dubey et al., 2008; Velmurugan et al., 2009). Also, it is not clear how specific genotypes become established in a particular host, because all strains are transmitted by oocysts shed by felids or by ingestion of infected tissues and studies involving *T. gondii* in wildlife are time consuming, expensive, and difficult. Additionally, permission is needed to collect tissues from certain wildlife, including swans. In the present study we had an opportunity to genetically characterize three isolates of *T. gondii* from mute swan (*Cygnus olor*).

2. Materials and methods

2.1. Naturally infected swans

Mute swans (*C. olor*) are native to Eurasia, and were introduced from Europe into the United States in the late 19th and early 20th centuries for use in ornamental ponds and lakes, zoos, and aviculture collections (Maryland Mute Swan Task Force, 2001; Ciaranca et al., 1997). Feral breeding is believed to have first started among escaped birds in the lower Hudson Valley in 1910 and on Long Island in 1912 (Atlantic Flyway Council, 2003). Since that time mute swans have expanded their range to many Eastern states several Midwestern states and portions of the western USA and Canada. Mute swans are a non-native species in North America that can have adverse impacts on aquatic habitats and compete with native waterfowl for food and resources and can damage property and agriculture (Atlantic Flyway Council, 2003; MDNR 2002, 2012, Craves and Susko, 2010). Mute swans are also a hazard to human health and safety because of aggressive behavior by territorial or food-habituated birds, and fecal contamination of water sources and areas with high recreational use. Because of these reasons, the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services program (WS) manages mute swan populations in several states in the Great Lakes Region and the Atlantic Coast.

From March 2011 through September 2012, 632 mute swans were removed by Wildlife Services program from the Great Lakes region and in other northeastern states for damage management purposes. The swans were shot legally by USDA personal trained in the safe use of firearms. Samples were opportunistically collected from the mute swans post-mortem and submitted for various disease testing including *T. gondii*. Blood was collected from a jugular vein by making a small cut in the jugular vein and then lowering the head below the body to allow the blood to flow into a blood collection tube. The tube was identified with a barcode number unique to each swan and then the tube was placed in a cooler with ice packs. The blood was allowed to clot for at least 4 h and then centrifuged at 1500 rpm for 15 min. The serum was separated using a disposable pipette and stored refrigerated until shipping.

The entire heart was collected by making a 10–12 cm cut in the abdomen directly below the sternum and subsequently pulling the heart back out the cavity. The heart was placed in a Ziploc bag and assigned the same barcode number as the serum sample. It was then placed in a cooler with ice packs and stored in a refrigerator until shipping to the testing laboratory. Collection site-specific data including GPS coordinates, county, state (Table 1), and date were recorded for each site and a unique barcode number for each swan on a standardized datasheet. Samples were shipped within 3 days of collection to the Animal Parasitic Diseases Laboratory, United States Department of Agriculture in Beltsville, Maryland for *T. gondii* examination.

Table 2
Isolation of *T. gondii* from mute swan in the USA.

Swan no.	MAT	Location	Date trapped	Bioassay[a]		Oocysts shedding by cat no.	Isolate designation	Genotypes (ToxoDB PCR-RFLP Genotype)
				SW	KO			
231	100	Province-RI	8/11/2011	1/3	1/2	46	TgSwanUs1	III (#2)
673	50	Wayne-MI	1/27/2012	3/4	0/1	21	TgSwanUs2	III (#2)
740	≥200	Monro-MI	3/29/2012	3/3[b]	2/2	22	TgSwanUs3	Atypical (#216)

[a] SW: Swiss Webster; KO: knockout. Mice infected with *T. gondii*/no. of mice inoculated.
[b] Mice died day 15,16,17 p.i.

2.2. Serology

Sera from animals were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Sera were diluted 2-fold serially from 1:25 to 1:200.

2.3. Bioassay in mice

Hearts of 14 seropositive swans were homogenized individually, digested in acidic pepsin, washed, and aliquots of homogenates were inoculated subcutaneously into two to five outbred Swiss Webster (SW) mice and/or two gamma interferon gene knockout (KO) mice (Dubey, 2010) (Table 2). Tissue imprints of lungs and brains of inoculated mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 45 days post-inoculation (p.i). and a 1:25 dilution of serum was tested for *T. gondii* antibodies by MAT. Mice were killed 46 days p.i. and brains of all mice were examined for tissue cysts as described (Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

2.4. Pathogenicity of oocysts derived from swan T. gondii strains in mice

Pathogenicity of oocysts of the three *T. gondii* isolates derived from swans was done in SW mice (Table 3). For this, *T. gondii*-free cats were fed tissues of mice infected with the swan isolates, and oocysts collected from the feces of cats (Dubey, 2010). Oocysts were sporulated in 2% sulfuric acid for a week on a shaker at room temperature, washed, counted, and diluted serially 10-fold from 10^{-1} to 10^{-7} to reach an end point of ≅1 oocyst. Oocysts from the last dilutions calculated to contain <1 oocysts were also bioassayed in KO mice (Table 3). Mortality was recorded, and after 2 months mice were tested for *T. gondii* infection (Dubey, 2010). Mice were considered uninfected when antibodies to *T. gondii* were not demonstrable in their sera and parasites were not found.

2.5. Pathogenicity of tachyzoites derived from T. gondii strain TgSwanUs3 in SW mice

For pathogenicity of tachyzoites, mesenteric lymph node of a SW mouse euthanized 5 days after oral inoculation of 100,000 oocysts was homogenized in saline with pestle and mortar, and filtered through gauze. The suspension was passed through a 5-μm micropore filter to remove any intact host cells and to ensure extracellular tachyzoites. The filtrate was diluted serially 10-fold to reach an end point of ≅1 tachyzoite (Table 3).

2.6. In vitro cultivation

Mouse tissues infected with swan *T. gondii* isolates were seeded on to CV1 cell culture flasks and tachyzoites were harvested from the medium.

2.7. Genetic characterization

T. gondii DNA was extracted from cell-cultured tachyzoites and strain typing was performed using the genetic

Table 3
Pathogenicity of oocysts or tachyzoites derived from *T. gondii* isolates from mute swans to Swiss Webster mice.[a]

Dose[c]	TgSwanUs1 (Cat 46) Oocysts	TgSwanUs2 (Cat 21) Oocysts	TgSwanUs3 (Cat 22) Oocysts	Tachyzoites
1000,000	Not done	5 (5,5,5,5,5)[b]	5 (5,5,6,6,6)	Not done
100,000	Not done	5 (6,6,6,9)	5 (6,6,6,6,9)	Not done
10,000	5 (5,5,5,6,6)	5(7,7,9)	5 (6,7,7,9,10)	Not done
1000	5 (6,6,6,6,6)	5	5 (8,8,8,8,8)	Not done
100	5 (8,8,8,10,10)	5	5 (9,9,9,9,9)	Not done
10	5 (10,10,11,11,11)	2	3 (12,12,12)	4 (19,19,21,22)
1	5	0	2 (16,34)	1 (17)
<1	0 (KO 0/5)[d]	0 (KO 0/5)[d]	0 (KO-0/5)[d]	0

[a] Five mice per group. Oocysts were inoculated orally, tachyzoites were inoculated subcutaneously.
[b] No. of mice infected with *T. gondii* of 5 mice inoculated. Day of death of each mouse is in parenthesis. Mice not shown in parenthesis did not die.
[c] Based on estimation that the last infective dilution has 1 infective organism.
[d] None of the five knockout (KO) mice were infected, indicating no infectious organisms.

Table 4
Genetic characterization of *T. gondii* isolates from mute swan in USA.

Strain ID	Genotypes (ToxoDB PCR-RFLP Genotype)	Genetic markers										
		SAG1	(5′ + 3′) SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
GT1	I (#10)	I	I	I	I	I	I	I	I	I	I	I
PTG	II (#1)	II	II	II	II	II	II	II	II	II	II	II
CTG	III (#2)	II or III	III	III	III	III	III	III	III	III	III	III
MAS	Atypical (#17)	u-1	I	II	III	III	III	u-1	I	I	III	I
TgCgCa1	Atypical (#66)	I	II	II	III	II	II	II	u-1	I	u-2	I
TgCtBr5	Atypical (#19)	I	III	III	III	III	III	I	I	I	u-1	I
TgCtBr64	Atypical (#111)	I	I	u-1	III	III	III	u-1	I	III	III	I
TgRsCr1	Atypical (#52)	u-1	I	II	III	I	III	u-2	I	I	III	I
Present study												
TgSwanUs1,2	III (#2)	1I/III	IIII	III	III	III	IIII	III	III	III	III	III
TgSwanUs3	Atypical (#216)	I	I	I	III	III	I	III	III	III	I	III

markers SAG1, 5′- and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico as described previously (Su et al., 2010). Appropriate controls were included (Table 4).

2.8. Ethics

All investigations reported here were approved by the institutional animal use protocol committee of the United States Department of Agriculture.

3. Results

Antibodies to *T. gondii* were found in 54 (8.5%) of 632 swan sera (Table 1), in titers of 25 in 28 sera, 50 in 22 sera, 100 in three sera, and 200 or higher in one serum.

Viable *T. gondii* was isolated from hearts of three of 14 seropositive swans by bioassay in mice (Table 2). The 3 SW mice inoculated with heart homogenate of swan #740 died of acute toxoplasmosis 15, 16, 17 days p.i. (Table 2). Tachyzoites were found in smears made from lungs of these mice; cat #22 fed tissues of these infected mice shed oocysts. SW mice inoculated with heart homogenates of swans #231 and 673 remained asymptomatic, developed antibodies to *T. gondii* and tissue cysts were found in the brains of seropositive mice. All KO mice that became infected after inoculation with swan tissues died of acute toxoplasmosis.

The TgSwanUs3 strain was virulent for SW mice (Table 3). All infected mice died of acute toxoplasmosis, irrespective of the dose and the stage of the parasite inoculated. Mice inoculated orally with 10 oocysts died day 12 p.i. whereas mice inoculated subcutaneously with tachyzoites died 19–22 day p.i.; there were no infected survivors (Table 3).

The TgSwanUs2 strain was less pathogenic than the TgSwanUs1; 10 oocysts of TgSwanUs1 were lethal versus more than 10,000 of TgSwanUs2 (Table 3).

None of the 15 KO mice inoculated orally with the last dilution of oocysts of the three isolates became infected with *T. gondii*, indicating no viable oocysts in the last dilution (Table 3).

Genetic typing revealed that two isolates (TgSwanUs1, TgSwanUs2) were Type III (ToxoDB genotype #2) and one was a new genotype (TgSwanUs3) designated as ToxoDB genotype #216 (Table 4).

4. Discussion

This is the first report of *T. gondii* infection in mute swan. Most samples were from three states with occurrence of antibodies in 12% of 291 swans from Michigan, in 15% of 187 from New Jersey and in 25% of 127 from Rhode Island.

Viable *T. gondii* was isolated from only three of the 14 seropositive swans. Two of these three isolates were type III, the type most prevalent in animals. The third isolate was atypical genotype that was identified for the first time. Our results add evidence that atypical strains are circulating in wildlife in the USA. As stated earlier atypical strains are thought to cause clinical toxoplasmosis in humans in certain countries, and these strains are pathogenic to mice. In the present study, TgSwanUs3 was lethal for outbred SW mice; all infected mice died of acute toxoplasmosis, irrespective of the dose. This result indicates that highly virulent *T. gondii* strains are circulating in the environment and they may cause more severe toxoplasmosis when spread into human population. The other two strains (TgSwanUs1, TgSwanUs2) were less pathogenic. Although the isolates TgSwanUs1, and TgSwanUs2 are both Type III but, their pathogenicity was different; 10 oocysts of TgSwanUs1 were lethal to SW mice whereas 1 million oocysts of the TgSwanUs2 isolate were needed to kill all infected mice (Table 3). Traditionally, pathogenicity has been evaluated based on parenteral inoculation of mice with tachyzoites, and Type III strains are relatively non pathogenic (Howe et al., 1996, 1997). We compared pathogenicity of strains using oral route (which is natural route of infection) and oocysts which are more stable at room temperature than the tachyzoites. We realize that at present there is no clear evidence that the mouse pathogenicity can be equated with pathogenicity in humans and livestock. Also, we show here that mouse pathogenicity and genetic typing are not strictly correlated even in mice because 10 oocysts of the Type III strain TgSwanUs1were lethal for mice whereas lethal dose for Type III strain TgSwanUs2 was 1000,000 oocysts. These

results indicate that better genetic markers are needed to correlate pathogenicity of *T. gondii* in animals.

Conflict of interest

None.

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