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Genetic characterisation of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type

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ABSTRACT

Little is known of the genetic diversity of *Toxoplasma gondii* circulating in wildlife. In the present study wild animals, from the USA were examined for *T. gondii* infection. Tissues of naturally exposed animals were bioassayed in mice for isolation of viable parasites. Viable *T. gondii* was isolated from 31 animals including, to our knowledge for the first time, from a bald eagle (*Haliaeetus leucocephalus*), five gray wolves (*Canis lupus*), a woodrat (*Neotoma micropus*), and five Arctic foxes (*Alopex lagopus*). Additionally, 66 *T. gondii* isolates obtained previously, but not genetically characterised, were revived in mice. *Toxoplasma gondii* DNA isolated from these 97 samples (31 + 66) was characterised using 11 PCR-restriction fragment length polymorphism (RFLP) markers (SAG1, 5′- and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22–8, c29–2, L358, PK1 and Apico). A total of 95 isolates were successfully genotyped. In addition to clonal Types II, and III, 12 different genotypes were found. These genotype data were combined with 74 *T. gondii* isolates previously characterised from wildlife from North America and a composite data set of 169 isolates comprised 22 genotypes, including clonal Types II, III and 20 atypical genotypes. Phylogenetic network analysis showed limited diversity with dominance of a recently designated fourth clonal type (Type 12) in North America, followed by the Type II and III lineages. These three major lineages together accounted for 85% of strains in North America. The Type 12 lineage includes previously identified Type A and X strains from sea otters. This study revealed that the Type 12 lineage accounts for 46.7% (79/169) of isolates and is dominant in wildlife of North America. No clonal Type I strain was identified among these wildlife isolates. These results suggest that *T. gondii* strains in wildlife from North America have limited diversity, with the occurrence of only a few major clonal types.

1. Introduction

The protozoan *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). Most of the research on *T. gondii* has been focused on humans or domestic animals. The increasing urbanisation of the US landscape has resulted in greater interaction between humans and wildlife, including raccoons (*Procyon lotor*), coyotes (*Canis latrans*) and white-tailed deer (*Odocoileus virginianus*), that have adapted to urban habitats. Wildlife species that live in urban areas are increasingly likely to come into contact with both domestic cats and the

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large population of feral cats which exist in some cities. In other areas, large mammals such as white-tailed deer and black bears (*Ursus americanus*) are popular game animals both for sport and meat hunting. Little is known concerning the prevalence and distribution of genotypes of *T. gondii* in these wildlife species and free-living dolphins.

Isolation of *T. gondii* from wildlife is time consuming, expensive and difficult. The quality of DNA from naturally-infected wildlife is often poor, because the density of *T. gondii* in tissues of asymptomatic animals is low and most tissues are often collected long after death. We have begun to characterise *T. gondii* isolates from livestock, free-living marine mammals and wildlife from different sources to potentially identify the reservoirs that transmit *T. gondii* to humans. Initially, genotyping was attempted using only one restriction fragment length polymorphism (RFLP) marker (SAG2), and many isolates were not individually designated (Dubey et al., 2004a,b). In the present study, we designated and genotyped numerous *T. gondii* strains that were obtained from wildlife or feral animals at the Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD, USA using a suite of 11 PCR-RFLP markers.

2. Materials and methods

2.1. Toxoplasma gondii isolates from previously reported studies but not genetically characterised

2.1.1. Toxoplasma gondii isolates from rodents and cats from pig farms in Illinois, USA

During the course of studies to determine sources of *T. gondii* infection on pig farms (Dubey et al., 1995), and methods to prevent *T. gondii* infection (Mateus-Pinilla et al., 1999), feral rodents and cats on these farms were examined for *T. gondii* infection. A decade later, some of these isolates were revived for the present study. Details of viable *T. gondii* isolates obtained are given in Supplementary Table S1.

2.1.2. Toxoplasma gondii isolates from wild animals in Mississippi and Georgia, USA

During an initial survey of wildlife from Georgia and Mississippi, viable *T. gondii* was isolated from white-tailed deer, raccoons, bobcats, foxes and coyotes and only preliminary genotyping was performed on these samples (Dubey et al., 2004a). Details of these isolates are given in Supplementary Tables S2 and S3.

2.1.3. Toxoplasma gondii isolates from miscellaneous wild animals

During the past two decades, *T. gondii* was isolated from tissues from wildlife. Details of these *T. gondii* isolates used in the present study are shown in Supplementary Table S4.

2.2. Toxoplasma gondii isolates from wildlife not previously reported

During the last 7 years, viable *T. gondii* was isolated from several species of wild animals. Details of sources of animals, number of sera and tissues examined for *T. gondii* infection are shown in Table 1.

2.3. Serology

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

2.4. Bioassay in mice

Samples were shipped overnight with cold packs to the APDL. Tissues were homogenised, digested in acidic pepsin, washed and aliquots of homogenates were inoculated s.c. into two to five outbred Swiss Webster (SW) mice (Dubey et al., 2009) and/or two knockout (KO) mice (Dubey et al., 2008c). 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 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 previously described (Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

2.5. Immunohistochemical examination

Tissues of sea otters submitted to the United States Geological Survey-National Wildlife Health Center (NWHC), Wisconsin, USA were examined at the APDL using immunohistochemistry as described previously (Thomas et al., 2007).

2.6. Genetic characterisation

Toxoplasma gondii DNA was extracted from the tissues of infected mice or cell-cultured tachyzoites and strain typing was

Table 1
Details of animals examined for *Toxoplasma gondii* infection not previously reported.

Species	USA State[a]	Year	Sera		Tissues	
			No. tested	No. positive (no-MAT titer)	No. bio-assayed	No. positive
Opossum (*Didelphis virginiana*)	GA	2008	3	1 (1-400)	1	1
Raccoon (*Procyon lotor*)	GA	2008	7	6 (3-200, 2-800, 1-1600)	6	1
	TX	2008	20	7 (5-50, 3-200, 1-400)	7	1
Coyote (*Canis latrans*)	GA	2008	6	0	0	0
	MN	2006	4	2 (2-25)	0	0
	WI	2006	10	4 (3-40,1-400)	4	1
Woodrat (*Neotoma micropus*)	TX	2008	66	3 (2-25,1-400)	74	1
Wolf (*Canis lupus*)	MN	2006	41	27 (3-20, 3-40, 9-80, 9-160, 3-320)	30	3
	WI	2006	11	9 (1-25,1-40, 5-80, 2-160)	9	2
	AK	2008–2010	53	14 (5-25,1-50,4-100,4-200)	14	1
Brown bear (*Ursus arctos horribis*)	AK	2007	3	2 (1-200, 1-800)	4	1
Sea otter (*Enhydra lutris kenyoni*)	WA	2009–2010	13	10 (1-25,1-160, 2-200,2-400,1-1600, 2-3200,1-12,800)	7	5
Arctic fox (*Vulpes argopus*)	AK	2010	27	16 (2-50,4-100,7-200,1-400,2-800)	14	5
Red fox (*Vulpes vulpes*)	AK	2010	9	3 (2-100,1-200)	3	2
Bald eagle (*Haliaeetus leucocephalus*)	WI	2005–2006	5	0	5	1
Red-tailed hawk (*Buteo jamaicensis*)	WI	2004	1	1 (1-100)	1	1
Bottle-nosed dolphin (*Tursiops truncatus*)	SC	2007–2011	107	18 (4-50, 2-100, 4-200, 2-400, 2-800, 4-3200)	18	4

MAT, modified agglutination test.
[a] AK, Alaska; GA, Georgia; MN, Minnesota; SC, South Carolina; WI, Wisconsin; TX, Texas.

performed using the genetic markers SAG1, 5′- and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22–8, c29–2, L358, PK1 and Apico as described previously (Su and Dubey, 2009; Su et al., 2010). NeighborNet phylogenetic networks were inferred using the software SplitsTree4 (Huson, 1998; Huson and Bryant, 2006; Pena et al., 2008).

2.7. Animal ethics

All experiments performed in mice were in accordance with a protocol approved by the Beltsville Area Animal Care and Use Committee, United States Department of Agriculture, Beltsville, Maryland, USA.

3. Results

3.1. Prevalence of Toxoplasma gondii

Antibodies to *T. gondii* were detected in one (33.3%) of three opossums, 13 (48.1%) of 27 raccoons, six (30%) of 20 coyotes, three (4.5%) of 66 woodrats, 50 (47.6%) of 105 gray wolves, 16 (59.3%) of 27 Arctic foxes, and 14 (18.4%) of 107 bottle-nosed dolphins (Table 1).

Viable *T. gondii* was isolated from 30 feralanimals including one each of opossum, woodrat, brown bear, red-tailed hawk, bald eagle, coyote, two raccoons, two red foxes, four dolphins, four Arctic foxes, five sea otters, six wolves and one captive Tammar wallaby (Table 2).

3.2. Genetic typing

The 95 *T. gondii* isolates in this study were grouped into 14 genotypes including clonal Types II and III, and 12 atypical types. These results were combined with those of 74 wildlife isolates previously reported in North America. The composite data set of 169 isolates consisted of a total of 22 genotypes (Table 3). Genotype #1, the most common type, accounted for 36.0% (61/169) of the isolates. This genotype could be further distinguished as Type X and Type A based on DNA sequencing typing (Miller et al., 2004; Sundar et al., 2008). The second most common type was Type II lineage which accounted for 27.8% (47/169) of the isolates. The Type II lineage includes two haplotypes that only differ at the locus Apico (allele I or II). The third and fourth most common types were genotype #2 and Type III with 10.6% (18/169) and 10.1% (17/169) of the total isolates, respectively. Genotypes #1 and #2 have recently been designated as the fourth clonal lineage (Type 12) in North America, based on extensive DNA sequencing and phylogenetic analysis (Khan et al., 2011). Phylogenetic network analysis of the 22 genotypes is summarised in Fig. 1.

Together, Type 12, Type II and Type III strains account for 84.6% (143/169) of total isolates. Geographic distribution of these major genotypes in North America is summarised in Fig. 2. These

Table 2
Details of isolation of *Toxoplasma gondii* from animals not previously reported.

Species (ID)	Date killed	USA State[a]	MAT	Bioassay[b]		Strain designation	Genotype
				Brain	Others		
Opossum (*Didelphis virginiana*)	1-25-2008	GA	400	0/4	H,T 4/4	TgOpGa1[c]	#4
Raccoon (*Procyon lotor*) (216)	1-25-2008	GA	800	0/4	H,T 2/4	TgRaGa8[c]	#1 (Type 12)
(2)	7-27-2008	TX	200	5/5	H,T 5/5	TgRaTX1[c]	#1 (Type 12)
Woodrat (*Neotoma micropus*)	5-6-2010	TX	<25	1/2	H,T 1/2	TgNlTX1[c]	#12
Brown bear (*Ursus arctos horribis*) (07106)[b]	8-30-2007	AK	800	0/4	H 4/4	TgBbAk1[c]	#1 (Type 12)
Red-tailed hawk (*Buteo jamaicensis*) (19281)	11-10-2004	WI	100	0/5	H 2/5	TgBjUS1[c]	#7
Bald eagle (*Haliaeetus leucocephalus*) (22855)	11-15-2009	CA	<25	not done	H 2/5	TgHlUs1[c]	II
Sea otter (*Enhydra lutris*) (22470)[f]	3-8-2009	WA	12800	4/4	H 4/4	TgSoUs40[c]	#1 (Type 12)
(22777)	9-21-2009	WA	400	1/5	H,M,T 0/15	TgSoUs41[c]	II
(22789)	9-29-2009	WA	1600	3/3	H,M,T 0/5	TgSoUs42[c]	II[d]
(22825)	10-20-2009	WA	800	5/5	H,M,T /9	TgSoUs43[c]	II[d]
(23023)[g]	4-28-2010	WA	>3200	Not done	H,T,M 4/5	TgSoUs44[c]	#1 (Type 12)
Wolf (*Canis lupus*) (GMU20A)	11-6-2006	AK	>200	1/4	T 1/5	TgWolfAk1	#9
(EF06 – 003)	5-10-2006	WI	160	NS	T 5/5	TgWolfWI1	#1 (Type 12)
(WJP – 927)	6-5-2006	MN	80	NS	T 2/4	TgWolf MN1	#1 (Type 12)
(EF06 – 009)	7-3-2006	WI	<25	NS	T 1/4	TgWolfMN2	Partial data
(JPG – 298)	8-3-2006	MN	NS	NS	T 5/5 (pathogenic)	TgWolfMN3	#1 (Type 12)
(JPG – 303)	8-11-2006	MN	>25	NS	T 4/4	TgWolfMN4	#1 (Type 12)
Coyote (*Canis latrans*) (HW06 – 005)	10-26-2006	WI	400	NS	T4/4	TgCoWI1	II
Tammar wallaby (*Macropus eugenii*)	4-25-1994	National Zoo, DC	NS	1/1[e]		TgWyUs4	#6
Bottle nosed dolphin (*Tursiops truncatus*) (SC0834)	8-25-2008	SC	>3200	5/5	H 1/5 M 5/5	TgDoUs4[c]	#1 (Type 12)
(SC0902)	1-6-2009	SC	400	1/5	M 0/5	TgDoUs5	II
(SC01003)	2-7-2010	SC	200	0/5	H½	TgDoUs6	#1 (Type 12)
(SC1133)	4-19-2011	SC	800	2/5	NS	TgDoUs7	III
Arctic fox (*Vulpes argopus*) (TUTDS-1)	5-9-2010	AK	100	Not done	H 1/4	TgVaUS1[c]	#11
(TUTDS-10)	5-10-2010	AK	>200	Not done	H 3/4	TgVaUS2	II
(TUTDS-12)	5-10-2010	AK	>200	Not done	H 2/4	TgVaUS3[c]	#1 (Type 12)
(TUTDS-19)	5-12-2010	AK	>200	Not done	H 1/4	TgVaUS4[c]	II[d]
(619AC02)	6-23-2010	AK	>800	Not done	H 1/5	TgVaUS5[c]	#1 (Type 12)
Red fox (*Vulpes vulpes*) (10128)	7-27-2010	AK	200	Not done	H 4/4	TgVaUS6[c]	II[d]
(AIABM061010A)	6-10-2010	AK	100	Not done	H 5/5	TgVvUS1[c]	II[d]

NS, no sample; MAT, modified agglutination test.
[a] AK, Alaska; CA, California; GA, Georgia; MN, Minnesota; WI, Wisconsin; TX, Texas.
[b] No. of mice infected/No. of mice inoculated. B, brain; H, heart; M, muscle; T, tongue; ND, not done.
[c] DNA from cell cultured organisms.
[d] At Apico Type I.
[e] Brain, heart, liver, lungs, and lymph node were pooled, and homogenate inoculated into 4 Swiss Webster mice. Three mice died due to bacterial infection within 4 days p.i. and were discarded. The fourth mouse survived and had tissue cysts in the brain when killed 6 months p.i.
[f] Died of *Sarcocystis neurona* encephalitis.
[g] Died of toxoplasmic encephalitis.

Table 3
Summary of *Toxoplasma gondii* isolates from wildlife in North America.

	SAG1	5′ + 3′SAG2	alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	*T. gondii* strain IDs	Comments
	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
Type II (*n* = 26)	II or III	II	II	II	II	II	II	II	II	II	II	TgMmUs2, TgCatUs1, TgCoGa1, TgBbPa3, 5, TgSoUS41, TgDoUS5, TgHlUs1, TgCoWl1, TgVaUS2, TgBsCoUS2, TgBsCoUS3, TgBrCoUS1	This study. Dubey et al. (2007d, 2008a,d,e)[a] Also identified from chickens and goose (Dubey et al., 2007e), sheep (Dubey et al., 2008c) and pigs (Velmurugan et al., 2009) in USA.
												[b]**TgRaW1, TgCyW4, 5, TgDoUs2, 3, TgWtdUs1, 3, 4, 5, TgSoUs5, 20, 27, 36**	
												TgMmUs3, 4, TgCatUs8, TgMmUs1, TgSoUS43, TgSoUS42, TgVaUS4, TgVvUS1, TgVaUS6	This study. Dubey et al. (2007d)[a]; Sundar et al. (2008)
Type II (*n* = 20)	II or III	II	II	II	II	II	II	II	II	II	I	**TgSoUs 3, 4, 18, 19, 21, 26, 29, 30, 37, TgCyW3, TgSkW1**	[a]Also identified from sheep (Dubey et al., 2008c) and pigs (Velmurugan et al., 2009) in USA.
Type II (*n* = 1)	II or III	II	II	II	II	II	II	II	II	II	Nd	TgRrUs1	This study
Type III (*n* = 17)	II or III	III	III	III	III	III	III	III	III	III	III	TgMmUs6, TgWtdUs32, TgRaGa1, TgWyUs1, 2, 3, TgBbPa1, 4, TgEsMt1, TgWfmMt1, 2, TgGoMs1, TgSkMs1, TgBlUS1, **TgWtdUs13, 15,TgDoUs7**	This study. Dubey et al. (2008e)[a] Also identified from sheep (Dubey et al., 2008c) and pigs (Velmurugan et al., 2009) in USA.
#1 (*n* = 61) Type 12	u-1	II	II	II	II	II	II	II	I	II	I	TgWtdUs16, 17, 18, 19, 20, 22, 23, 24, 26, 27, 28, 29, 30, 31, 33, 34, 35, 36, TgCatGa1, 2, 3, 4, TgCatMs1, TgRaTx1, TgRaGa8, TgSoUS40, TgDoUS4, TgTaUS1, TgBbAk1, TgWolfWl1, TgWolfMN1, TgWolfMN3, TgWolfMN4, TgDoUS6, TgVaUS3, TgVaUS5, TgSoUS44, **TgSoUs6, 7, 9, 10, 12, 13, 14, 22, 23, 24, 25, 32, 33, 34, TgSoUs8, 11, 15, 31, TgSoUs16, 17, 38, TgCatCa2, TgRaCa2, TgSkCa1**	This study. Dubey et al. (2007c)[a]; Sundar et al. (2008)[a] Also identified from sheep (Dubey et al., 2008c) and pigs (Velmurugan et al., 2009) in USA.
#2 (*n* = 18) Type 12	II or III	II	II	II	II	II	II	II	I	II	I	TgMmUs5, TgCatUs2, TgBbPa2, TgCatUs3, 4, 5, 6, **TgWtdUs2, 6, 7, 9, 11, 12, 14, TgSoUs35, TgRaW5, TgCyW1, 2**	This study. Dubey et al. (2007d)[a]; Dubey et al. (2008d)[a]; Sundar et al. (2008)[a], Also identified from sheep (Dubey et al., 2008c) and pigs (Velmurugan et al., 2009) in USA.
#3 (*n* = 2)	I	III	III	III	III	III	III	III	III	III	I	TgRaGa2, **TgRaW4**	This study. Dubey et al. (2007c)[a] Also identified from sheep (Dubey et al., 2008c) and pigs (Velmurugan et al., 2009) in USA.
#4 (*n* = 2)	I	III	III	III	III	III	II	III	III	III	III	TgCatUs7, TgOpGa1	This study. Also identified from pigs (Velmurugan et al., 2009.) in USA.
#5 (*n* = 1)	I	III	III	I	I	I	II	III	III	I	I	TgSKMs3	This study.
#6 (*n* = 1)	II or III	III	III	III	III	I	I	I	III	I	III	TgWyUs4	This study.
#7 (*n* = 2)	u-1	I	II	III	III	III	III	I	I	III	I	TgBjUS1, **TgDoUs1**	This study. Dubey et al. (2008e)[a]
#8 (*n* = 1)	I	III	III	III	I	III	I	III	III	III	III	TgFsUS1	This study.
#9 (*n* = 1)	u-1	II	II	III	III	II	II	III	II	II	I	TgWolfAK1	This study. Also identified from a sheep in USA (Dubey et al., 2008c).
#10 (*n* = 2)	I	I	I	I	I	I	I	III	III	I	III	TgHcUS1, TgHcUS2	This study.
#11 (*n* = 2)	II or III	I	I	I	I	I	I	III	III	III	III	TgVaUS1, TgBsCoUS1	TgGoatUS26 (Dubey et al., 2011)
#12 (*n* = 1)	I	I	II	III	III	III	I	III	I	II	III	TgNlTX1	Pena et al. (2008)
#13 (*n* = 2)	I	III	III	III	III	II	I	I	I	I	I	**TgRaW2, 3**	Dubey et al. (2007d)[a]
#14 (*n* = 1)	I	III	III	III	III	III	III	I	III	III	III	**TgCyW6**	Dubey et al. (2007d)[a]

#15 (n = 1)	I	I	I	III	III	III	III	I	III	**TgBBeCa1**	Dubey et al. (2008b)[a]
#16 (n = 1)	I	II	II	III	II	II	u-1	I	u-2	I	**TgCgCa2**	Dubey et al. (2008b)[a]
#17 (n = 2)	II or III	II	II	III	III	III	III	III	III	II	**TgWtdUs10, TgSoUs39**	Dubey et al. (2008d)[a]; Sundar et al. (2008)[a]
#18 (n = 1)	II or III	III	III	III	II	II	III	II	II	I	**TgWtdUs8**	Dubey et al. (2008d)[a]
#19 (n = 2)	II or III	II	II	II	II	II	II	I	II	II	**TgSoUs1, 2**	Sundar et al. (2008)[a]
#20 (n = 1)	I	I	II	I	III	III	I	I	III	I	**TgBbUS1**	Dubey et al. (2010b)[a]
Partial data	u-1	II	II	II	II	II	nd	I	nd	I	TgWolfMN2	This study
Patial data	I	nd	nd	nd	nd	I	I	III	nd	nd	TgMsUS1	This study

[a] References for isolates genotyped and published previously.
[b] *Toxoplasma gondii* isolates genotyped in previous studies are in bold.

genotypes are grouped by the state from which the sample was obtained.

4. Discussion

4.1. Biology and prevalence

4.1.1. Opossum

In the present study, *T. gondii* was isolated from a single seropositive opossum from Georgia, USA. Smith and Frenkel (1995) isolated *T. gondii* from one of three dye test positive opossums from Kansas, and to our knowledge this isolate was not genotyped.

4.1.2. Raptors

Toxoplasma gondii was isolated from a bald eagle for the first time. Previously, Dubey et al. (2010a) had isolated *T. gondii* from a ferruginous hawk, a barn owl, an American kestrel, rough-legged hawk, red-tailed hawk and two Swainson's hawks. These raptor isolates were genotyped in the current study which represents the first genotyping of raptor isolates. Although Lindsay et al. (1993) isolated viable *T. gondii* from 41% of 27 red-tailed hawks, one of three kestrels from Alabama, USA these isolates were not genotyped. Prevalence of *T. gondii* in raptors likely indicates the consumption of infected rodents. Raptors are generally resistant to clinical toxoplasmosis but a case of fatal toxoplasmosis was diagnosed immunohistochemically in an immature bald eagle that was hospitalised at a pet hospital in Pennsylvania, USA (unfixed tissues were not available for isolation of viable *T. gondii*) (Szabo et al., 2004).

4.1.3. Marine mammals

Toxoplasmosis in marine mammals is epidemiologically and clinically important. The Southern sea otter is listed as a threatened species under the Endangered Species Act in the USA. *Toxoplasma gondii* can cause fatal illness in sea otters and other marine mammals (Kreuder et al., 2003; Conrad et al., 2005; Honnold et al., 2005; Thomas et al., 2007; Dubey, 2010; Gibson et al., 2011). Sea otters are presumed to acquire *T. gondii* infection from the contamination of marine waters with oocysts washed from land (Miller et al., 2008b). Historically, two new genetic types, Type X (Miller et al., 2004) and Type A (Sundar et al., 2008), have been described from sea otters. In one report, Type X was associated with increased mortality in sea otters (Conrad et al., 2005). Types A and X have identical RFLP profiles and belong to Type 12 in this study.

In the present study, all five isolates from sea otters were either Type II (TgSoUs41, 42, 43) or Type 12 (TgSoUs40, 44, either Type A or X). Of the five sea otters from whose tissues viable *T. gondii* was isolated in the present study, only one was considered to have active toxoplasmosis. This sea otter (No. 23023) was a young female pup that was found dead on the beach. It still had some natal pelage but juvenile teeth were erupting. The pup appeared to be 1–3 months old. Neonatal toxoplasmosis in sea otters is rarely reported. Miller et al. (2008a) described generalised toxoplasmosis in a neonatal pup in California, USA. The genotype of viable *T. gondii* recovered from this pup was Type X.

It is of interest that most *T. gondii* isolates from marine mammals were either Type II or Type 12; Types I and III were absent. Honnold et al. (2005) described clinical toxoplasmosis in a Hawaiian monk seal; DNA extracted from the tissues of the seal was considered Type III based on SAG2 typing. We re-characterised this *T. gondii* DNA using 10 additional markers and it had a Type I allele at SAG1, GRA6 and c22–8, and a Type III allele at c29–2 and L358; there was no amplification at the remaining five loci.

Seroprevalence of *T. gondii* in bottle-nosed dolphins on both coasts of the USA is typically very high (Dubey, 2010), but viable

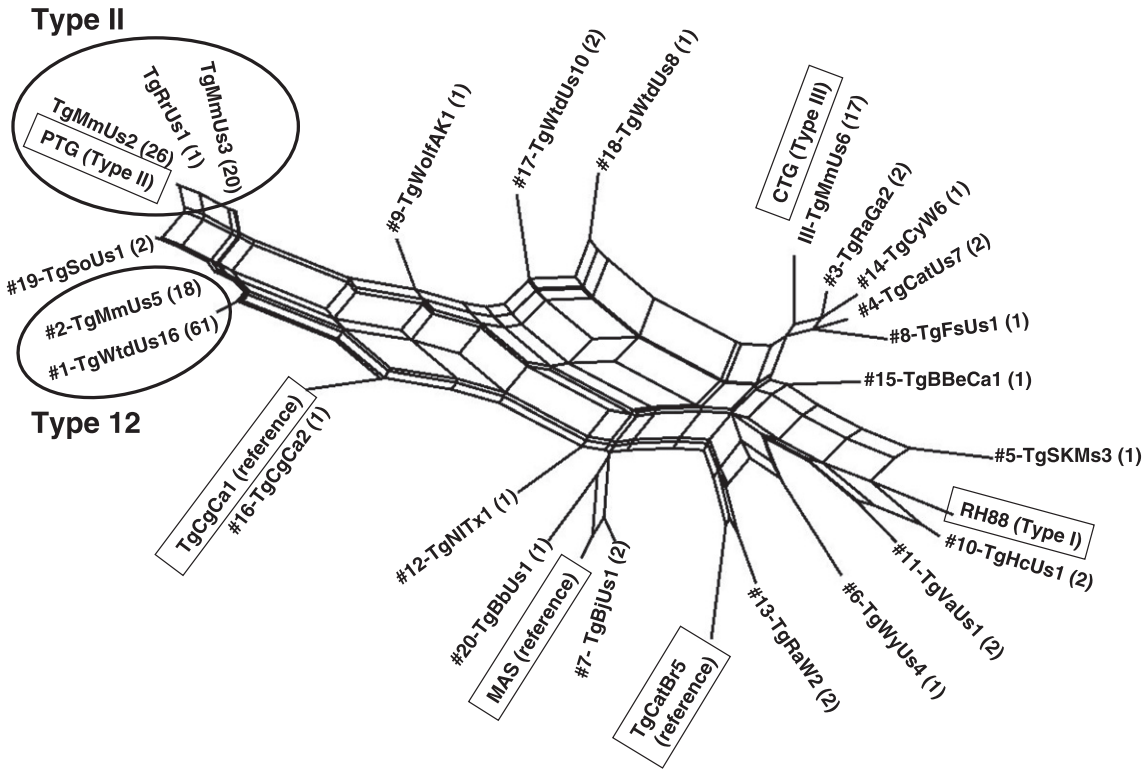

Fig. 1. Phylogenetic network analysis of *Toxoplasma gondii* from wildlife in North America. Genotype number (#) and the representative strain are listed for each taxonomic branch. The numbers in parentheses indicate the number of isolates belonging to that genotype. Genotype #1 and #2 belong to the Type 12 group (Khan et al., 2011). The Type II strains include two genotypes with either type I or II alleles at the locus Apico. Together, Type 12, Type II, and Type III strains accounted for 85% of total samples. Reference strains are indicated in boxes.

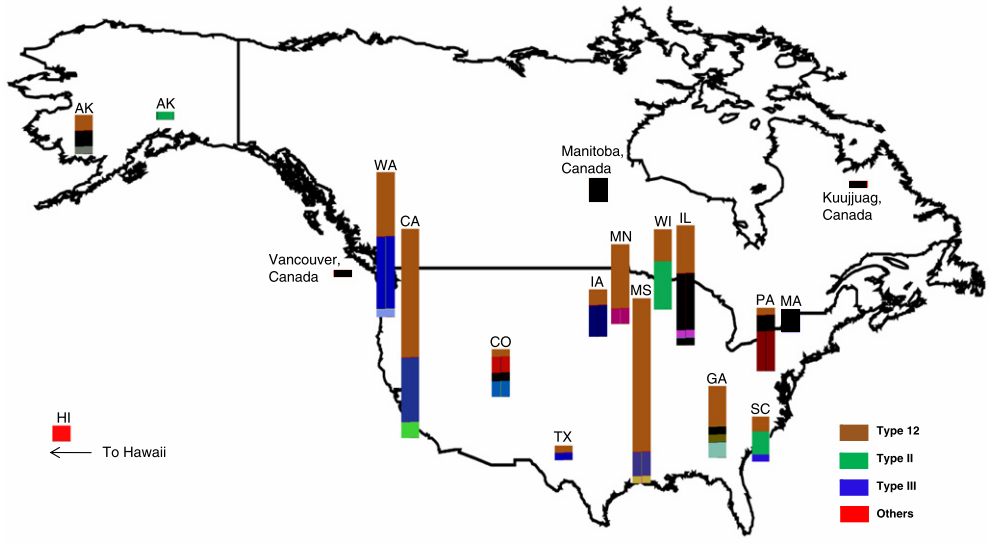

Fig. 2. Geographical distribution of the major genotypes of *Toxoplasma gondii* from wildlife in North America. Samples are grouped by states. Sample size is represented by the size of the bar. The smallest bar represents one isolate. Colour code: brown, green, blue and red are for Type 12, Type II, Type III and others, respectively. AK, Alaska; Co, Colorado; Ia, Iowa; IL, Illinois; Ga, Georgia; MA, Massachusetts; MN, Minnesota; MS, Mississippi; SC, South Carolina; TX, Texas; WI, Wisconsin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parasites have rarely been isolated. In the present study, *T. gondii* antibodies were found in only 16.8% of dead, stranded dolphins in South Carolina, USA and viable *T. gondii* was isolated from four of 18 dolphins. This seroprevalence is much lower than two previous studies conducted between 1999 and 2003 that found 96.8% of 94 dolphins from California and 100% of 47 dolphins from Florida were seropositive (Dubey et al., 2003). In a subsequent study, 100% of 146 free-ranging dolphins from 2003 and 2004 from Florida and South Carolina were seropositive (Dubey et al., 2005). In a more recent report, *T. gondii* antibodies were found in 51.9% of 52 dolphins from South Carolina that were stranded between 2005–2007, and viable *T. gondii* was isolated from three of 32 dolphins (Dubey et al., 2008e). Reasons for differences in *T. gondii* seroprevalence in live versus dead stranded dolphins are unknown; data

were obtained using standardised MAT tests and all were analysed by a single person. From the combined data of seven dolphin isolates from South Carolina, three were the clonal Type II strains (TgDoUs2, 3, 5), one was clonal Type III (TgDoUs7), two were Type 12 strains (TgDoUs4, 6) and one was type #7 strain (TgDoUs1). The former two genotypes are common in other wildlife such as in sea otters. To our knowledge, this is the first time that the Type III strain was isolated from a marine mammal.

4.1.4. Macropods

Macropods are highly susceptible to clinical toxoplasmosis but little is known of the epidemiology of the parasite in the wild. The macropodid marsupials in the present study were captive; there are no free-living macropods in the USA. The archived strains from the three wallabys from Pennsylvania (Dubey and Crutehley, 2008) were classified as clonal Type III. Recently, Parameswaran et al. (2010) characterised *T. gondii* DNA amplified directly from tissues of naturally-infected kangaroos in Australia and reported the presence of both archetypal Types I and II, and non-archetypal lineage alleles among strains circulating in Australian macropods. Of the 13 specimens PCR-positive with the B1 gene, DNA sequencing identified five with the type I allele, two with the type II or III allele, and seven had new alleles; viable *T. gondii* were not available for further genotyping. Genotyping of two archived strains (one *Macropus rufogriseus,* one *Vombatus ursinus*) from tachyzoites grown in cell culture, revealed an atypical genotype (Parameswaran et al., 2010). Moré et al. (2010) cultivated viable *T. gondii* from two macropods (*Macropus rufus, Macropus giganteus*) that had died in a zoo in Argentina. Using the same 11 RFLP markers used in the current study, these two isolates were classified as clonal Type II (*M. giganteus*) and Type III (*M. rufus*).

4.1.5. Wolves

In the present study, *T. gondii* was isolated from six wolves from Alaska, Wisconsin and Minnesota for the first time. Antibodies to *T. gondii* were reported previously in 9% of 125 (Zarnke et al., 2000) and 17.8% of 320 (Stieve et al., 2010) wolves from Alaska. Five of these wolf *T. gondii* strains were successfully genotyped, with four belonging to Type 12 and one to genotype #9 (Table 2).

4.1.6. Foxes

Very little information is available concerning *T. gondii* infection in red foxes in the USA (Dubey et al., 2009). Red foxes are present throughout the country and infection in this host indicates *T. gondii* infection in local small mammals and birds. Viable *T. gondii* was isolated from a red fox from Georgia (Dubey et al., 2004a) and one of four red foxes from Kansas (Smith and Frenkel, 1995); the isolate from Georgia was Type II based on SAG2 and the isolates from Kansas were not genotyped. Here, we isolated viable *T. gondii* from two seropositive red foxes from Alaska; these isolates were Type II (with a type I allele at the Apico locus).

Transmission of *T. gondii* in Arctic foxes is of special interest due to the high seroprevalence of *T. gondii* in this animal as well as the absence of cats in certain areas of the Arctic. Previously, antibodies to *T. gondii* were found in 43% of 594 Arctic foxes from Norway, and viable *T. gondii* (Type II) was isolated from a seropositive fox from Svalbard, Norway in the absence of any felids (Prestrud et al., 2008a). Subsequently, Prestrud et al. (2008b) directly amplified *T. gondii* DNA from the brains of 55 of 167 seropositive Arctic foxes from Svalbard, Norway; 46 were Type II, seven were Type III and two had atypical genotypes. In the present study, seroprevalence was 51.8% and viable *T. gondii* was isolated from five of 14 seropositive Arctic foxes from Alaska. This isolation rate is likely an underestimate because most tissues of foxes were badly decomposed (as many as 17 days elapsed between tissue collection and their receipt at APDL for bioassay).

4.1.7. Domestic cats from rural environment

Cats are central to the transmission of *T. gondii,* especially with respect to food animals. Unfortunately, nothing is known of the *T. gondii* genotypes circulating in domestic cats in the USA. The only *T. gondii* isolates we are aware are those isolated from cats on pig farms in Illinois (Dubey et al., 1995; characterised in the present study), and two cats from Mississippi (Dubey et al., 2004b). Five of eight isolates from cats in Illinois were Type 12, as was one of the two isolates from cats in Mississippi. In contrast, only one of the seven isolates from rodents on pig farms in Illinois was Type 12. These studies are relevant with respect to *T. gondii* transmission in domestic pigs and eventually to humans, as part of the food chain. Unfortunately, an attempt was not made to isolate *T. gondii* from pigs on the farms where rodents and cats were surveyed. These epidemiological studies are very expensive and difficult because viable *T. gondii* was isolated from only 10 of 1,676 rodents bioassayed (Dubey et al., 1995).

4.2. Genetic types

Genetic analyses of 169 *T. gondii* isolates from wildlife in this study revealed limited diversity with a few dominant genotypes in North America. The same dominant genotypes were also identified in domestic animals including pigs and sheep in this region (Dubey et al., 2008b; Velmurugan et al., 2009). Of these genotypes, Type 12 was the most common type in wildlife. This genotype includes the Type X and Type A *T. gondii* strains reported in sea otters from California and Washington State (Miller et al., 2004; Sundar et al., 2008). Although Type 12 has been identified from pigs and sheep in the USA, the frequency is low, and the dominant genotype in these domestic animals is Type II (Dubey et al., 2008b; Velmurugan et al., 2009). It is not clear why there is a difference in genotype distribution among wildlife versus domestic animals. It may be due to sampling variation or adaptation of biological traits in different genotypes. Overall, the results of this study clearly showed that there are three wide-spread genotypes with a number of rare *T. gondii* strains circulating in wildlife of North America. Recent phylogenetic study of *T. gondii* in wildlife from North America identified the clonal lineage designated as Type 12 (Khan et al., 2011). The Type 12 lineage includes genotypes #1 and #2 in this current study. It is shown in this study that Type 12 is widespread and is the most common lineage in wildlife from North America.

The genetic relationship among the 169 *T. gondii* isolates is presented as a NeighborNet phylogenetic network in Fig. 1. Instead of forcing a strictly bifurcating topology in a conventional single phylogenetic tree, the phylogenetic network allows a phylogenetic tree with reticulations. Reticulation topology presents mutually incompatible trees simultaneously; such relationships may be due to recombination, gene conversion or a lack of genetic information to resolve the conflict of a number of equally supported phylogenetic trees. A phylogenetic network is preferred to the traditional bifurcating phylogenetic tree in describing and visually presenting complex relationships in population biology (Morrison, 2005). Here, the phylogenetic network of *T. gondii* isolates from North America is reticulated, suggesting either the lack of genetic information to resolve the trees or some level of recombination in the parasite population. Given the number of isolates analysed, the network revealed much lower diversity in North America than that of Brazil in South America (Pena et al., 2008).

4.3. Genetic diversity and epidemiological significance

Historically, *T. gondii* was considered to be clonal with low genetic diversity (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002a,b, 2004; Lehmann et al., 2003; Khan et al., 2005; Aubert et al., 2010). 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 (Lehmann et al., 2006; Dubey et al., 2002, 2007a,b; Dubey and Su, 2009). Humans can 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 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 and Sundar, 2009). Severe cases of toxoplasmosis have been reported in immunocompetent patients in association with atypical *T. gondii* genotypes (Ajzenberg et al., 2004; Demar et al., 2007; Elbez-Rubinstein et al., 2009; Grigg and Sundar, 2009; Vaudaux et al., 2010; Wendte et al., 2010).

Results of the present study and other recent studies indicate that atypical genotypes of *T. gondii* circulate in the animal food chain in the USA. Unfortunately, there are only limited data on genotypes circulating in humans in the USA and the data are limited to sick patients. *Toxoplasma gondii* infection in wildlife is important because people can become infected directly by eating undercooked game meat, occasionally with serious consequences (Dubey et al., 2009). Wildlife carcasses left unattended and viscera from game animals are a source of infection for free-ranging domestic cats and for other wild felids. Millions of deer and bears are hunted annually in the USA and there are many thousands of bobcats and cougars that may have access to these carcasses. A single felid can excrete millions of oocysts and thus can rapidly contaminate the environment which may lead to infections in many other hosts. In addition to the presence of *T. gondii* in the terrestrial environment, the very high seroprevalence of *T. gondii* in sea otters and dolphins suggests contamination is also common in the marine environment. Our data suggest that deer and marine mammals might have host-adapted *T. gondii* genotypes. Our data on deer were limited to one state (Mississippi) but because white-tailed deer are present throughout much of the USA, this species should be a suitable candidate for further studies on the population biology of *T. gondii* isolates circulating in wildlife.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.06.005.

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