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Transplacental toxoplasmosis in naturally-infected white-tailed deer: Isolation and genetic characterisation of *Toxoplasma gondii* from foetuses of different gestational ages

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Abstract

Clinical toxoplasmosis is most severe in congenitally-infected hosts. In humans, transmission of *Toxoplasma gondii* from the mother to the foetus is considered to be most efficient during the last trimester of pregnancy but clinical congenital toxoplasmosis is more severe if transmission occurs during the first trimester. However, there are no data on the rate of congenital transmission of *T. gondii* with respect to gestational age in any host during natural infection. In the present study, attempts were made to isolate *T. gondii* by bioassay in mice inoculated with tissues from foetuses of 88 naturally-exposed white-tailed deer from Iowa and Minnesota. Viable *T. gondii* was isolated from foetuses of six of 61 deer in early pregnancy (45–85 days of gestation) from Iowa and foetuses of nine of 27 deer from Minnesota in mid-gestation (130–150 days) of a gestational period of 7 months. The 15 *T. gondii* isolates obtained from foetal deer were PCR-restriction fragment length polymorphism genotyped using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22–8, c29–2, L358, PK1 and an apicoplast marker, Apico. Five genotypes were revealed, including the clonal Type II and III lineages, and three non-clonal genotypes. DNA sequencing analysis of representative isolates at loci SAG2, c22–8, L358 and PK1 revealed that the three non-clonal genotypes are closely related to the clonal Type I, II and III lineages. It is very likely that these non-clonal genotypes were derived from genetic crosses among the three clonal Type I, II and III lineages. The most common genotype was Type II, commonly found in humans in North America and Europe, suggesting the possible link of transmission from game animals to humans.
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Keywords: Transplacental; *Toxoplasma gondii*; White-tailed deer; *Odocoileus virginianus*; USA; Genotype

1. Introduction

Toxoplasma gondii is transmitted to humans and other animals by the ingestion of food or water contaminated by environmentally resistant oocysts, by the ingestion of tissues of infected animals or transplacentally from the mother to the foetus. The greatest economic or medical impact of *T. gondii* is on hosts transplacentally infected with this parasite (Wolf et al., 1939; Roberts et al., 1994). Transplacental transmission occurs when tachyzoites from the dam (during parasitaemia) infect the foetus. In most hosts, transmission of the parasite only occurs once, during

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primary infection. However, in certain strains of mice *T. gondii* can be transmitted for several generations and repeatedly by the same dam (Beverley, 1959). In humans, transmission of *T. gondii* from the mother to the foetus is considered to be most efficient during the last trimester of pregnancy due to permeability of the placenta but congenital toxoplasmosis is more severe if transmission occurs during the first trimester (Desmonts and Couvreur, 1974; Systematic Review on Congenital Toxoplasmosis Study Group, 2007). However, we are not aware of any studies documenting the isolation of *T. gondii* from different gestational age foetuses from clinically normal hosts. Here, we report isolation of viable *T. gondii* from foetal tissues of apparently healthy white-tailed deer at different stages of gestation.

2. Materials and methods

2.1. Naturally-infected deer

Two populations of pregnant deer (from Iowa (IA) and Minnesota (MN), USA) were used in the present study. After contracting with Iowa City, IA (N 41.5918, W 91.6264), 84 deer were killed by sharpshooters in January 2007, during the annual herd control. Deer were killed with single head shots and brought nightly to a skilled butcher for meat processing for city charities. During this time researchers were present to draw blood and collect foetuses from the female deer. These deer were estimated to be approximately 2 months gestational age (Table 1).

Sixty-one deer from Minnesota were killed in March, 2007, and these deer were considered to be about 4–5 months gestational age; the gestational period in white-tailed deer is 7 months. These deer were shot as part of a program to reduce wild deer potentially infected with bovine tuberculosis in a 200 km^2 area centred at Skime, MN (N 48.54694, W 95.60250).

Blood or clot was collected from the heart or chest cavity of deer soon after death. All foetuses (singles or twins) were removed. Whole foetuses and blood samples were sent with cold packs to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD for *T. gondii* examination.

2.2. Serological examination

Sera of deer were tested for *T. gondii* antibodies using twofold dilutions, from 1:25 to 1:3200 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

2.3. Bioassay of foetal tissues for T. gondii

Foetuses from Iowa deer were small (about the size of a mouse) and bones were not ossified. The whole foetus (or foetuses if twins) were homogenised in approximately 10 vol. of 0.9% NaCl solution (saline), centrifuged and the sediment was suspended in 5 vol. of antibiotic saline containing 1000 U of penicillin and 100 μg of streptomycin per ml of saline. Approximately 1 ml of foetal homogenate was inoculated s.c. into two to four IFNγ gene knock-out (KO) mice from Jackson Laboratories (Dubey and Lindsay, 1998) and four to eight out-bred Swiss Webster (SW) mice from Taconic Farms, German Town, New York.

For Minnesota deer, only foetal brain was bioassayed. Whole brain of the foetus (or foetuses) was homogenised in saline, centrifuged and the sediment was suspended in saline containing 0.25% trypsin. After incubation at 37 °C for 60 min, the brain homogenate was centrifuged. The sediment was suspended in saline and centrifugation was repeated two more times to remove the trypsin. The final sediment was suspended in antibiotic saline and inoculated s.c. into four to eight SW mice and/or SW mice that were given dexamethasone in drinking water (Romand et al., 1998) for 1-month starting on the day of deer tissue inoculation (Table 2). The number of mice inoculated varied depending on the availability of mice on the day foetuses arrived.

All mice that were inoculated with deer tissues were examined for *T. gondii* infection. Lungs and brains of mice that died were examined for *T. gondii* parasites. The remaining mice were bled on day 45 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 49 days p.i. and brains of all mice were examined microscopically 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.

To exclude the possibility of mixed infection with a morphologically related parasite, *Neospora caninum* (Dubey et al., 2002a) infected mouse brains were fed to *T. gondii*-free cats as described (Dubey, 1995); *T. gondii* oocysts are produced by cats and *N. caninum* oocysts are produced in dogs. Faeces of cats were examined for shedding of *T. gondii* oocysts 3–14 days post-ingesting deer tissues. Faecal floats were incubated for 1 week at room temperature to allow sporulation of oocysts and were bioassayed in SW mice (Dubey and Beattie, 1988). The inoculated mice were examined for *T. gondii* infection. Strains derived from oocysts from cats were used in the present study for characterisation of *T. gondii* isolates.

Mice often become ill or die of acute toxoplasmosis during the first week after ingesting *T. gondii* oocysts. For the present study, mesenteric lymph nodes of mice fed oocysts were examined for tachyzoites 4–5 days after being fed oocysts (Tables 1 and 2). After ascertaining the presence of tachyzoites, mesenteric lymph node homogenates of mice were sub-inoculated into new SW mice to exclude *Hammondia hammondi* infection (Dubey and Sreekumar, 2003); *H. hammondi* cannot be transmitted by sub-inoculation of tissue stages.

Table 1
Isolation of *Toxoplasma gondii* from foetal tissues of deer from Iowa (IA) and Minnesota (MN)

Deer No.	Gestational age (days)	Modified agglutination test	Bioassay in mice		*T. gondii* isolation in cats[a]			Strain designation
			No. *T. gondii*-positive mice/No. inoculated		Cat No.	Oocyst fed to mice[b]	Sub-passage[c]	
			KO	SW				
IA 965	61–65	1600	ND	1/4	395	DK4, DK4	K36, K36	TgWtdUs1
IA 822	66–75	50	0/2	1/2	394	DK4, DK4	K36, K36	TgWtdUs2
IA 784	61–65	100	1/2	0/2	387	DK4, DK4	K36, K36	TgWtdUs3
IA 858	76–85	800	1/2	1/2	384	DK4, DK4	K36, K36	TgWtdUs4
IA 870	66–75	<25	2/2	0/2	389	DK4, DK4	K36, K36	TgWtdUs5
IA 802	45–52	25	1/2	0/2	214	DK4, DK4	K36, K36	TgWtdUs6
MN 282	134–141	<25	1/4	ND	19	DK4, DK4	K49, K49	TgWtdUs7
MN 240	133–140	<25	2/4	0/4	15	D5, D5	K49, K49	TgWtdUs8
MN 247	133–140	800	ND	1/4	1	DK4, DK4	K49, K49	TgWtdUs9
MN 467[d]	136–143	200	ND	1/8	12	DK4, DK4	K49, K49	TgWtdUs10
MN 491[d]	135–142	100	ND	1/8	9	DK4, DK4	D19, DK49	TgWtdUs11
MN 451[d]	137–144	<25	ND	1/8	7	DK4, DK4	K49, K49	TgWtdUs12
MN 568	141–148	800	2/4	0/4	11	DK4, DK4	K49, K49	TgWtdUs13
MN 321	143–150	<25	1/4	2/4	6	DK4, DK4	K49, K49	TgWtdUs14
MN 318	142–150	400	2/4	0/4	42	DK4, DK4	K49, K49	TgWtdUs15

[a] Cats were fed tissues from infected mice.
[b] DK = killed when ill. K = killed. D = died. The number is the day mice were killed or died.
[c] Mice were inoculated with homogenates of mesenteric lymph nodes of mice fed oocysts.
[d] Brain was trypsinised before bioassay in mice.

All mice and cats used in experiments were handled using procedures approved by the Animal Care Program, US Department of Agriculture.

2.4. Genetic characterisation for T. gondii

Toxoplasma gondii DNA was extracted from the tissues of infected mice from each isolate and strain typing was performed using 10 PCR-restriction fragment length polymorphism (RFLP) genetic markers SAG1, SAG2, SAG3, BTUB, GRA6 c22–8, c29–2, L358, PK1, and Apico (Dubey et al., 2006, 2007a; Su et al., 2006). Seven reference strains including RH88, PTG, CTG, TgCgCa1 (also known as Cougar or COUG), MAS, TgCatBr5 and TgDgCo11, a dog isolate from Colombia (Dubey et al., 2007c), were used in the genotyping. PCR products for representative isolates of each genotype were sequenced from both ends at loci SAG2, c22–8, L358 and PK1. The primers used for DNA sequencing are: SAG2-SqF, TAGCTTT CAAGACCGCACCT and SAG2-SqR, CTGCTTGC GATTCTGTGTGT for locus SAG2; c22–8SqF, AAGG ATCGGGGAAAGTGTCT and c22–8SqR, GCGAACC TTCTGTCATCTCC for locus c22–8; L358-SqF, ATG TCCTCTTTCTGCCTTCG and L358-SqR, GGAGAAA GCGAAACCTTCCT for locus L358; and PK1-SqF, GGC ACAATGGAAGACGATTT and PK1-SqR, GTACCA GGCCACCAAACATT for locus PK1. DNA sequence data were processed using BioEdit software, freely available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html. The DNA sequence lengths for SAG2, c22–8, L358 and PK1 are 471, 479, 363 and 846 bp, respectively. Phylogenetic network analysis was performed for each locus using the program SplitsTree4 (Huson and Bryant, 2006).

3. Results

Fifty-four (64.2%) of 84 deer from Iowa were seropositive with MAT titres of 1:25 in five, 1:50 in five, 1:100 in 16, 1:200 in eight, 1:400 in four, 1:800 in five, 1:1600 in five, and 1:3200 in six deer. Foetuses from 61 deer (18 seronegative, 43 seropositive) were bioassayed in mice and *T. gondii* was isolated from six (Table 1); one of these isolates was from a seronegative deer.

From the Minnesota deer, foetuses from 27 deer (15 seropositive, 12 seronegative) were bioassayed. *Toxoplasma gondii* was isolated from foetuses of nine deer (Table 1); four of these deer were seronegative.

In total *T. gondii* was isolated from 15 deer and these isolates were designated TgWtdUs1–15 (Tables 1 and 2). Cats fed infected tissues from all 15 deer shed oocysts. Mice fed sporulated oocysts (number not determined) of all isolates became ill and had to be killed between 4–6 days p.i. However, mice inoculated with tachyzoites (number not determined) of these isolates generally remained asymptomatic, except the isolate from deer no. 491 (Tables 1 and 2). The isolate from deer no. 491 was pathogenic. Of the two mice fed oocysts, one died on day 19 (mouse A) and the other had to be killed on day 49 because it was ill; the four SW mice inoculated with lung homogenate from mouse A died between 10 and 13 days p.i.

Genotyping of the 15 deer isolates revealed that nine isolates were *T. gondii* clonal Type II strains, two isolates were clonal Type III strains, and four isolates belong to three

Table 2
Genotyping of *Toxoplasma gondii* isolates from foetal deer from Iowa and Minnesota

Genotype	SAG1	5′ + 3′ SAG2[a]	SAG2[b]	SAG3	BTUB	GRA6	c22–8	c29–2	L358	PK1	Apico	Isolate IDs
Reference	I	I	I	I	I	I	I	I	I	I	I	RH88
Reference	II or III	II	II	II	II	II	II	II	II	II	II	PTG
Reference	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
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
Reference	I	I	II	III	I	III	II	I	I	u-3	I	TgDgCo11
#1 (Type II)	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs1
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs3
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs4
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs5
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs7
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs9
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs11
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs12
	II or III	II	II	II	II	II	II	II	II	II	II	TgWtdUs14
#2 (Type III)	II or III	III	III	III	III	III	III	III	III	III	III	TgWtdUs13
	II or III	III	III	III	III	III	III	III	III	III	III	TgWtdUs15
#3	II or III	II	II	II	II	II	II	II	I	II	I	TgWtdUs2
	II or III	II	II	II	II	II	II	II	I	II	I	TgWtdUs6
#4	II or III	III	III	III	II	II	II	III	II	II	I	TgWtdUs8
#5	II or III	II	II	III	III	III	III	III	III	III	II	TgWtdUs10

[a] The SAG2 marker based on 5′- and 3′-end DNA sequence polymorphisms of the SAG2 gene (Howe et al., 1997).
[b] The SAG2 marker recently developed, based on 5′-end DNA sequence of the SAG2 gene, is able to identify additional alleles often seen in atypical *T. gondii* strains (Su et al., 2006).

non-clonal genotypes (Table 2). DNA sequencing analysis of representative isolates (TgWtdUs2, 4, 5, 6, 8, 10, 13 and 15) at loci SAG2, c22–8, L358 and PK1 revealed that the sequence data of the deer isolates match perfectly with PCR-RFLP genotyping data at all four loci (Table 2). It indicates that the three atypical genotypes of deer isolates are closely related to the clonal Type I, II and III lineages, and they are likely derived from genetic recombination among these clonal lineages.

4. Discussion

Antibodies to *T. gondii* in white-tailed deer are widely prevalent in the USA. Using a titre of 1:25 in MAT, antibodies to *T. gondii* were found in 44% of 106 deer from Kansas (Brillhart et al., 1994), 30% of 1367 deer in Minnesota (Vanek et al., 1996), 44% of 16 deer from Alabama (Lindsay et al., 1991), 60% of 593 deer from Pennsylvania (Humphreys et al., 1995) and 46.5% of deer from Mississippi (Dubey et al., 2004). Lindsay et al. (1991) isolated *T. gondii* from four of 19 adult deer. Deer are popular game animals in the USA. During the 2006 deer hunting seasons in Iowa and Minnesota, 150,552 and 270,778 deer were harvested in the respective states (http://files.dnr.state.mn.us/outdoor_activities/hunting/deer/2006_harvestreport.pdf). The deer population in Minnesota is estimated to be about one million animals and on average about 200,000 deer are harvested annually. In the present study, MAT antibodies were found in 64.2% of 84 deer from Iowa and 31.1% of 61 deer from Minnesota. However, unlike previous surveys the present study included only adult females. Although the sample size is small, the 31% seroprevalence from Minnesota deer is similar to 30% of 1367 deer from Minnesota from a previous study (Vanek et al., 1996) and is half the seroprevalence from the Iowa deer. It is noteworthy that in all of these surveys the same MAT procedure was used and one of us (JPD) was involved. Cases of clinical toxoplasmosis (Sacks et al., 1983), including ocular manifestations (Ross et al., 2001), have been documented in humans who had consumed undercooked venison.

In the USA, poultry, pork and beef are the main meat types consumed. In a recent nationwide study of the prevalence of *T. gondii* in retail meat, viable *T. gondii* was isolated from only seven of 2094 pork samples but none from 2094 beef or 2094 chicken meat samples (Dubey et al., 2005). Thus, while the scope of human infection resulting from meat sources remains undetermined, the low *T. gondii* infection in market pigs alone cannot account for the 10–40% seroprevalence in humans in the USA (Jones et al., 2003, 2007). We are not aware of a risk assessment study in the USA but 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* is one of three pathogens (along with *Salmonella* and *Listeria*) which account for >75% of all deaths due to foodborne disease in the USA and economic figures to care for congenitally-infected children are high (Roberts et al., 1994; Mead et al., 1999).

The isolation rate of *T. gondii* from 2-month gestational age deer foetuses from Iowa (six of 61, 10%) is about one-third of the isolation from Minnesota deer (nine of 27,

33%); these findings might have been related to gestatioal age; the data are not definitive because different isolation procedures were used for the Iowa deer and the Minnesota deer. All foetuses appeared to be healthy. It is noteworthy that the foetus from deer no. 802 from Iowa was about 50 days old gestational age. It is surprising that five of the 15 isolates of *T. gondii* were from seronegative deer. It is possible, but unlikely, that the foetuses and dams were not properly identified or the quality of serum or body fluid was poor so that antibodies had degraded or only IgM antibodies were present; MAT only detects IgG antibodies. These data suggest that *T. gondii* can infect the foetus before the dam develops IgG antibodies. We are not aware of any reports of *T. gondii*-associated abortions in deer. Results of the present study indicate that transplacental transmission of *T. gondii* in deer is high and perhaps the highest among all hosts of *T. gondii*. Transplacental transmission is considered to be <1% of all *T. gondii* infections in higher mammals including sheep and humans (Dubey and Beattie, 1988). Recently a high rate of congenital transmission of *T. gondii* was reported in certain flocks of sheep in England (Morley et al., in press).

Most *T. gondii* isolates from human and animal sources in Northern America and Europe have been grouped into one of three clonal lineages by multi-locus enzyme electrophoresis, PCR-RFLP and microsatellite typing (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002a,b) with Type I strains being more virulent for mice. However, pathogencity can vary with the host and the stage ingested (Dubey 2006). In the present study, mice fed oocysts died or became ill with toxoplamosis, yet mice inoculated with tachyzoites of the same isolate remained asymptomatic.

Recent studies on *T. gondii* populations in animal populations started to reveal the diversity of the parasite. We have recently found that isolates of *T. gondii* from Brazil and Colombia are biologically and genetically different from those in North America and Europe (Dubey et al., 2002b, 2007b; Lehmann et al., 2006). *Toxoplasma gondii* isolates from asymptomatic chickens from Brazil were more pathogenic to mice than isolates from Europe or USA, irrespective of the genotype. Additionally, most isolates from chickens from Brazil were not clonal and Type II was absent (Dubey et al., 2007a). In this study, genotyping of the 15 deer isolates revealed that the majority were clonal Type II strains (60%, 9/15), and the Type III strains were found at a lower frequency (13%, 2/15). Three non-clonal genotypes were also identified (Table 2). DNA sequencing analysis of representative isolates (TgWtdUs2, 4, 5, 6, 8, 10, 13 and 15) at loci SAG2, c22–8, L358 and PK1 showed that there is no single nucleotide polymorphism between the deer isolates and the clonal Type I, II or III strains within the same allele types at a given locus. For example, isolate TgWtdUs10 has a type II RFLP allele at SAG2 locus (Table 2), and it has an identical DNA sequence to Type II strain PTG at the same locus (Fig. 1). However, TgWtdUs10 has type III RFLP alleles at loci c22–8, L358 and PK1 (Table 2), and it has identical DNA sequences to Type III strain CTG at these loci (Fig. 1). Taken together, our data indicate that the three non-clonal genotypes of deer isolates are closely related to the clonal Type I, II and III lineages, and they were likely derived from natural recombination among the three clonal lineages. Since no clonal Type I strain was identified from any of these deer, whereas two of the three non-clonal genotypes have

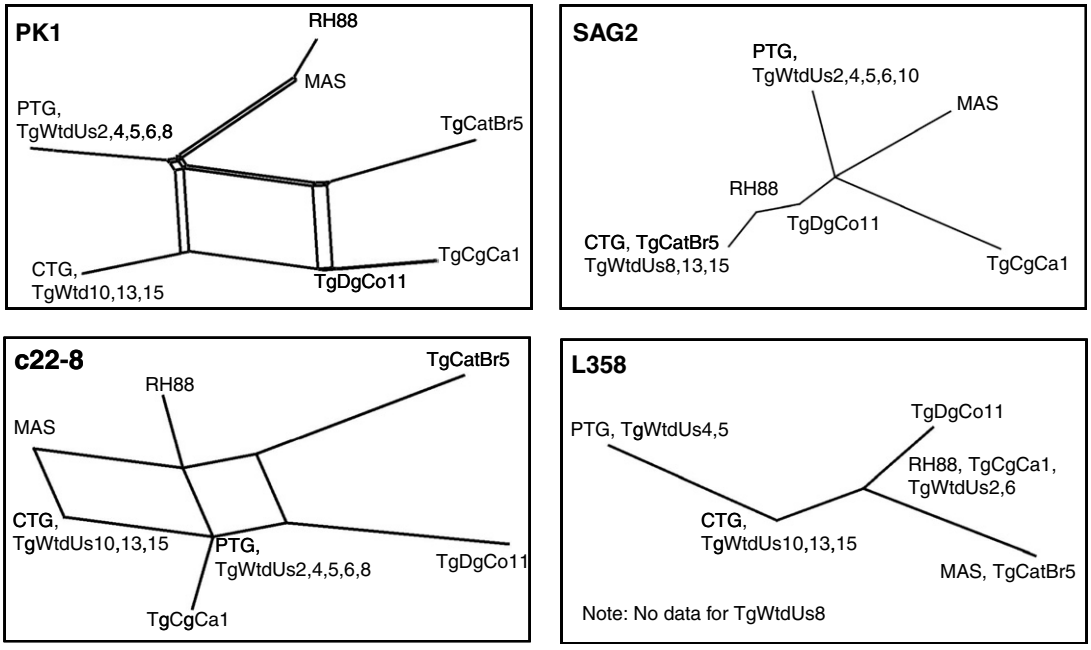

Fig. 1. Phylogenetic network analysis of representative T. gondii isolates..

type I alleles in some of the markers used, we can exclude the possibility that these recombinant genotypes were experimentally generated during the process of isolating *T. gondii* in cats.

As the deer, common game animals in USA, have very high seropositive rates of *T. gondii* infection, they may be important in transmission of the parasite to humans. High prevalence of clonal Type II strains both in humans and deer in the USA suggests the possible link of transmission from game animals to humans. This is important in public health and would suggest that more attention should be paid in preparing meat from game animals for human consumption.

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