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A NEW ATYPICAL HIGHLY MOUSE VIRULENT *TOXOPLASMA GONDII* GENOTYPE ISOLATED FROM A WILD BLACK BEAR IN ALASKA

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ABSTRACT: Most strains of *Toxoplasma gondii* isolated in North America and Europe are grouped into 3 (Types I, II, III) genotypes and are considered clonal. Recent evidence suggests that illness due to toxoplasmosis in immunocompetent persons may be related to infection with an atypical genotype; these strains are mouse virulent. In the present study, a new mouse-virulent atypical *T. gondii* genotype was isolated from an asymptomatic black bear (*Ursus americanus*) from Alaska. The bear had a titer of 1:1,600 using the modified agglutination test for *T. gondii*. Swiss Webster out-bred mice inoculated with bear heart homogenate died of acute toxoplasmosis, 12 days post-inoculation (PI). Cats fed tissues from chronically infected animals (day 30 PI) shed oocysts, but only 1 of 3 cats fed acutely infected mice (12, 16, 18 days PI) shed oocysts. The isolate (designated TgBbUS1) was mouse virulent; mice inoculated with 1 oocyst or 1 tachyzoite died of acute toxoplasmosis. The restricted fragment length polymorphism using 10 markers revealed that the strain possessed an atypical genotype: type I allele at loci SAG1, (5′-3′)SAG2, SAG3, c22-8, c29-2, L358, and Apico; type II allele at locus alt.SAG2; and type III allele at loci BTUB, GRA6, and PK1. DNA sequencing at intron loci EF1, HP2, and UPRT1 revealed that the TgBbUS1 is a divergent *T. gondii* strain. These results indicate that mouse-virulent atypical *T. gondii* genotypes are also circulating in wildlife in North America.

The protozoan *Toxoplasma gondii* infects virtually all species of warm-blooded animals, including humans, livestock, and marine mammals (Dubey, 2009). Toxoplasmosis is an important cause of morbidity and mortality in immunosuppressed individuals and can cause serious health problems in healthy adults. Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or accidentally ingesting oocysts from the environment. 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 variability, or to other factors. Recently, attention has been focused on the genetic variability among *T. gondii* isolates from apparently healthy and sick hosts (Howe et al., 1997; Grigg et al., 2001). Severe cases of toxoplasmosis reported in immunocompetent patients were considered to be due to infection with atypical *T. gondii* genotypes (Ajzenberg et al., 2004; Demar et al., 2007; Elbez-Rubinstein et al., 2009; Grigg and Sundar, 2009).

Most *T. gondii* isolates from human and animal sources have been grouped into 1 of 3 clonal lineages by multi-locus enzyme electrophoresis, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), and microsatellite typing (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg, Bañuls et al., 2002; Ajzenberg, Cogné et al., 2002). We have recently found that the isolates of *T. gondii* from Brazil are biologically and genetically different from those in North America and Europe (Dubey et al., 2002; Lehmann et al., 2006; Dubey and Su, 2009). *Toxoplasma gondii* isolates from asymptomatic chickens from Brazil were more pathogenic to mice than were isolates from Europe or North America, irrespective of the genotype (Dubey et al., 2006). Additionally, most isolates from chickens from Brazil were not clonal, and Type II was absent (Dubey et al., 2007). Here, we report the isolation of a new *T. gondii* genetic type strain from a black bear (*Ursus americanus*) in Alaska.

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MATERIALS AND METHODS

Naturally infected bears

Hearts and sera were collected from 7 black bears killed near Anchorage, Alaska. The seropositive bear was killed 11 July 2009 at Alexander Lake, Alaska (latitude 61.72669, longitude 150.84667).

Serological examination

Sera were tested for *T. gondii* antibodies with the modified agglutination test (MAT), as described by Dubey and Desmonts (1987).

Bioassay of bear tissues for *T. gondii* infection

Fifty grams of myocardium from the bear with MAT titer of 1:1,600 were homogenized in 250 ml of aqueous 0.85% NaCl solution (saline), and homogenate was incubated with an acid pepsin solution for 1 hr at 37 C, then centrifuged and neutralized (Dubey, 2009). The homogenate was inoculated subcutaneously into 2 interferon gamma gene knockout (KO) mice and 4 out-bred female Swiss Webster (SW) mice, as previously described (Dubey and Lindsay, 1998; Dubey et al., 2002).

For bioassays in cats, infected tissues were fed to 5 *T. gondii*–free cats (Table I) (Dubey, 2009). Feces of cats were examined daily for shedding of *T. gondii* oocysts, beginning on day 3 through day 14 after feeding on infected tissues. 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, 2009).

Inoculated mice were examined for *T. gondii* infection. Tissue imprints of mesenteric lymph nodes, lungs, and/or brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 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 43 days PI and brains of all mice were examined for tissue cysts, as described (Dubey, 2009). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Mouse virulence of the *T. gondii* isolate from the bear

To assess pathogenicity of oocysts of the bear isolate, oocysts from cat 91 and cat 54 were counted in a disposable hemocytometer, diluted 10-fold, until the last dilution did not contain oocysts. Aliquots from each of the last 6 dilutions were fed to 5 SW mice (Table II). A similar experiment was performed with tachyzoites. For this, lymph nodes of 2 mice that were killed 5 days after feeding on oocysts from cat 54 were homogenized in saline, passed through a 27-gauge needle, and filtered through a 5-μm filter (PALL, Gelman Laboratories, Port Washington, New York) to remove

TABLE I. Shedding of oocysts by cats fed tissues infected with the bear *Toxoplasma gondii* isolate.

	Tissues fed		Oocysts	Antibodies to *T. gondii* in cats	
Cat no.	Host	Day PI	shed	Day PI	MAT titer
30	Mouse	12	No	30	800
96	Mouse	16	Yes	Not done	Not done
34	Mouse	18	No	37	>3,200
54	Mouse	83	Yes	31	>3,200
91	Cat	37	Yes	33	>3,200

TABLE II. Infectivity of the bear isolate of *Toxoplasma gondii* to SW mice.*

Dose†	Oocysts (cat 91)	Oocysts (cat 54)	Tachyzoites
10,000	5 (7 or 8)‡	5 (5)	Not done
1,000	5 (7 or 8)	5 (7–8)	5 (12–13)
100	5 (8 or 9)	5 (9)	5 (13–14)
10	5 (9–12)	5 (10)	4 (14–16)
1	1 (10)	2 (10, 13)	1 (14)
<1	0§	0§	0§

* Five mice inoculated in each group.
† Estimate based on that 1 organism is infective.
‡ Day of death.
§ No *T. gondii* antibody and no tissue cysts.

host cells from free tachyzoites. The filtrate was diluted 10-fold serially in saline and aliquots from 10^{-3} to 10^{-8} dilutions were inoculated subcutaneously (s.c.) into 5 SW mice for each dilution. The mice were observed for 2 mo. Carcasses of 2 mice that died on day 16 PI (Table II) were fed to cat 96.

Genetic characterization for *T. gondii*

Toxoplasma gondii DNA was extracted from the tissues of infected mice and strain typing was performed using the 10 PCR-RFLP markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su et al., 2006; Su and Dubey, 2009) (Table III). To further characterize this *T. gondii* strain, intron sequences were generated at loci EF1, HP2, and UPRT1, based on a previously published method (Khan et al., 2007). The target sequences were amplified by PCR. The PCR products of HP2 and EF1 were sequenced from one end using the forward sequencing primers. For UPRT1, a new sequence primer, 5′-TCAACCGAAGTTTGCTTTCC-3′, was used. Sequences were processed using BioEdit (available free at http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with previously published sequences (Khan et al., 2007).

RESULTS

Antibodies to *T. gondii* were detected in 1 of 7 bears; the seropositive bear had a MAT titer of 1:1,600.

The 2 KO mice inoculated with bear heart died on day 11 PI and the SW mice died on day 12 PI; the cat fed tissues of these mice did not shed oocysts (Table I).

Tachyzoites found in homogenates of the lungs of a SW mouse that died on day 12 PI were cryopreserved in liquid nitrogen and sub-cultured in African Green Monkey cells.

Tachyzoites revived from the cryopreserved material were inoculated s.c. into 8 SW mice; all mice died 17–22 days PI. Tissues of 3 mice dead on day 18 PI were fed to a cat; the cat did not shed oocysts (Table I).

Homogenate of lung tissue from mice that died on day 18 PI were inoculated s.c. into another group of 8 SW mice. These mice were medicated with sulfadiazine sodium (1 mg/ml) from day 6 to 17 days PI and 6 died 11–28 days PI. The 2 surviving mice were killed on day 83 PI because they were comatose; tissue cysts were found in their brains and the cat fed tissues shed oocysts (Table I). The cat fed tissues of 2 mice infected for 16 days shed oocysts (Table II).

All mice fed viable oocysts died of acute toxoplasmosis during the second week PI and tachyzoites were found in their tissues. Neither antibodies to *T. gondii* (MAT 1:25) nor tissue cysts were found in mice that survived 2 mo after being fed oocyst dilutions (Table II). Similar results were obtained with tachyzoites (Table II).

Genotyping revealed that the strain possessed an atypical genotype with a combination of Type I, II, and III alleles at different loci (Table III). Intron sequences for TgBbUS1 were 490, 486, and 397 base pairs for EF1, HP2, and UPRT1, respectively. These sequences were compared with previously published data (Khan et al., 2007). The TgBbUS1 strain is identical to strain RUB (a human isolate from French Guiana) at locus EF1, identical to strain GPHT (a human isolate from France) at locus HP2, and closely related to strain CAST (a human isolate from the United States) with 6 SNPs at locus UPRT1. For comparison, a neighbor-joining phylogenetic tree based on the concatenated intron sequences of HP2, EF1, and UPRT1 from previously published data (Khan et al., 2007) is presented in Figure 1.

TABLE III. Genetic characterization of the bear isolate of *Toxoplasma gondii*.

	PCR-RFLP markers											
Genotype	SAG1	5′+3′ SAG2	alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Isolate designation
Type I	I	I	I	I	I	I	I	I	I	I	I	RH
Type II	II or III	II	II	II	II	II	II	II	II	II	II	PTG
Type III	II or III	III	III	III	III	III	III	III	III	III	III	CTG
Reference	I	II	II	III	II	II	II	u-1	I	u-2	I	TgCgCa1 (Cougar)
Reference	u-1	I	II	III	III	III	u-1	I	I	III	I	MAS
Reference	I	III	III	III	III	III	I	I	I	u-1	I	TgCatBr5
Present study	I	I	II	I	III	III	I	I	I	III	I	TgBbUS1

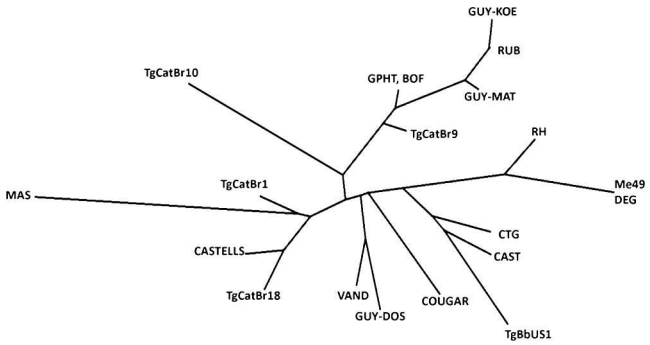

FIGURE 1. Neighbor-joining phylogenetic tree based on intron sequences of HP2, EF1, and UPRT1. DNA sequences of representative isolates were retrieved from previously published data (Khan et al., 2007).

DISCUSSION

Most *T. gondii* isolates from human and animal sources in North America and Europe have been grouped into 1 of 3 clonal lineages, including Types I, II, and III (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg, Bañuls et al., 2002; Ajzenberg, Cogné et al., 2002). When tachyzoites are used to infect out-bred mice, Type I strains are uniformly lethal. In contrast, Type II and III strains are significantly less virulent (Howe et al., 1996). However, pathogenicity is also dependent on the stage of the parasite, host, and the route of inoculation. In the present study, mice infected with tachyzoites or oocysts died of acute toxoplasmosis, whereas cats fed tissue cysts became infected but remained asymptomatic.

The epidemiology of *T. gondii* in nature is complex because the parasite is transmitted by several mechanisms and virtually all warm-blooded animals are considered potential hosts. Traditionally, a mouse-cat cycle of *T. gondii* is considered important in a domestic urban setting. The efficiency of this rodent-cat cycle is dependent on the prevalence of *T. gondii* in mice and the opportunity for cats to catch them. Nothing is known of the mortality or illness in mice due to toxoplasmosis in nature. Under experimental conditions, cats can excrete millions of oocysts after ingestion of even a few bradyzoites (Dubey, 2001) and bradyzoites are formed in mice as early as 3 days PI (Dubey and Frenkel, 1976). In this respect, it is of interest that the 2 cats fed mice that were inoculated 12 or 18 days previously with the bear isolate of *T. gondii* became infected but did not shed oocysts, and only few oocysts were shed by cat 96 that was fed mice infected for 16 days. These data indicate that acutely infected mice can die of toxoplasmosis before bradyzoites are formed and the mouse-cat cycle may not be efficient with certain strains of *T. gondii*. RFLP genotyping and DNA sequencing at 3 intron loci revealed that the TgBbUS1 is an atypical strain. These results indicate that divergent parasite strains are circulating in wildlife in North America.

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