Available online at www.sciencedirect.com
ScienceDirect
International Journal for Parasitology 38 (2008) 999–1006
www.elsevier.com/locate/ijpara

High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA

J.P. Dubey [a,*], N. Sundar [a], D. Hill [a], G.V. Velmurugan [a], L.A. Bandini [a], O.C.H. Kwok [a], D. Majumdar [b], C. Su [b]

[a] *United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705-2350, USA*
[b] *Department of Microbiology, The University of Tennessee, Knoxville, TN 37996-0845, USA*

Received 11 September 2007; received in revised form 26 November 2007; accepted 27 November 2007

Abstract

Little information is available on the presence of viable *Toxoplasma gondii* in tissues of lambs worldwide. The prevalence of *T. gondii* was determined in 383 lambs (<1 year old) from Maryland, Virginia and West Virginia, USA. Hearts of 383 lambs were obtained from a slaughter house on the day of killing. Blood removed from each heart was tested for antibodies to *T. gondii* by using the modified agglutination test (MAT). Sera were first screened using 1:25, 1:50, 1: 100 and 1:200 dilutions, and hearts were selected for bioassay for *T. gondii*. Antibodies (MAT, 1:25 or higher) to *T. gondii* were found in 104 (27.1%) of 383 lambs. Hearts of 68 seropositive lambs were used for isolation of viable *T. gondii* by bioassay in cats, mice or both. For bioassays in cats, the entire myocardium or 500 g was chopped and fed to cats, one cat per heart and faeces of the recipient cats were examined for shedding of *T. gondii* oocysts. For bioassays in mice, 50 g of the myocardium was digested in an acid pepsin solution and the digest inoculated into mice; the recipient mice were examined for *T. gondii* infection. In total, 53 isolates of *T. gondii* were obtained from 68 seropositive lambs. Genotyping of the 53 *T. gondii* isolates using 10 PCR–restriction fragment length polymorphism markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) revealed 57 strains with 15 genotypes. Four lambs had infections with two *T. gondii* genotypes. Twenty-six (45.6%) strains belong to the clonal Type II lineage (these strains can be further divided into two groups based on alleles at locus Apico). Eight (15.7%) strains belong to the Type III lineage. The remaining 22 strains were divided into 11 atypical genotypes. These results indicate high parasite prevalence and high genetic diversity of *T. gondii* in lambs, which has important implications in public health. We believe this is the first in-depth genetic analysis of *T. gondii* isolates from sheep in the USA.
Published by Elsevier Ltd on behalf of Australian Society for Parasitology Inc.

Keywords: *Toxoplasma gondi*; Lambs; *Ovis aris*; Genotype; Bioassay; PCR–RFLP; USA

1. Introduction

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 USA, 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 human infants. Toxoplasmosis is an important cause of morbidity and mortality in immunosuppressed individuals, and can cause serious health problems in healthy adults (Luft et al., 1993; Montoya and Liesenfeld, 2004). *Toxoplasma gondii* is one of three pathogens (along with *Salmonella* and *Listeria*) which account for >75% of all deaths due to food-borne disease in the USA (Mead et al., 1999).

There are two major modes of transmission of *T. gondii*. Infection may occur by ingestion of food or water contaminated with oocysts excreted by infected cats or by inges-

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
E-mail address: jitender.dubey@ars.usda.gov (J.P. Dubey).

0020-7519/$34.00 Published by Elsevier Ltd on behalf of Australian Society for Parasitology Inc.
doi:10.1016/j.ijpara.2007.11.012

tion of uncooked or undercooked meat containing tissue cysts of *T. gondii*. The proportion of the human population that acquires infection by ingestion of oocysts in the environment or by eating contaminated meat is not known and currently there are no tests available that can determine the infection source. However, sero-epidemiological data suggest that ingesting improperly cooked meat containing *T. gondii* is a major source of infection for humans in the USA (Kimball et al., 1974; Dubey and Beattie, 1988).

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 seven of 2094 pork samples but not from 2094 beef or 2094 chicken meat samples (Dubey et al., 2005b). Thus, while the scope of human infection resulting from meat sources remains undetermined, the low prevalence of *T. gondii* infection in market pigs alone cannot account for the seroprevalence in humans in the USA. In the present study, we determined the prevalence of *T. gondii* infection in lambs slaughtered for human consumption and genetically characterised the isolates.

2. Materials and methods

2.1. Naturally-infected lambs

Between November 2006 and April 2007 hearts of 383 lambs from a slaughter house in Baltimore, Maryland were obtained for the present study. The lambs were raised as small flocks in Maryland, Virginia and West Virginia, and were between six and 12 months old. No other information could be gathered for these animals. The heart of each lamb was placed in a ziplock bag and kept at ambient temperature until transport to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland. Hearts were obtained in nine batches of 31–47 animals. Blood or

Table 1
Isolation of *T. gondii* from hearts of lambs from Maryland, Virginia, and West Virginia

Lamb No	MAT titre	*T. gondii* isolation in cats			*T. gondii* isolation in mice[b]	Isolate designation
		Cat No.	Oocyst fed to mice	Sub-passage to mice[a]		
421	100	344	No oocysts	Not done	2/2 KO	TgShUs1
430	200	312	DK4,DK4	K59,K59	2/2 KO	TgShUs2
431	50	333	DK5,DK5	K59,K59	0/2 KO	TgShUs3
446	400	336	DK5,DK5	K59,K59	0/2 KO	TgShUs4
458	400	316	DK4,DK4	K59,K59	0/2 KO	TgShUs5
425	1600	330	DK5,DK5	K59,K59	2/2 KO	TgShUs6
471	3200	359	DK4,DK4	K57,K57	1/2 KO	TgShUs7
475	3200	356	DK4,DK4	K57,K57	1/2 KO	TgShUs8
476	1600	358	DK4,DK4	K57,K57	0/2 KO	TgShUs9
478	3200	368	DK4,DK4	K57,K57	0/2 KO	TgShUs10
490	3200	350	DK5,DK5	K57,K57	0/2 KO	TgShUs11
491	100	361	DK4,DK4	K48,K48	0/2 KO	TgShUs12
529	400	371	DK4,DK5	K59,K59	Not done	TgShUs13
531	400	362	DK5,DK5	K59,K59	2/2 SW	TgShUs14
532	800	364	DK4,DK5	K59,K59	2/2 SW	TgShUs15
566	800	367	DK4,DK4	K59,K59	2/2 SW	TgShUs16
584	800	373	DK4,DK4	K59,K59	2/2 SW	TgShUs17
551	400	375	DK4,DK4	K59,K59	0/2 SW	TgShUs18
568	800	365	DK4,DK4	K59,K59	0/2 SW	TgShUs19
571	1600	370	DK4,DK4	K59,K59	2/2 SW	TgShUs20
585	800	369	DK4,DK4	K59,K59	0/2 SW	TgShUs21
553	100	377	K52,K52	K36,K36	0/2 SW	TgShUs22
596	1600	376	DK4,K47	K44,K44	0/2 SW	TgShUs23
592	3200	379	DK4,DK4	K44,K44	2/2 SW	TgShUs24
602	1600	372	DK3,DK3	K44,K44	2/2 SW	TgShUs25
616	3200	390	DK4,DK4	K44,K44	2/2 SW	TgShUs26
593	800	378	DK3,DK3	K44,K44	2/2 SW	TgShUs27
642	200	396	DK4,DK4	D11,D11	0/2 SW	TgShUs28
646	200	380	DK5,DK5	K48,K48	0/2 SW	TgShUs29
649	200	382	DK4,DK4	K29,K29	0/2 SW	TgShUs30
652	400	388	DK4,DK4	K48,K48	2/2 SW	TgShUs31
653	400	395	DK4,DK4	K48,K48	0/2 SW	TgShUs32
660	200	391	DK4,DK4	K48,K48	0/2 SW	TgShUs33
641	800	398	DK4,DK4	K48,K48	1/2 SW	TgShUs34
670	3200	383	DK3,DK3	K42,K42	0/2 SW	TgShUs35
687	800	397	DK3,DK3	K35,K35	2/2 SW	TgShUs36
694	800	386	No oocysts	Not done	1/2 SW	TgShUs37

MAT, modified agglutination test; K, killed; D, died; DK, killed when ill; KO, IFNγ knockout mice.
[a] Oocysts were fed to two Swiss Webster (SW) mice and results for both mice are given.
[b] Number of mice *T. gondii*-positive from the number inoculated with lamb heart.

blood clot from the heart, or fluid in the bag, was removed, centrifuged and serum was separated.

2.2. Serological examination

Sera of lambs were tested for *T. gondii* antibodies with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Sera were first screened at dilutions of 1:25, 1:50, 1:100 and 1:200 on the day lambs were killed. Sera were then stored at −20 °C. Positive sera were end titrated until a dilution of 1:3200 was reached.

2.3. Bioassay of lambs for T. gondii infection

2.3.1. Seropositive lambs

Hearts of 68 seropositive lambs were bioassayed for *T. gondii* in cats, mice or both (Tables 1 and 2). The number of lambs selected for bioassay in each of the nine batches depended on the availability of cats and the number of seropositive animals in a batch. Hearts from 51 (50 lambs in the first eight batches and one from batch 9) were bioassayed in cats and mice. Hearts from the remaining 17 seropositive lambs in batch 9 were bioassayed in mice only. After removing fat, auricles and blood, the myocardium from each heart was chopped and gently ground in a blender without any fluid. The ground heart was removed for feeding to a cat. It was estimated that 5–10 g of heart tissue still remained in the blender. Fifty millilitres of aqueous 0.85% NaCl solution (saline) were then poured in the blender and the remaining heart tissue was homogenised for 30 s at top speed. This homogenate was incubated with an acid pepsin solution for 1 h at 37 °C, centrifuged and neutralised (Dubey, 1998). The homogenate was inoculated s.c. into two IFNγ gene knockout (KO) mice (Dubey and Lindsay, 1998; Dubey et al., 2005a) obtained from Jackson Laboratories, New York or two outbred female Swiss Webster (SW) mice (Taconic Farms, Germantown, New York), as previously described (Dubey et al., 2002).

Hearts from 17 lambs (MAT titres of 1:50 or higher) from batch 9 were bioassayed only in mice. For this, 50-g portions of myocardium from each of the 17 lambs were homogenised in saline, digested in pepsin, centrifuged, neutralised, suspended in antibiotic saline and inoculated s.c. into 10 SW mice.

For bioassays in cats, approximately 500 g of myocardium from each of the 51 lambs was fed separately to 51 *T. gondii*-free cats (Dubey et al., 2002). Faeces of cats were examined daily for shedding of *T. gondii* oocysts, beginning on day three through day 14 after feeding of lamb hearts. Faecal floats were incubated in 2% sulphuric acid for one week at room temperature on a shaker to allow sporulation of oocysts, and were bioassayed by oral administration to mice (Dubey and Beattie, 1988). Four to seven days after feeding of 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 SW mice to exclude *Hammondia hammondi* infection (Dubey and Beattie, 1988).

Inoculated mice were examined for *T. gondii* infection. Tissue imprints of lungs and brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 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 43 days p.i. 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.

2.3.2. Seronegative lambs

Hearts of 44 lambs with MAT titres of <1:25 were bioassayed in cats. For this, 20–50 g of myocardium from each of the 44 lambs from batches 8 and 9 were mixed in pools of 13, 14, 8 and 9, and fed to four cats over a period of three–six days; in the interim the meat was stored at 4 °C. Faeces of the recipient cats were examined microscopically for *T. gondii* oocysts up to 11 days after the last meal of heart meat.

2.4. Mouse virulence of the ovine T. gondii isolates

To compare pathogenicity of tachyzoites with oocysts of the isolate TgShUs28 (lamb 642), four mice were fed oocysts and killed four days later. Mesenteric lymph nodes from these mice were homogenised in saline and the homogenate was filtered through a 5 μm filter (PALL, Gelman Laboratories). The filtrate was diluted 10-fold serially

Table 2
Isolation of *T. gondii* in mice inoculated with heart tissue from lambs from Maryland, Virginia and West Virginia

Lamb No.	MAT titre	Isolation in mice	
		No. infected[a]	Isolate designation[b]
704	800	3	TgShUs38
705	200	2	TgShUs39
707	50	5	TgShUs40a, 40b
710	1600	2	TgShUs41a, 41b
713	50	5	TgShUs42a, 42b
714	1600	8	TgShUs43
716	200	1	TgShUs44
718	200	4	TgShUs45
719	>3200	10	TgShUs46
723	1600	9	TgShUs47
727	800	5	TgShUs48
728	400	2	TgShUs49a, 49b
729	200	1	TgShUs50
730	>3200	10	TgShUs51
734	400	1	TgShUs52
735	1600	9	TgShUs53

MAT, modified agglutination test.
[a] Out of 10 mice inoculated with lamb heart tissue.
[b] Isolate designation followed by a or b indicates that two different genotypes were seen in different groups of mice that were inoculated with the same tissue digest.

in saline and aliquots from 10^{-2} to 10^{-7} dilutions were inoculated s.c. into four SW mice for each dilution. Oocysts of this isolate were also end-titrated in mice. Oocysts were counted and diluted 10-fold from 10^{-1} to 10^{-6} to reach an end point of ≅1 oocyst. Aliquots (0.5 ml) from each dilution were orally inoculated in to four mice each.

2.5. Animal ethics approval

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

2.6. Genetic characterisation for T. gondii

Toxoplasma gondii DNA was extracted from the tissues of infected mice and strain typing was performed using the 10 PCR–restriction fragment length polymorphism (RFLP) markers SAG1, SAG2, SAG3, BTUB, GRA6 c22-8, c29-2, L358, PK1 and Apico (Dubey et al., 2006; Su et al., 2006; Dubey et al., 2007b, d). For the strains isolated as oocysts, DNA was obtained from the mesenteric lymph nodes of mice that had died (or were killed) after being fed oocysts. Phylogenetic network analysis of the lamb *T. gondii* isolates was carried out using SplitsTree4 (Huson, 1998; Huson and Bryant, 2006).

3. Results

3.1. Serological prevalence

Antibodies to *T. gondii* were found in 104 (27.1%) of 383 lambs with MAT titres of 1:25 in 20, 1: 50 in 21, 1: 100 in 10, 1:200 in 10, 1:400 in 12, 1: 800 in 12, 1:1, 600 in nine, and 1:3200 or higher in 10. Seropositivity in lambs varied from a low of 3.3% in batch 1 to a high of 71.7% in batch 6.

3.2. Isolation of T. gondii

Toxoplasma gondii was isolated from 53 of 68 (77.9%) seropositive lambs, from three of four lambs with titres of 1:50, from three of nine lambs with titres of 1:100, from nine of 10 lambs with titres of 1:200, from nine of 12 lambs with titres of 1:400, from 11 of 13 lambs with MAT of 1:800, from nine of nine lambs with titres of 1:1600, and nine of 10 lambs with titres of 1:3200. *Toxoplasma gondii* was not isolated from the lamb with a titre of 1:25. In summary, the parasite was isolated from six of 13

Table 3
Summary of genotyping of *T. gondii* isolates from lambs

Genotype	PCR–RFLP markers											Isolate designation
	SAG1	5′+3′ SAG2*	SAG2**	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	
Type I	I	I	I	I	I	I	I	I	I	I	I	RH
Type II	IIorIII[a]	II	II	II	II	II	II	II	II	II	II	PTG
Type III	IIorIII	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
Lamb#1	IIorIII	II	II	II	II	II	II	II	II	II	II	TgShUs6, 7, 15, 16, 18, 19, 20, 33, 41a, 42b, 44, 46, 48, 49b, 50, 51, 53
Lamb #2	IIorIII	II	II	II	II	II	II	II	II	II	I	TgShUs4, 9, 10, 12, 14, 23, 24, 25, 26
Lamb #3	IIorIII	II	II	II	II	II	II	II	I	II	I	TgShUs3, 8, 11, 27,29, 30, 31, 34, 38, 45
Lamb #4	IIorIII	II	II	II	II	II	II	II	I	II	II	TgShUs47, 52
Lamb #5	IIorIII	III	III	III	III	III	III	III	III	III	III	TgShUs13, 17, 21, 36, 40b, 41b, 42a, 43
Lamb #6	IIorIII	III	III	I	I	I	III	III	III	I	I	TgShUs22, 28
Lamb #7	IIorIII	III	III	II	II	I	III	III	II	II	I	TgShUs32
Lamb #8	IIorIII	II	II	III	III	III	III	III	III	III	II	TgShUs2
Lamb #9	IIorIII	III	III	III	II	II	II	III	II	II	I	TgShUs39
Lamb #10	IIorIII	III	III	III	II	II	II & III	III	III	III	II	TgShUs35
Lamb #11	I	III	III	III	III	III	III	III	III	u-2	III	TgShUs5
Lamb #12	I	I	II	III	III	III	u-1	III	I	III	I	TgShUs49a
Lamb #13	I	III	III	III	III	III	III	III	III	III	I	TgShUs40a
Lamb #14	u-1	II	II	II	II	II	II	II	I	II	I	TgShUs37
Lamb #15	u-1	II	II	III	III	II	II	III	II	II	I	TgShUs1

* 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).

(46.1%) with MAT titres of 1:50 and 1:100, and from 47 of 54 (87%) with titres of 1:200 or higher. Of the 17 lamb hearts that were bioassayed in mice, *T. gondii* was isolated from 16 (Table 3).

3.3. Mouse virulence of the ovine T. gondii isolates

Of the 53 isolates of *T. gondii*, 33 isolates were obtained primarily by bioassay in cats. Oocysts of 32 of these 33 isolates were pathogenic to mice; mice fed oocysts (number of oocysts fed was not determined) became ill or died (Table 1). However, mice inoculated with tachyzoites of these 32 isolates became infected but remained asymptomatic.

In the initial trial tachyzoites derived from oocysts from the isolate TgShUs28 (lamb 642) were pathogenic to mice (Table 1). Results of oocyst and tachyzoite titrations revealed that mice in terminal dilutions containing few or one organism died of acute toxoplasmosis; the last dilution was not infective as revealed by serological and parasitological examination. Oocyst dilutions 10^{-1} to 10^{-4} were lethal to all 16 mice. The 10^{-5} dilution of oocysts was infective to one of four mice and the infected mouse died of acute toxoplasmosis. The 10^{-6} dilution containing one or no oocyst was not infective to mice. All 12 mice inoculated with a 10^{-2} to 10^{-4} dilution of tachyzoites died of acute toxoplasmosis between 14 and 19 days p.i. One of the four mice inoculated with 10^{-5} dilution died of toxoplasmosis on day 24 p.i. Antibodies to *T. gondii* were not found in the 1:25 dilution of serum of any mouse that survived more than one month and tissue cysts were not found in mouse brains.

For two isolates (TgShUs1, TgShUs37), cats fed lamb hearts did not shed oocysts but the mice inoculated with lamb heart became infected with *T. gondii* (Table 2). None of the 16 isolates primarily obtained in mice were pathogenic to mice (Table 2).

The four cats fed hearts from seronegative (MAT $<$ 1:25) lambs did not shed oocysts.

3.4. Genotyping

Genotyping of the 53 *T. gondii* isolates using the 10 PCR–RFLP markers revealed 57 strains with 15 genotypes (designated as lamb#1 to #15, Table 3). Genotyping of DNA samples from mice infected with hearts of lamb No. 707, 710, 713 and 728 identified mixed *T. gondii* infection, and these samples are designated with "a" or "b" following the isolate designation (Tables 2 and 3).

Lamb#1 had 17 strains with type II alleles at all 10 loci and these were considered clonal Type II. Lamb#2 had nine strains that had type II alleles at all loci except a type I allele at locus Apico; these strains were also considered as Type II. Therefore, there were 26 Type II strains, accounting for 45.7% of the 57 strains. Lamb#3 had 10 strains with type II alleles at all loci except type I alleles for loci L358 and Apico. Lamb#4 had two strains that had type II alleles at all loci except type I allele for locus L358. Lamb#5 had eight strains that had type III alleles at all loci; these were considered clonal Type III strains, this lineage accounted for 15.7% of strains identified in this study. Lamb#6 had two isolates that had a combination of type I and III alleles at different loci. Lamb#7 to #15 each had one strain, and each genotype had a combination of different alleles at different loci, except for Lamb#10 which may have had a mixed infection. Phylogenetic network analysis of these lamb genotypes is presented in Fig. 1. Genotype Lamb#10 was excluded due to its mixed infection. The result of phylogenetic network analysis showed that genotypes from Lambs#1, 2, 3, 4 and 14, accounting for 68% (39/57) of the isolates identified in this study, form a cluster with the Type II strain PTG, indicating these lamb genotypes are closely related to the clonal Type II lineage (Fig. 1).

4. Discussion

The MAT has been used extensively to detect IgG antibodies to *T. gondii* in sera of animals but has only been validated in pigs using the isolation of the parasite as the reference (Dubey et al., 1995). Recently, *T. gondii* was isolated from eight of 30 (26.6%) ewes from France with MAT titres of 1:20 or higher; from none of two sheep with titres of 1:20, from one of eight with titres of 1:80, and from seven of 11 ewes with titres of 1:160 or higher (Dumètre et al., 2006; A Dumètre, personal communication). The differences in isolation rates between the French study (26.6%) and the present investigation (94%, 16 of 17 in batch 9) may in part be due to the age (lambs versus ewes) and the number of mice used for bioassay (five versus 10 mice). It is noteworthy that in both studies, 50 g of myocardium was digested using an identical digestion procedure. Although the present study was not designed as a validation study, isolation of *T. gondii* from lambs with a MAT titre of 1:50 and the lack of oocyst shedding by four cats fed hearts from 44 lambs with a MAT titre of $<$1:25 supports the validity of MAT.

Dubey and Kirkbride (1989a) isolated *T. gondii* from eight of eight naturally-infected lambs from a flock in South Dakota, USA. The lambs were from a flock that had aborted due to toxoplasmosis. *Toxoplasma gondii* was found histologically in 11 of 30 lambs that were born dead. Lambs that survived the first week after birth remained asymptomatic and were bled when three to four month old; antibodies (MAT 1:1024 or higher) were found in 67 of 112 lambs. Eight of these lambs with MAT titres of 1:4096 or higher were slaughtered when they were seven months old. *Toxoplasma gondii* was isolated from the hearts of three, tongues of seven, leg of lamb in eight, and lamb chops of seven; 100 g of each tissue was bioassayed in mice (Dubey and Kirkbride, 1989a).

The MAT titre that should be considered specific for the detection of antibodies to *T. gondii* in sheep has not been determined. We use a 1:25 serum dilution to screen sheep sera for detecting *T. gondii* antibodies. Although the present study was not designed as a validation study,

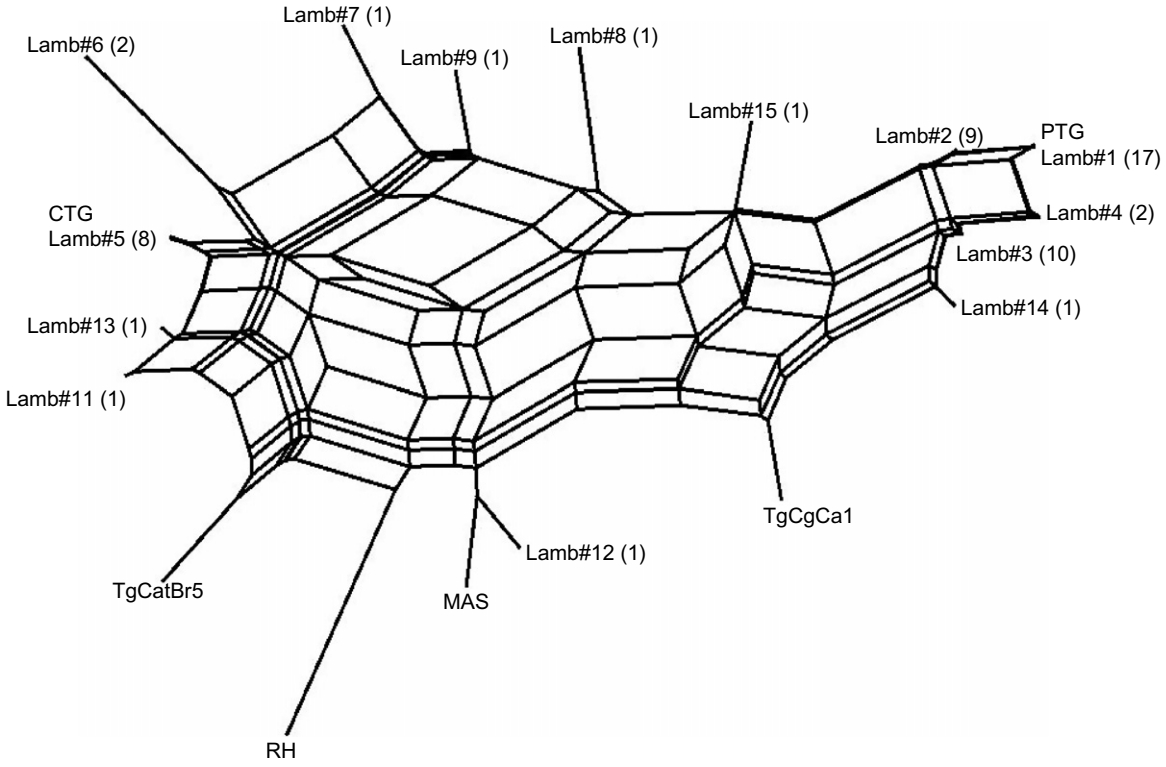

Fig. 1. Phylogenetic network (NeighborNet) of lamb isolates. RH, PTG, CTG, TgCgCa1 (Cougar), MAS and TgCatBr5 are reference strains. The number of isolates belonging to each genotype is indicated in parentheses.

the isolation of viable *T. gondii* from three of four lambs with a MAT titre of 1:50 suggests that one could rely on this as a screening dilution.

For the present study, heart tissues were selected for bioassay for convenience and availability, since matching serum and tissue from the same animal is difficult in a commercial slaughterhouse because of the speed with which animals are processed. In the current study blood was removed from the heart, thus minimising chances of error in matching tissue and serum. Lamb hearts are edible and are sold commercially.

In the USA, *T. gondii* was isolated four decades ago from two of 50 lamb chops from retail meat stores in California (Remington, 1968) and from the diaphragms of eight of 86 (9.3%) sheep from a slaughterhouse in Baltimore, Maryland (Jacobs et al., 1960). Dubey and Beattie (1988) summarised previous worldwide reports of the isolation of *T. gondii* from sheep. In one survey, indirect haemagglutination antibodies to *T. gondii* were found in 8% of 1056 lambs in a California slaughterhouse (Riemann et al., 1977). In another study, MAT antibodies were found in 65.5% of 1564 ewes from 33 farms in northwest USA (Dubey and Kirkbride, 1989b). Malik et al. (1990) reported ELISA antibodies to *T. gondii* in 42% of 345 lambs and 80% of adult sheep from five slaughterhouses in northeastern USA. According to US Department of Agriculture regulations, sheep <1 year old (without permanent teeth) are classified as lambs and slaughtered for human consumption, while older animals are classified as sheep and their meat (mutton) is sold for pet food and export. In the USA lambs and sheep are slaughtered in separate commercial slaughter facilities. Between three and 3.5 million lambs are slaughtered in the USA for food each year, and the per capita consumption of lamb meat in the USA is approximately 0.5 kg per year (NASS Agricultural Statistics, 2005 http://www.usda.gov/nass/pubs/agr05/agstats2005.pdf).

Results of the present study and previous surveys indicate the prevalence of *T. gondii* in lambs can be high but the role of ingestion of infected lamb in the epidemiology of toxoplasmosis in humans remains to be determined. Cook et al. (2000) identified eating uncooked lamb as a risk factor for *T. gondii* infection in pregnant women in Europe. We are not aware of a similar 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).

Most *T. gondii* isolates from human and animal sources in Northern America and Europe have been grouped into one of three clonal lineages including Types I, II and III (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002a, b). When tachyzoites were used to infect outbred mice, Type I strains are uniformly lethal. In contrast, Type II and III strains are significantly less virulent (Howe et al., 1996). In the present study, mice infected with tachyzoites and bradyzoites of all 32 isolates from lambs were not virulent for mice, suggesting these isolates are not the virulent Type I strains. This is confirmed by our genotyping

result in that no Type I strain was found (Table 3). In this study, two genetically similar isolates including TgShUs22 and TgShUs28 had different pathogenicity for mice, with the latter being more virulent (Table 2). This was unexpected and further study of multilocus DNA sequencing is needed to determine if these two strains are genetically identical or just closely related.

Toxoplasma gondii was considered to be clonal with low genetic diversity (Howe and Sibley, 1995). However, we recently found that the isolates of *T. gondii* from Brazil and Colombia are biologically and genetically different from those in North America and Europe (Dubey et al., 2002, 2007a, 2007b, 2007c, 2007d; Lehmann et al., 2006). *Toxoplasma gondii* isolates from asymptomatic chickens from Brazil were more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype. Additionally, most isolates from chickens in Brazil were different from the major clonal lineages in North America and Europe, and the Type II strain was absent (Dubey et al., 2007a, 2007c). All these data suggest that the overall diversity of *T. gondii* is much higher than was previously believed.

Little information is available concerning genotypes of *T. gondii* circulating in sheep worldwide. Using PCR–RFLP based on the 5′ and 3′ ends of the SAG2 locus, Howe and Sibley (1995) first reported that the ME49 strain of *T. gondii* isolated from diaphragm of a sheep from Maryland in 1958 was genotype II. They also listed the M7741 *T. gondii* strain as Type III; this strain was also from the diaphragm of a sheep from Baltimore and was used extensively by Dubey and Frenkel (1972) to describe the life cycle of *T. gondii*. Owen and Trees (1999) found that DNA amplified directly from the placentas of 13 aborted sheep from 10 widely separated farms in the United Kingdom and two isolates from the hearts of lambs from an undefined location were all Type II, based on the SAG2 locus. Jungersen et al. (2002) reported that 11 isolates of *T. gondii* from Denmark (six from aborted lambs, five from healthy sheep) were Type II. The results from the United Kingdom and Denmark are of interest because there was no difference in genotype based on health (abortion) status of the animals. Recently, Dumètre et al. (2006) found that all eight *T. gondii* isolates from adult sheep from France were clonal Type II, using the SAG2 locus and five satellite markers (TUB2, TgM-A, W35, B17, B18). Using the same markers as reported by Dumètre et al. (2006), Zia-Ali et al. (2007) found that of the four isolates of *T. gondii* from adult sheep in Iran, two isolates were Type II and two were Type III. In summary, previously published data indicated that Type II are the predominant strains in sheep. Interestingly, no Type I isolates of *T. gondii* has been isolated from sheep so far.

In the present study, 15 genotypes were identified from 57 *T. gondii* isolates using 10 PCR–RFLP markers, suggesting high genetic diversity of the parasite in lambs from Maryland, Virginia and West Virginia (Table 3, Fig. 1). Phylogeneic network analysis indicated that the clonal Type II lineage and its closely related genotypes (Lamb#1, 2, 3, 4 and 14) accounted for 68% (39/57) of the isolates. Type III lineage accounted for 14% (8/57) of the strains and was the second most prevalent genotype. This data is in agreement with previous finding that the Type II and III lineages predominate in North America (Howe and Sibley, 1995). With the use of 10 PCR–RFLP markers, the resolution of genotyping was significantly improved and we were able to identify a variety of genotypes from the lamb isolates. The identification of unique alleles in several loci including SAG1 for genotypes Lamb#14 and 15, c22-8 for genotype Lamb#12, and PK1 for genotype Lamb#11, indicates that the genetic makeup of the non-clonal genotypes are quite diverse. Most of the non-clonal genotypes have a combination of alleles of Types I, II and III from different loci. It is not clear if those are simply recombinants from genetic crosses of clonal Type I, II and III strains, or are diverged lineages. We are in the process of carrying out multilocus DNA sequencing studies to address this question. Nevertheless, the high prevalence and high diversity of *T. gondii* in lambs are of importance for public health, as lamb meat can be an important source of *T. gondii* infection for humans and there is a potential that more virulent parasite strains may circulate in animal reservoirs and subsequently transmit to humans.

References

Ajzenberg, D., Bañuls, A.L., Tibayrenc, M., Dardé, M.L., 2002a. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. Int. J. Parasitol. 32, 27–38.

Ajzenberg, D., Cogné, N., Paris, L., Bessières, M.H., Thulliez, P., Filisetti, D., Pelloux, H., Marty, P., Dardé, M.L., 2002b. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J. Infect. Dis. 186, 684–689.

Boyer, K.M., Holfels, E., Roizen, N., Swisher, C., Mack, D., Remington, J., Withers, S., Meier, P., McLeod, R.The toxoplasmosis study group, 2005. Risk factors for *Toxoplasma gondii* infection in motheRefers of infants with congenital toxoplasmosis: implications for prenatal management and screening. Am. J. Obstet. Gynecol. 192, 564–571.

Cook, A.J.C., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. Br. Med. J. 321, 142–147.

Dardé, M.L., Bouteille, B., Perstreal, M., 1992. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiologic implications. J. Parasitol. 78, 909–912.

Dubey, J.P., Frenkel, J.K., 1972. Cyst-induced toxoplasmosis in cats. J. Protozool. 19, 155–177.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19, 337–339.

Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of animals and man. CRC Press, Boca Raton, Florida, pp.1–220.

Dubey, J.P., Kirkbride, C.A., 1989a. Economic and public health considerations of congenital toxoplasmosis in lambs. J. Am. Vet. Med. Assoc. 195, 1715–1716.

Dubey, J.P., Kirkbride, C.A., 1989b. Enzootic toxoplasmosis in sheep in North-Central United-States. J. Parasitol. 75, 673–676.

Dubey, J.P., Thulliez, P., Weigel, R.M., Andrews, C.D., Lind, P., Powell, E.C., 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. Am. J. Vet. Res. 56, 1030–1036.

Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. Vet. Parasitol. 74, 75–77.

Dubey, J.P., Lindsay, D.S., 1998. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. Int. J. Parasitol. 28, 1823–1828.

Dubey, J.P., Graham, D.H., Blackston, C.R., Lehmann, T., Gennari, S.M., Ragozo, A.M.A., Nishi, S.M., Shen, S.K., Kwok, O.C.H., Hill, D.E., Thulliez, P., 2002. Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from São Paulo, Brazil: unexpected findings. Int. J. Parasitol. 32, 99–105.

Dubey, J.P., Zarnke, R., Thomas, N.J., Wong, S.K., Van Bonn, W., Briggs, M., Davis, J.W., Ewing, R., Mensea, M., Kwok, O.C.H., Romand, S., Thulliez, P., 2003. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. Vet. Parasitol. 116, 275–296.

Dubey, J.P., Edelhofer, R., Marcet, P., Vianna, M.C.B., Kwok, O.C.H., Lehmann, T., 2005a. Genetic and biologic characteristics of *Toxoplasma gondii* infections in free-range chickens from Austria. Vet. Parasitol. 133, 299–306.

Dubey, J.P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., Marcet, P.L., Lehmann, T., Vianna, M.C.B., Miska, K., Sreekumar, C., Kwok, O.C.H., Shen, S.K., Gamble, H.R., 2005b. Prevalence of viable *Toxoplasma gondii* in beef, chicken and pork from retail meat stores in the United States: risk assessment to consumers. J. Parasitol. 91, 1082–1093.

Dubey, J.P., Patitucci, A.N., Su, C., Sundar, N., Kwok, O.C.H., Shen, S.K., 2006. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Chile, South America. Vet. Parasitol. 140, 76–82.

Dubey, J.P., Applewhaite, L., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Hill, R., Su, C., 2007a. Molecular and biological charactaerization of *Toxoplasma gondii* isolates from free-range chickens from Guyana, South America identified severeal unique and common parasite genotypes. Parasitology 134, 1–7.

Dubey, J.P., Sundar, N., Gennari, S.M., Minervino, A.H.H., Farias, N.A.R., Ruas, J.L., dos Santos, T.R.B., Cavalcante, G.T., Kwok, O.C.H., Su, C., 2007b. Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Pará state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. Vet. Parasitol. 143, 182–188.

Dubey, J.P., Gennari, S.M., Sundar, N., Vianna, M.C.B., Bandini, L.M., Yai, L.E.O., Kwok, O.C.H., Su, C., 2007c. Diverse and atypical genotypes identified in *Toxoplasma gondii* from dogs in São Paulo, Brazil. J. Parasitol. 93, 60–64.

Dubey, J.P., Cortés Vecino, J.A., Vargas-Duarte, J.J., Sundar, N., Velmurugan, G.V., Bandini, L.M., Polo, L.J., Zambrano, L., Mora, L.E., Kwok, O.C.H., Smith, T., Su, C., 2007d. Prevalence of *Toxoplasma gondii* in dogs from Colombia, South America and genetic characterization of *T. gondii* isolates.. Vet. Parasitol. 145, 45–50.

Dumètre, A., Ajzenberg, D., Rozette, L., Mercier, A., Dardé, M.L., 2006. *Toxoplasma gondii* infection in sheep from Haute-Vienne, France: seroprevalence and isolate genotyping by microsatellite analysis. Vet. Parasitol. 142, 376–379.

Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566.

Howe, D.K., Summers, B.C., Sibley, L.D., 1996. Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii*. Infect. Immun. 64, 5193–5198.

Howe, D.K., Honoré, S., Derouin, F., Sibley, L.D., 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J. Clin. Microbiol. 35, 1411–1414.

Huson, D.H., 1998. SplitsTree: A program for analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73.

Huson, D.H., Bryant, D., 2006. Application of Phylogenetic Networks in Evolutionary Studies. Mol. Biol. Evol. 23, 254–267.

Jacobs, L., Remington, J.S., Melton, M.L., 1960. A survey of meat samples from swine, cattle, and sheep for the presence of encysted Toxoplasma. J. Parasitol. 46, 23–28.

Jones, J.L., Kruszon-Moran, D., Wilson, M., McQuillan, G., Navin, T., McAuley, J.B., 2001. *Toxoplasma gondii* infection in the United States: seroprevalence and risk factors. Am. J. Epidemiol. 154, 357–365.

Jones, J.L., Kruszon-Moran, D., Wilson, M., 2003. *Toxoplasma gondii* infection in the United States, 1999-2000. Emerg. Infect. Dis. 9, 1371–1374.

Jones, J.L., Kruszon-Moran, D., Sanders-Lewis, K., Wilson, M., 2007. *Toxoplasma gondii* infection in the United States, 1999-2004, decline from the prior decade. Am. J. Trop. Med. Hyg. 77, 405–410.

Jungersen, G., Jensen, L., Rask, M.R., Lind, P., 2002. Non-lethal infection parameters in mice separate sheep type II *Toxoplasma gondii* isolates by virulence. Comp. Immunol. Microbiol. Infect. Dis. 25, 187–195.

Kimball, A.C., Kean, B.H., Fuchs, F., 1974. Toxoplasmosis: risk variations in New York City obstetric patients. Am. J. Obstet. Gynecol. 119, 208–214.

Lehmann, T., Marcet, P.L., Graham, D.H., Dahl, E.R., Dubey, J.P., 2006. Globalization and the population structure of *Toxoplasma gondii*. Proc. Natl. Acad. Sci. 103, 11423–114228.

Luft, B.J., Hafner, R., Korzun, A.H., Leport, C., Antoniskis, D., Bosler, E.M., Bourland, D.D., Uttamchandani, R., Fuhrer, J., Jacobson, J., Morlat, P., Vilde, J.L., Remington, J.S., 1993. Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. 329, 995–1000.

Malik, M.A., Dreesen, D.W., de la Cruz, A., 1990. Toxoplasmosis in sheep in northeastern United States. J. Am. Vet. Med. Assoc. 196, 263–265.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases. 5, 607–624.

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet. 363, 1965–1976.

Owen, M.R., Trees, A.J., 1999. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. J. Parasitol. 85, 382–384.

Remington, J.S., 1968. Toxoplasmosis and congenital infection. Birth Defects. 4, 49–56.

Riemann, H.P., Willadsen, C.M., Berry, L.J., Behymer, D.E., Garcia, Z., Franti, C.E., Ruppanner, R., 1977. Survey for *Toxoplasma* antibodies among sheep in the western United States. J. Am. Vet. Med. Assoc. 171, 1260–1264.

Su, C., Zhang, X., Dubey, J.P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. Int. J. Parasitol. 36, 841–848.

Zia-Ali, N., Fazaeli, A., Khoramizadeh, M., Ajzenberg, D., Dardé, M., Keshavarz-Valian, H., 2007. Isolation and molecular characterization of *Toxoplasma gondii* strains from different hosts in Iran. Parasitol. Res. 101, 111–115.