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Genotyping *Toxoplasma gondii* from wildlife in Pennsylvania and identification of natural recombinants virulent to mice

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Abstract

Recent studies indicated the predominance of *Toxoplasma gondii* haplogroup 12 in wildlife in USA. But still little is known of the genetic diversity of this parasite circulating in wildlife. In the present study, we tested coyotes (*Canis latrans*), red foxes (*Vulpes vulpes*), white-tailed deer (*Odocoileus virginianus*), and geese (*Branta canadensis*) from the state of Pennsylvania for *T. gondii* infection. Antibodies to *T. gondii* were found in 160 of 367 animals, including 92 (34.5%) of 266 coyotes, 49 (62.0%) of 79 white-tailed deer, 17 (85.0%) of 20 red fox, and two of two Canada geese tested by the modified agglutination test (cut off titer 1:25). Tissues from 105 seropositive animals were bioassayed in mice, and viable *T. gondii* was isolated from 29 animals, including 10 of 53 coyotes, 11 of 16 foxes, 7 of 49 deer, and one of one goose. DNA isolated from culture-derived tachyzoites of these isolates was characterized initially using multilocus PCR-RFLP markers. Nine genotypes were revealed, including ToxoDB PCR-RFLP #1 (4 isolates), #2 (2 isolates), #3 (4 isolates), #4 (6 isolates), #5 (4 isolates), #54 (1 isolate), #141 (1 isolate), #143 (1 isolate), and #216 (6 isolates), indicating high genetic diversity of *T. gondii* in wildlife in Pennsylvania. Pathogenicity of six *T. gondii* isolates (5 of #216 and #141) was determined in outbred Swiss Webster mice. Three of #216 and the #141 isolates were acute virulent to mice, and the other 2 #216 isolates were intermediate virulent. To determine the extent of genetic variation of these as well as a few recently reported virulent isolates from wildlife in North America, intron sequences were generated. Analysis of intron sequences and PCR-RFLP genotyping results

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Conflict of interest

None.

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indicated that the #216 isolates are likely derived from recombination of the clonal type I and III lineages. To determine if *T. gondii* virulence can be predicted by typing, we genotyped a collection of strains using PCR-RFLP markers for polymorphic genes *ROP5*, *ROP16*, *ROP18* and *GRA15*, which are known to interact with host immune response. The results showed that there is an association of genotypes of *ROP5* and *ROP18* with mouse-virulence, however, additional gene(s) may also contribute to virulence in distinct *T. gondii* genotypes.

Keywords

Toxoplasma gondii; Wildlife; Seroprevalence; Isolation; Genotyping; Pennsylvania; USA

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 antibodies to *T. gondii* (reviewed in Dubey and Jones, 2008). Humans become infected postnatally by ingesting tissue cysts from undercooked meat, or by consuming food or drink contaminated with oocysts. However, only a small percentage of seropositive 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 (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., 2011; Pomares et al., 2011; Sobanski et al., 2013). However, little is known of the association of genotype and clinical disease in animals and humans in the USA (Dubey, 2010). An atypical *T. gondii* genotype was isolated from a lamb aborted from a chronically infected sheep from Texas (Edwards and Dubey, 2013). A variant of Type II (NE-II) was recently found associated with prematurity and severe disease at birth in congenitally infected children in the USA (McLeod et al., 2012). Type II strains are the most prevalent in Europe, and are also abundant in North America, and cause congenital toxoplasmosis in children (Howe and Sibley, 1995).

Historically, *T. gondii* was considered to be clonal with low genetic diversity and grouped into 3 types, namely I, II, III (Howe and Sibley, 1995; Sibley and Ajioka, 2008). However, recent studies have revealed a greater genetic diversity of *T. gondii*, particularly isolates from Brazil (Khan et al., 2011; Su et al., 2012; Dubey et al., 2012, 2013a). A fourth clonal lineage (Type 12) was recently described, predominantly from wildlife (Dubey et al., 2011; Khan et al., 2011). 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 the Type II (Dubey et al., 2008a; Velmurugan et al., 2008). It is not clear why there is difference in genotype distribution among wildlife versus domestic animals, unless there is exclusive sylvatic cycling. Also, this

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trend was not seen in isolates from wild animals in France (Richomme et al., 2009; Aubert et al., 2010), and Norway (Prestrud et al., 2008). It may be due to sampling variation or adaptation of biological traits in different genotypes. Isolation of *T. gondii* from wildlife is time consuming, expensive, and difficult. Most of the data are derived from opportunistic sampling that may have limited availability due to the species habitat use, range, or season the species is available due to harvest season regulations. Although investigating wildlife it is not clear how a genotype will become established in a particular host.

In the present study we had an opportunity to survey different wildlife species in Pennsylvania. The species we examined are found statewide, across habitats, and have different exposure potentials due to their behavior, and may be important in the spread of *T. gondii* in both wildlife and human populations.

2. Materials and methods

2.1. Naturally infected animals

Wildlife samples were obtained through various methods with the majority of animals collected through hunter harvest or those taken to resolve wildlife damage issues conducted by USDA-APHIS-Wildlife Services. Heart, tongue, or brain (goose only) samples as well as blood samples were collected from each animal. Samples were collected within 48 h of mortality and refrigerated until they could be submitted for testing. Hunter harvested samples were taken using legal means and during approved seasons. All sampling or control activities were conducted under permits assigned to USDA-APHIS-Wildlife Services (USFWS permit MB 068253-0, Pennsylvania Special Use Permits 141-2010, 131-2011, 153-2012). All wildlife species taken to resolve wildlife damage conflicts were euthanized using approved AVMA methods (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). During 2007 and 2008 samples of blood, heart, and tongue were collected from hunted coyotes by one us (M. Weaver) as part of her Master's degree thesis; these samples were treated as the other samples described above. All samples were submitted to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture, Beltsville, Maryland for *T. gondii* examination. Samples were derived from most counties in Pennsylvania.

2.2. Serology

Sera from animals were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Sera were screened at 1:25, 1:50, 1:100, and 1:200 dilutions. Selected samples were titrated further.

2.3. Bioassay in mice

Tissues were homogenized in saline, digested in acidic pepsin, centrifuged, and aliquots of homogenates were inoculated subcutaneously into two to five outbred SW mice and/or one or two KO mice (Dubey, 2010). 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 described

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(Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

2.4. Pathogenicity of oocysts of T. gondii strains in mice

Pathogenicity of oocysts of the *T. gondii* isolates from seven isolates was done in SW mice. For this, *T. gondii*-free cats (Dubey, 1995) were fed tissues of infected mice and oocysts collected from the feces of cats (Dubey, 2010). Oocysts were sporulated in 2% sulfuric acid for a week on a shaker at room temperature, washed, counted, and diluted 10-fold from 10^{-1} to 10^{-7} to reach an end point of ≈1 oocyst. Aliquots from each dilution of oocysts were fed to five SW mice and the recipient mice examined for *T. gondii* infection. Mortality was recorded, and after two months mice were tested for *T. gondii* infection as described above.

To determine pathogenicity of tachyzoites from six isolates, SW mice fed oocysts were killed five to seven days p.i.; their mesenteric lymph nodes were homogenized in saline, filtered through 5-μm membrane filter to remove host cells, tachyzoites were counted, and diluted 10-fold to reach end point with <1 tachyzoite. Aliquots from each dilution were inoculated subcutaneously into five SW mice for each dilution.

2.5. In vitro cultivation

Infected mouse tissues were seeded on to CV1 cell culture flasks and tachyzoites were harvested from the medium as described (Dubey et al., 2013a).

2.6. Genetic characterization

T. gondii DNA was extracted from cell-cultured tachyzoites and strain typing was 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 et al., 2010). To further reveal the extent of genetic diversity of *T. gondii* isolates, four introns from three unlinked genes *UPRT*, *EF1* and *HP2* were sequenced as reported previously (Khan et al., 2007). Sequencing of introns was conducted by Genewiz (Genewiz Inc, NJ, USA). Sequences were aligned using ClustalX (Higgins et al., 1996) and nexus file was imported into Molecular Evolutionary Genetic Analysis (MEGA) Version 4.1 to identify all variable sites (Kumar et al., 2001). All variable sites from intron sequences and RFLP markers were concatenated and incorporated into SplitsTree v4.4 to generate unrooted neighbor-net analysis using 1000 bootstrap replicates (Huson and Bryant, 2006).

To determine if *T. gondii* virulence can be predicted by genotyping polymorphic genes *ROP5*, *ROP16*, *ROP18* and *GRA15*, which are known to interact with the host immune response (Melo et al., 2011), we conducted PCR-RFLP typing of the above loci for *T. gondii* strains with known virulent phenotypes. The typing was performed following previously reported method (Su et al., 2010). The primer sequences and condition for restriction digestion of PCR products for this assay are summarized in Table 1. Network analysis of these typing data was performed using SplitsTree4.4 as above.

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2.7. Ethical considerations

All experiments were performed according to approved protocols by all institutions involved.

3. Results

Between 2010 and 2012, tissues and serum from 265 animals, including 164 coyotes, 20 foxes, 79 deer, and two geese were collected by one of us (Van Why). The rest 102 samples were collected from coyotes in 2007. Antibodies to *T. gondii* were found in 160 of 367 animals with highest prevalence in red fox and geese (Table 2).

Viable *T. gondii* was isolated from 10 coyotes, 11 foxes, seven deer, and one goose (Tables 2 and 3). Of these, some or all SW mice inoculated with one isolate from coyote, four isolates from foxes, and two isolates from deer died of acute toxoplasmosis. Oocysts were obtained from seven mouse virulent isolates either by feeding tissues of mice that died after inoculation with the host tissues or by feeding mice sub-inoculated with the isolated strains (Table 3).

Oocysts of four *T. gondii* isolates from foxes were most virulent for SW mice (Table 4). All mice fed oocysts died of toxoplasmosis and the mortality was dose dependent. Most mice died of enteritis and pneumonia within 21 days p.i. Tachyzoites of three of these four isolates were also lethal for all mice; tachyzoites of the fourth isolate were not titrated when it was discovered that all isolates were the same genotype. Oocysts and tachyzoites of two isolates from deer were also virulent for SW mice (Table 5). Oocysts from the coyote isolate were only mildly pathogenic; lethal dose was more than 1000 oocysts (data not shown). This strain belongs to PCR-RFLP genotype #5.

PCR-RFLP analysis of 29 *T. gondii* isolates from wildlife in Pennsylvania by the genetic markers SAG1, 5′- and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico revealed nine genotypes (Table 6). Network analysis of concatenated intron sequences for three Pennsylvania isolates belonging to the genotype #216 and other 23 reference stains revealed that the genotype #216 is closely related to haplogroup 3 strains (Fig. 1). For 23 *T. gondii* strains with known virulence phenotypes (including 5 Pennsylvania isolates in this study), PCR-RFLP analysis of loci *ROP5*, *ROP16*, *ROP18* and *GRA15* are summarized in Table 7 and Fig. 2. The markers *ROP5*, *ROP16*, *ROP18* and *GRA15* revealed 5, 4, 2 and 2 alleles, respectively (Table 7). Eleven genotypes were revealed from the 23 *T. gondii* strains (Fig. 2). Due to low allele diversity for markers *ROP16* and *GRA15*, they contribute little to the genotyping of these *T. gondii* strains, whereas the combinations of *ROP5* and *ROP18* alleles identified 10 of the 11 genotypes. In addition, markers *ROP16* and *GRA15* alleles were not associated with virulence phenotypes. There are clusters of haplogroups: 2, 11 and 12; 3 and 9; 5 and 10; and 1, 4 and 7 that associated well with virulence phenotypes. The cluster related to haplogroup 6 has a mixed collection of virulent, intermediate virulent and non-virulent *T. gondii* strains.

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4. Discussion

4.1. Coyotes

Coyotes are considered resistant species to clinical toxoplasmosis, and we are not aware of any report of a clinical case of toxoplasmosis in this animal. Laboratory-reared coyotes fed *T. gondii* oocysts or tissue cysts became infected but remained asymptomatic (Dubey, 1982). In the present study, *T. gondii* antibodies were found in 34.5% of 266 coyotes, which is lower than 51.4% of 35 coyotes from Wisconsin (Dubey et al., 2007), 59% of 222 coyotes collected in Kentucky, Ohio, and Indiana (Dubey et al., 1999), and 62% of 52 coyotes from Texas (Lindsay et al., 1996). In the present study viable *T. gondii* was isolated from 10 (32.3%) of 53 seropositive coyotes. The majority of isolates that have been genotyped (18 in total) in the present or previous studies (Dubey et al., 2004a, 2007, 2011) comprise genotypes #1 and #3 are also known as the Type II, genotypes #4 and #5 are known as Type 12, both are dominant in wildlife in North America (Dubey et al., 2011).

Coyotes may be ideal as sentinels of *T. gondii* due to their habitat use and behavior (Aguirre, 2009). Coyotes are generalists that are able to exploit a variety of habitats and resources. Their populations have increased in many areas over the past 20 years, and they have colonized much of the eastern United States, using both rural and urban habitats (Anonymous, 1997; Bekoff and Gese, 2003; Gehrt and Riley, 2010; Mastro et al., 2012). Due to their diversity of diet and habitat use, the potential for coyotes to contract toxoplasmosis is high (Dubey et al., 1999). Because coyotes prey on or scavenge many species that may be harboring *T. gondii*, they could be the indicator of possible presence and exposure levels in an area.

4.2. Foxes

Very little information is available concerning *T. gondii* infection in red foxes in the USA (Dubey et al., 1999; Dubey, 2010). In the present study 85% of 20 foxes were seropositive, similar to results from other states (Dubey et al., 1999), and viable *T. gondii* was isolated from 11 (68.7%) of 16 seropositive foxes. *T. gondii* was previously isolated from a red fox from Georgia (Dubey et al., 2004a) and one fox from Kansas (Smith and Frenkel, 1995); the isolate from Georgia was Type II based on SAG2 and the isolates from Kansas were not genotyped. Two viable *T. gondii* from two red foxes from Alaska were genotype #3 (Type II with type I allele at the Apico locus) (Dubey et al., 2011). In the present study seven genotypes were identified from the 11 fox isolates, including genotype #1 of one isolate, #2 of two, #3 of one, #4 of one, #54 of one, #141 of one and #216 of four. It is interesting to see the relatively high frequency of #216 from foxes. Genotype #216 has been limited in North America, with one isolate from Grenada. Based on genotyping of ROP5, 16, 18 and GRA5, it is close to haplogroup 6 (Fig. 2). However, based on the 11 PCR-RFLP markers and intron sequences, it is closely related to haplogroup 3 (Fig. 1). It is likely a recombinant of the Type I and III strains.

Although it is difficult to determine the role that red foxes play in the spread of *T. gondii*, this species is more closely associated with human environments and resources than coyotes are (Soulsbury et al., 2010). Similar to coyotes, this species consumes prey and carrion that

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has been identified as carriers of *T. gondii* as well as preying on feral cats (Korschgen, 1959; Crossett and Elliott, 1991; Golightly et al., 1994) and possibly also exhibit coprophagy. Red fox may have more exposure potential to *T. gondii* due to a closer association with domestic/feral cat populations in rural, suburban, and urban environments (Soulsbury et al., 2010), providing a source for monitoring toxoplasmosis in the environment in areas where coyotes may not be available to sample.

4.3. Deer

In the present study, 62% of 79 deer were seropositive, indicating high seroprevalence. Humphreys et al. (1995) reported 60% of 593 deer from Pennsylvania sampled in 1991 had *T. gondii* antibodies. Samples collected in this study were from highly urbanized locations where deer densities are considered higher than normal and encounters with humans and domestic animals could be considered high. The areas where Humphreys et al. (1995) collected samples are from counties with much lower human density and with deer being collected from hunter harvest, it is likely that most deer were not found within a close proximity to a human occupied area. This observation indicates that deer densities or habitat may not be a limiting factor in exposure of deer to *T. gondii*.

In the present study, *T. gondii* was isolated from seven (20%) of 35 seropositive deer. *T. gondii* has been isolated previously from WTD in the USA (Lindsay et al., 1991, 1997; Dubey et al., 2004b, 2008b, 2013b; Yu et al., 2013), and some of these isolates have been genotyped using the 11 markers used in the present study (Dubey et al., 2011; Yu et al., 2013). Of the 44 *T. gondii* isolates (6 from Iowa, 9 from Minnesota, 19 from Mississippi, 9 from New Jersey, 1 Alabama), four were ToxoDB PCR-RFLP genotype #1 (Type II clonal), five were #2 (Type III), one was #3 (Type II variant), nine were #4 (Type 12), 18 were #5 (Type 12), one was #54, and one was #74, one was #216, one was #220, and three were #221. It is intriguing that 18 of 19 isolates from WTD from Mississippi State were genotype #5 (Type 12) although these deer were from six sites over a broad geographic area (Dubey et al., 2004a). Thus, there was a greater genetic variability than previously realized, although type 2 and 12 still predominate as seen in other wildlife species in North America.

4.4. Pathogenicity of natural isolates

Historically, *T. gondii* strains were grouped as virulent or avirulent, based on mortality in outbred mice. Pathogenicity is dependent on the stage of the parasite, dose, and the route of inoculation. Oocyst induced infections are more pathogenic, irrespective of the dose (Dubey, 2010). Therefore, we determined pathogenicity of the strains isolated here using both tachyzoites and oocysts, and inocula were titrated to reach an end point. In the present study, two of the seven *T. gondii* isolate from WTD were mild to high virulent for mice, irrespective of the dose or the stage inoculated (Tables 4 and 5). We further examined their genetic relationship with a collection of well characterized strains, using a set of intron markers that together with RFLP genotypes, has been used to cluster related strains into haplotypes (Khan et al., 2007; Su et al., 2012). Three representatives (TgFoxPa7, TgFoxPa8 – genotype #216; and TgFoxPa9 – genotype #141), together with four virulent strains reported recently, were sequenced for four introns at three genetic loci as described (Khan et al., 2007). The four recently reported virulent strains are TgSwanUs3 (genotype #216)

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(Dubey et al., 2013a), TgBbUs1 (genotype #147) (Dubey et al., 2010), TgShUs28 (genotype #73) (Dubey et al., 2008a), and TgShUs55 (genotype #32) (Edwards and Dubey, 2013). The analysis of composite data of intron sequences and PCR-RFLP showed that, the Pennsylvania isolates TgFoxPa7, TgFoxPa8, TgFoxPa9 and the isolate from mute swan TgSwanUs3 have the combination of type I and III alleles at different PCR-RFLP loci and the type III sequences for the four introns. All these isolates are more closely related to haplopgroup 3 (also called Type III) than haplogroup 1 (also called Type I, ToxoDB PCR-RFLP genotype #2) (Fig. 1).

To determine if *T. gondii* virulence can be predicted by typing, we genotyped a number of *T. gondii* strains using markers for polymorphic genes *ROP5*, *ROP16*, *ROP18* and *GRA15*, which are known to interact with host immune response (Melo et al., 2011; Hunter and Sibley, 2012). The serine/threonine kinase, ROP18, has been identified as a major determinant of virulence (Saeij et al., 2007; Taylor et al., 2006). ROP18 phosphorylates and inactivates a family of host derived immunity-related p47 GTPases (IRGs), thereby protecting the parasite from clearance by innate immune effectors (Fentress et al., 2010; Fentress and Sibley, 2011). The ROP18 alleles in Type I and II strains are important for virulence, whereas the Type III allele is not expressed in Type III strains, therefore making these strains non-virulent. *ROP5* locus encodes a pseudokinase, it consists of a family of 6–10 tandem repeats. The alleles in Type I and III strains are important for virulence, whereas Type II allele is non-virulent (Behnke et al., 2011; Reese et al., 2011). ROP5 controls virulence in *T. gondii* by facilitating ROP18 to phosphorylate IRG protein (Behnke et al., 2012; Fleckenstein et al., 2012). Therefore, the interaction of different alleles of ROP5 and ROP18 may have differential consequence of virulence. The results of genotyping *T. gondii* strains with different virulent phenotypes by markers *ROP5*, *ROP18*, *ROP16* and *GRA15* indicate that allele types of ROP16 and GRA15 have little contribution to virulence, whereas there is association of allele types of ROP5 and ROP18 with virulence (Table 7 and Fig. 2). However, additional gene(s) may also contribute to virulence in distinct *T. gondii* genotypes. Future study with a large sample size is needed to verify this finding.

4.5. Significance for human toxoplasmosis

Results of the present study, and other recent studies, indicate that a variety of *T. gondii* genotypes circulate in the food animal chain in the USA. Some of these genotypes may have potential to be highly virulent. *T. gondii* infection in wildlife is important because people can become infected directly by eating undercooked game meat, occasionally with serious consequences (Dubey, 2010). Both deer and geese are important game species, so animals harvested through recreational activities in addition to animals obtained through depredation activities and road kill collection provides considerable human exposure opportunities. Study of *T. gondii* infection rate in hunters or people consuming games may provide useful information to evaluate such risk.

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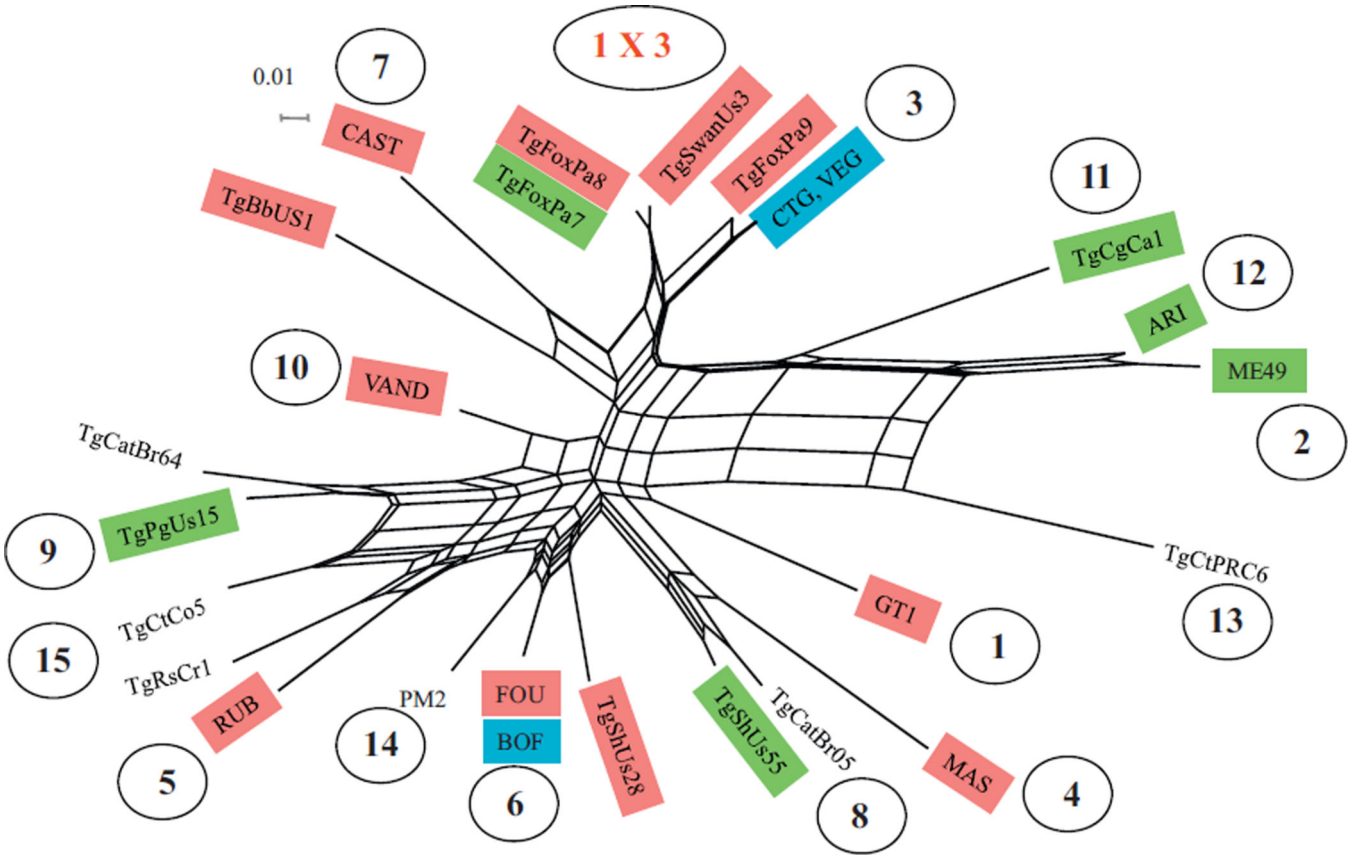

Fig. 1.

Neighbor-net analysis using concatenated 4 intron sequence markers comprising 1775 bp and 10 RFLP markers. The, wildlife isolates (TgFoxPa7, 8, 9) of *T. gondii* from Pennsylvania are closely related to haplogroup 3 strains. *T. gondii* haplogroups are shown in the circle numbers as designated previously (Su et al., 2012). Mouse-virulent strains are in red boxes, intermediate virulent in, green, and non-virulent strains in blue, the unknowns are in black. Samples TgFoxPa7, TgFoxPa8 and TgFoxPa9 are from this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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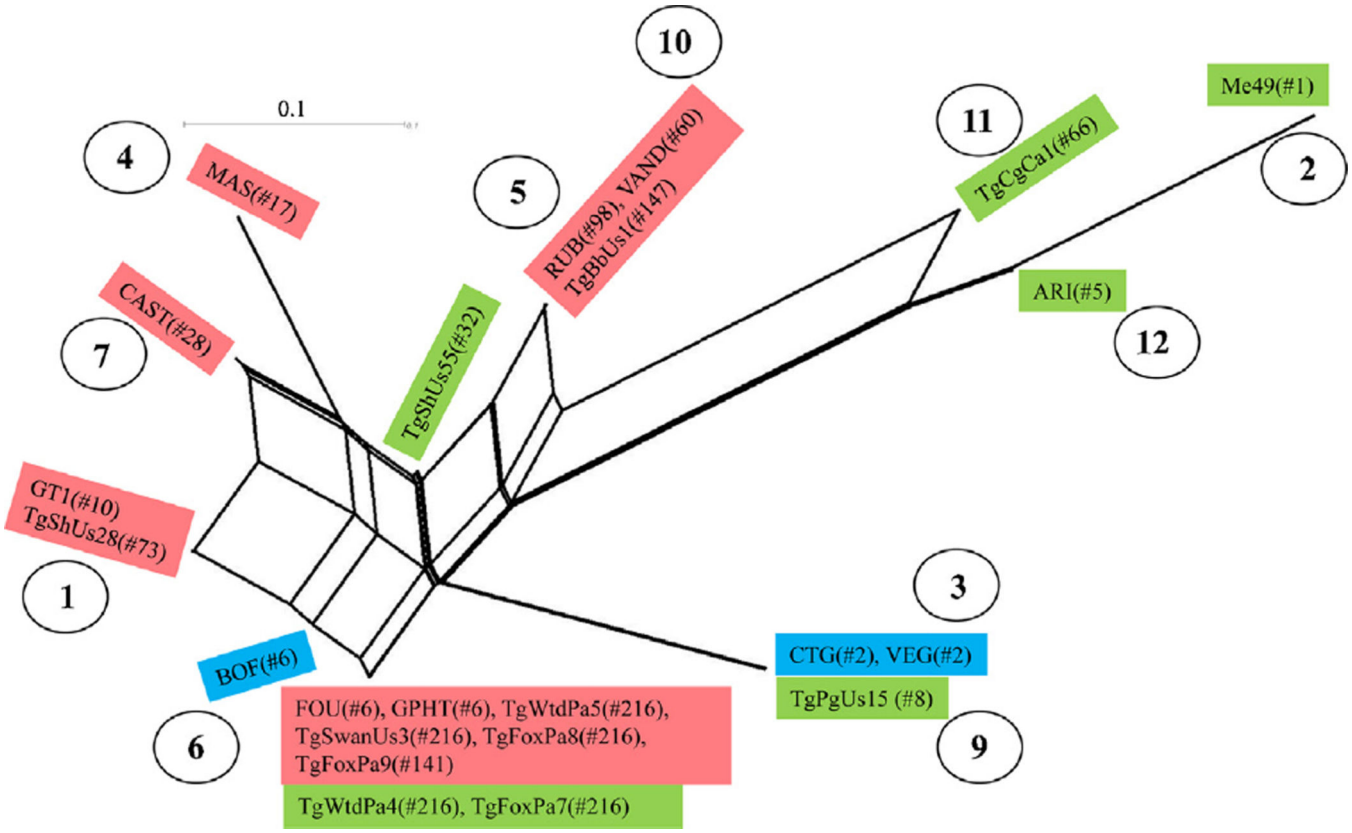

Fig. 2.

Genetic relationship of 23 *T. gondii* strains based on *ROP5*, *ROP16*, *ROP18* and *GRA15* PCR-RFLP polymorphisms. Ten, genotypes were identified among these strains. Mouse-virulent strains are in red boxes, intermediate virulent in green, and non-virulent, strains in blue. The number in the parenthesis is ToxoDB PCR-RFLP genotypes. *T. gondii* haplogroups are shown in the circle numbers, as designated previously (Su et al., 2012). Isolates TgWtdPa4, TgWtdPa5, TgFoxPa7, TgFoxPa8 and TgFoxPa9 are from current study. The result shows potential association of genotypes with virulence phenotype. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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Table 1

PCR primes for genotyping ROP5, ROP16, ROP18 and GRAG15.

Markers	External primers for multiplex PCR	Internal primers for nested PCR	NEB Restriction enzymes, buffers, incubation temperature and time, and gel electropheresis[a]
ROP5	ROP5-Fext GGACAGACGCAGGCTTTTAC ROP5-Rext TCAAACGTCCTGACACTTCG	ROP5-Fint: TGTGGCAGTTCAGTCTCAGC ROP5-Rint: TCGAAGTTGAGGAACCGTCT	FspBI digestion: 37 °C, 1 h, 2.5% agarose gel
ROP18	ROP18-DelFext: CTCGTCGACCACACAGCTAA ROP18-UPSFext: TTTTATCGACATCCCGCTTC ROP18-UPSRext: GAGTGCTTTCTGTCGCTCCT	Rop18-UPSFint: CACAGCATGAGCTTAAGAGTTG ROP18-UPSRint: CACCGCAAGACAGGCTGTCTTC	No enzyme treatment. Type III has positive PCR, Type I and II are negative. 1.5% agarose gel.
		ROP18-DelFint: AGTTCCCTTCCCTGGTGTCT ROP18-DelRint: ACAAACTGGACTGGGGTGAG	Type III has no PCR products, others have PCR products for RFLP analysis. ScrFI + MfeI, NEB4, BSA, 37 °C, 1 h, 2.5% gel
ROP16	ROP16-Fext ATCTGCTTATCCGGCGACTA ROP16-Rext TCCGTTGGCATTTATCATCA	ROP16-Fint: TACCAAACCCAGCTTTCACC ROP16-Rint: TCGTCAACAGCTGACTCCAC	NlaIII + TaqαI, NEB4, BSA, 37 °C 30 min, 65 °C 30 min, 2.5% agarose gel
GRA15	GRA15-Fext CACGTACACAACCCATCTCG GRA15-Rext2 CCTTTGAACGGGTAATGGAA	GRA15-Fint: GGACCACCCAGAACAGAAAA GRA15-Rint2: CCCTTATCGGTTTTTGGTCA	No restriction enzyme needed. 1.5% agarose gel

[a] Restriction enzyme FspBI was purchased from Fisher Scientific, Pittsbergh, PA, all other enzymes were purchased from the New England BioLab.

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Table 2

Prevalence of *T. gondii* in wildlife in Pennsylvania.

Host	No. tested	No. seropositive (%)	No. of animals with MAT titers of:				No. bioassayed	No. *T. gondii* isolated
			25	50	100	≥200		
Coyote	266	92 (34.5)	25	33	18	16	53[a]	10[b]
Fox	20	17 (85.0)	1	2	2	12	16	11
Deer	79	49 (62.0)	4	11	8	26	35	7
Goose	2	2 (100.0)	0	1	0	1	1	1
Total	367	160 (43.6%)	30	47	28	55	105	29

[a] 22 coyotes from 2007 survey.

[b] 2 from 2007 survey.

Vet Parasitol. Author manuscript; available in PMC 2015 August 05.

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Table 3

Isolates of viable *T. gondii* from wildlife in Pennsylvania.

Host	County	Date	MAT	Bioassay[a]		Isolate ID	Cat oocysts	ToxoDB-PCR-RFLP genotype #
				SW[b]	KO[c]			
Coyote								
23	Susquehanna	2/8/2012	>200	1/5	0/0	TgCoPa1		1
42	Sullivan	2/8/2012	≥200	5/5	0/0	TgCoPa2		5
53	Wyoming	2/10/2011	≥200	2/4 (29)	1/1	TgCoPa3	Cat #50	5
76	Clearfield	2/16/2011	50	2/2	1/1	TgCoPa4		5
137	Clearfield	3/3/2011	50	4/4	1/1	TgCoPa5		3
164	Mercer	3/3/2011	50	4/4	1/1	TgCoPa6		4
198	Erie	3/3/2011	50	1/4	1/1	TgCoPa7		4
201	Lycoming	3/3/2011	100	4/4	0/1	TgCoPa8		1
45	Potter	2/23/2008	100	2/2	Not done	TgCoPa9		5
78	Bradford	2/23/2008	100	1/2	Not done	TgCoPa10		1
Fox								
13	Adams	2/3/2011	≥200	1/1	1/1	TgFoxPa1		3
89	Adams	2/16/2011	≥200	3/3	1/1	TgFoxPa2		54
90	Adams	2/16/2011	≥200	0/2	1/1	TgFoxPa3		2
91	Bradford	2/16/2011	50	2/2	1/1	TgFoxPa4		216
95	Adams	2/16/2011	50	3/3	1/1	TgFoxPa5		1
96	Adams	2/16/2011	100	3/3 (16, 16, 20)	1/1	TgFoxPa6	Cat #53	216
238	Philadelphia	4/22/2011	200	4/4 (14, 14, 13, 9)	1/1	TgFoxPa7	Cat #70	216
239	Philadelphia	4/22/2011	100	1/4 (13)	1/1	TgFoxPa8	Cat #74	216
240	Philadelphia	4/22/2011	400	2/4 (19, 20)	1/1	TgFoxPa9	Cat #42	141
241	Philadelphia	4/22/2011	800	2/4	1/1	TgFoxPa10		4
242	Philadelphia	4/22/2011	400	4/4	1/1	TgFoxPa11		2
Goose 9	Philadelphia	7/6/2011	50	1/3	0/2	TgGoosePa1		143
White-tailed deer								
28	St. Clair	2/9/2011	≥200	3/3	2/2	TgWTDPa1		3
111	Allegheny	2/24/2011	≥200	3/3	0/0	TgWTDPa2		3

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Host	County	Date	MAT	Bioassay[a]		Isolate ID	Cat oocysts	ToxoDB-PCR-RFLP genotype #
				SW[b]	KO[c]			
114	Allegheny	2/24/2011	≥200	1/3	0/0	TgWTDPa3		4
118	Philadelphia	2/24/2011	≥200	3/3 (18, 18, 19)	0/0	TgWTDPa4	Cat #64	216
119	Philadelphia	2/24/2011	≥200	1/3 (41)	0/0	TgWTDPa5	Cat #58	216
122	Philadelphia	2/24/2011	≥200	3/3	0/0	TgWTDPa6		4
129	Philadelphia	2/24/2011	≥200	3/3	0/0	TgWTDPa7		4

[a] No of mice infected/No. of mice inoculated. SW = Swiss Webster, KO = knockout. MAT = modified agglutination test.

[b] Day of death is parenthesis.

[c] All infected KO mice died of toxoplasmosis.

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Table 4

Pathogenicity of oocysts of *T. gondii* isolates from wildlife in Pennsylvania to Swiss Webster mice[a].

Dose[c]	TgFoxPa9 (Cat #42, Fox 240)		TgFoxPa7 (Cat #70, Fox 238)		TgFoxPa8 (Cat #74, Fox 239)		TgFoxPa6 (Cat #53, Fox 96)[f]
	Oocysts	Tachyzoites	Oocysts	Tachyzoites	Oocysts	Tachyzoites	Oocysts
100,000	5 (5, 5, 5, 5, 5)[b]	Not done	Not done	Not done	Not done	Not done	5 (6, 6, 6, 9, 10)
10,000	5 (7, 7, 7, 7, 9)	Not done	Not done	Not done	Not done	Not done	5 (6, 6, 8, 8, 9)
1000	5 (8, 8, 8, 8, 8)	Not done	Not done	Not done	5 (8, 8, 8, 8, 10)	5 (17, 17, 17, 17, 17)	5 (8, 8, 9, 9, 9)
100	5 (9, 9, 10, 10, 10)	5 (15, 20, 20, 20, 21)	5 (8, 10, 10, 10, 12)	5 (18, 19, 19, 20, 20)	5 (10, 10, 10, 10, 10)	5 (17, 18, 18, 20, 26)	5 (9, 9, 10, 10, 10)
10	4 (11, 11, 14, 17)	3 (24, 24, 28)	5 (10, 11, 11, 11, 11)	5 (21, 21, 25, 26, 39[d])	2 (11, 12)	5 (20, 20, 21, 25, 25)	3 (12, 12, 12)
1	2 (11, 11)	1 (28)	4 (13, 16, 16, 21)	1 (25)	1 (11)	1 (25)	1 (122[e])
<1	0	0	0	0	0	0	0

[a] Five mice per group. Oocysts were inoculated orally, tachyzoites were inoculated subcutaneously.

[b] No. of mice infected with *T. gondii* of five inoculated (day of death of each mouse is in parenthesis). S = survived, infected-killed day 39.

[c] Based on estimation that the last infective dilution has 1 infective organism.

[d] Killed day 39. Many tissue cysts.

[e] Killed day 122. Many tissue cysts.

[f] Tachyzoites not titrated

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Table 5

Pathogenicity of oocysts of *T. gondii* isolates from 2 WTD in Pennsylvania to Swiss Webster mice[a].

Dose[b]	TgWTDPa5 (Cat #58, WTD #119)		TgWTDPa4 (Cat #64, WTD #118)	
	Oocysts	**Tachyzoites**	**Oocysts**	**Tachyzoites**
100,000	Not done	Not done	Not done	Not done
10,000	5 (6, 7, 7, 7, 7)[c]	Not done	5 (7, 7, 7, 7, 7)	Not done
1000	5 (11, 11, 11, 11, 11)	5 (18, 18, 18, 19, 19)	5 (8, 8, 8, 8, 9)	5 (15, 18, 20, 20, 20)
100	5 (11, 11, 11, 11, 11)	5 (18, 19, 21, 21, 21)	5 (9, 9, 9, 10, 10)	5 (19, 20, 21, 21, 25)
10	5 (11, 11, 11, 11, 12)	5 (18, 19, 19, 19, 20)	4 (10, 12, 12, 17)	5 (19, 19, 22, 54)
1	4 (11, 17, 21, 32)	2 (27, 27)	3 (10, 12, 12)	3 (19, 25, 32)
<1	0	0	0	Not done

[a] Five mice per group. Oocysts were inoculated orally and tachyzoites were inoculated subcutaneously.

[b] Based on estimation that the last infective dilution has 1 infective organism.

[c] No. of mice dead/infected with *T. gondii* of five inoculated (day of death of each mouse is in parenthesis).

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Table 6

Genotyping of *T. gondii* isolates from Wildlife in Pennsylvania.

Strain ID (Number of isolates)	Genotypes (ToxoDB PCR-RFLP genotypes)	Genetic markers (Su et al., 2010)										
		SAG1	(5′+3′) SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
GT1	Type I (#10)	I	I	I	I	I	I	I	I	I	I	I
PTG	Type II (#1)	II/III	II	II	II	II	II	II	II	II	II	II
CTG	Type III (#2)	II/III	III	III	III	III	III	III	III	III	III	III
MAS	Atypical (#17)	u-1	I	II	III	III	III	u-1	I	I	III	I
TgCgCa1	Atypical (#66)	I	II	II	III	II	II	II	u-1	I	u-2	I
TgCtBr5	Atypical (#19)	I	III	III	III	III	III	I	I	I	u-1	I
TgCtBr64	Atypical (#111)	I	I	u-1	III	III	III	u-1	I	III	III	I
TgRsCr1	Atypical (#52)	u-1	I	II	III	I	III	u-2	I	I	III	I
Present study												
TgCoPa1, 8, TgFoxPa5, TgCoPa10 (*N* = 4)	Type II (#1)	II/III	II	II	II	II	II	II	II	II	II	II
TgFoxPa3, 11 (*N* = 2)	Type III (#2)	II/III	III	III	III	III	III	III	III	III	III	III
TgCoPa5, TgFoxPa1 TgWTDPa1, 2 (*N* = 4)	Type II variant (#3)	II/III	II	II	II	II	II	II	II	II	II	I
TgCoPa6, 7, TgFoxPa10, TgWTDPa3, 6, 7 (*N* = 6)	Type 12 (#4)	II/III	II	II	II	II	II	II	II	I	II	I
TgCoPa2, 3, 4, 9 (N = 4)	Type 12 (#5)	u-1	II	II	II	II	II	II	II	I	II	I
TgFoxPa2 (*N* = 1)	Atypical (#54)	II/III	II	II	III	III	III	III	III	III	III	II
TgFoxPa9 (*N* = 1)	Atypical (#141)	II/III	III	III	III	III	III	III	III	III	I	III
TgGoosePa1 (*N* = 1)	Atypical (#143)	I	I	I	III	I	III	III	I	III	I	III
TgFoxPa4, 6, 7, 8, TgWTDPa,4, 5 (*N* = 6)	Atypical (#216)	I	I	I	III	III	I	III	III	III	I	III

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Table 7

Genotyping of *T. gondii* isolates with polymorphic loci of ROP5, ROP16, ROP18 and GRAG15.

Strain ID	ToxoDB#	ROP5	ROP18[a]	GRA15	ROP16	Virulence[b]	Accumulative mortality%	References for virulence determination in mice
FOU	6	III	I (I*)	I/III	I/III	Vir	100	Khan et al. (2007) and Khan et al. (2009)
GPHT	6	III	I	I/III	I/III	Vir	100	Khan et al. (2007)
GT1	10	I	I (I*)	I/III	I/III	Vir	100	Khan et al. (2007) and Khan et al. (2009)
TgShUs28	73	I	I	I/III	I/III	Vir	100	Dubey et al. (2008a)
MAS	17	u-1	u1 (I*)	I/III	I/III	Vir	100	Khan et al. (2007) and Khan et al. (2009)
CAST	28	I	u1 (I*)	I/III	I/III	Vir	100	Khan et al. (2007) and Khan et al. (2009)
VAND	60	u-2	u1 (I*)	I/III	I/III	Vir	100	Khan et al. (2007) and Khan et al. (2009)
RUB	98	u-2	u1 (I*)	I/III	I/III	Vir	100	Khan et al. (2007) and Khan et al. (2009)
TgBbUs1	147	u-2	u1	I/III	I/III	Vir	100	Dubey et al. (2010)
TgFoxPa9	141	III	I	I/III	I/III	Vir	100	This study
TgFoxPa8	216	III	I	I/III	I/III	Vir	100	This study
TgSwanUs3	216	III	I	I/III	I/III	Vir	100	Dubey et al. (2013a)
TgWtdPa5	216	III	I	I/III	I/III	Vir	100	This study
TgWtdPa4	216	III	I	I/III	I/III	Int	83	This study
TgFoxPa7	216	III	I	I/III	I/III	Int	91	This study
P89 (TgPgUs15)	8	III	III (III*)	I/III	I/III	Int	76	Khan et al. (2007) and Khan et al. (2009)
TgShUs55	32	III	u1	I/III	I/III	Int	80	Edwards and Dubey (2013)
ARI	5	II	II	I/III	II	Int	60	Khan et al. (2011)
Me49	1	II	II (II*)	II	II	Int	40	Khan et al. (2007) and Khan et al. (2009)
TgCgCa1	66	u-2	II (II*)	I/III	II	Int	90	Khan et al. (2007) and Khan et al. (2009)
BOF	6	nd	I (I*)	I/III	I/III	Non	8	Khan et al. (2007) and Khan et al. (2009)
CTG	2	III	III (III*)	I/III	I/III	Non	0	Khan et al. (2007) and Khan et al. (2009)
VEG	2	III	III (III*)	I/III	I/III	Non	13	Khan et al. (2007) and Khan et al. (2009)

[a] ROP18 alleles in the parenthesis are based on intron sequence data. The three distinct allele groups were designated as I*, II* and III*, respectively (Khan et al., 2009).

[b] Virulence of *T. gondii* is determined based on accumulative mortality of infected outbred mice. Mice were infected with a series of low does (10, 100 and 1000 tachyzoites) by intraperitoneal injection. Mortality of mice was determined at day 30 post infection. *T. gondii* strains cause 100% mortality in mice are considered acute virulent (Vir), 99–30% are intermediately virulent (Int), and <30% are non-virulent (Non) (Su et al., 2002).