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Sequence variation in ROP8 gene among *Toxoplasma gondii* isolates from different hosts and geographical localities

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ABSTRACT. The protozoan parasite *Toxoplasma gondii* has a worldwide distribution; it can cause serious diseases in humans and almost all other warm-blooded animals. Different genotypes of *T. gondii* result in different lesions in the same host. *T. gondii* rhoptry protein 8 (TgROP8) is a major factor of *T. gondii* acute virulence. We examined sequence variation in the TgROP8 gene among *T. gondii* isolates from different hosts and geographical localities. The TgROP8 gene was amplified from individual isolates and sequenced. A phylogenetic tree was constructed using Bayesian inference, maximum parsimony, and maximum likelihood based on the sequences obtained plus TgME49 from the ToxoDB database. The TgROP8 gene was 1728 bp in length for all the examined *T. gondii* strains, and their A+T contents were 45.37-45.95%. Sequence analysis detected 140 (0.06-

5.56%) variable nucleotide positions resulting in 96 (0-10.78%) amino acid substitutions. Sequence variations in the TgROP8 gene resulted in polymorphic restriction sites for endonucleases *Bst*BI, *Bsa*I, and *Xho*I, which allowed the differentiation of the three classical genotype strains (types I, II, and III) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). However, phylogenetic analyses indicated that the TgROP8 gene is not a suitable genetic marker for population studies of *T. gondii*.

Key words: *Toxoplasma gondii*; Toxoplasmosis; ROP8; Genotyping; Sequence diversity

INTRODUCTION

The important zoonotic and obligate intracellular protozoan parasite *Toxoplasma gondii*, a member of the phylum Apicomplexa, infects almost all warm-blooded animals, including an estimated one-third of the world's human population (Montoya and Liesenfeld, 2004; Nardoni et al., 2011; Schlüter et al., 2014). As the causative agent of toxoplasmosis, *T. gondii* can cause serious diseases in pregnant women and immunocompromised individuals, e.g., AIDS patients, tumor sufferers, and those recovering from transplant operations (Kim and Weiss, 2008; Weiss and Dubey, 2009; Silva et al., 2014). Moreover, *T. gondii* can also result in abortion or congenital toxoplasmosis, especially in livestock, leading to considerable economic losses (Fayer et al., 2004; Dubey et al., 2005; Innes, 2010; McAuley, 2014). Additionally, clonal *T. gondii* strains have an uneven geographical distribution, which leads to different toxoplasmosis in humans and animals (Sibley and Ajioka, 2008; Robert-Gangneux and Dardé, 2012).

During infection, several rhoptry proteins (ROPs) have been shown to be key virulence factors (Peixoto et al., 2010; Yuan et al., 2011; Talevich and Kannan, 2013), and play an important role in disrupting signaling and defense mechanisms, and in recruiting organelles (Morrissette and Sibley, 2002; Hunter and Sibley, 2012). *T. gondii* ROP8 (TgROP8), a ROP2-related ROP, is one of the major mediators of acute virulence, and can offer a template for homology modeling of active kinase ROP18 (Bradley and Sibley, 2007; Boothroyd and Dubremetz, 2008; Qiu et al., 2009; Parthasarathy et al., 2013). However, little is known about its sequence diversity among different *T. gondii* strains, despite its important biological impact. Therefore, the objective of this research was to examine sequence diversity in the ROP8 gene among *T. gondii* isolates from different hosts and from different geographical regions.

MATERIAL AND METHODS

T. gondii isolates

Sixteen *T. gondii* strains from different hosts and geographic locations and one strain called *T. gondii* ME49 (ToxoDB: TGME49_215775) were used in this study (Table 1). Genomic DNA was obtained as described previously (Su et al., 2010; Zhou et al., 2009, 2010) (Table 1).

Table 1. Details of *Toxoplasma gondii* isolates used in this research.

No.	Isolate	Host	Geographical location	Genotype*
1	RH	Human	France	Reference, Type I, ToxoDB#10
2	TgPLH	Pig	Henan, China	Type I, ToxoDB#10
3	GT1	Goat	USA	Reference, Type I, ToxoDB#10
4	MAS	Human	France	Reference, ToxoDB#17
5	TgCgCa1	Cougar	Canada	Reference, ToxoDB#66
6	TgCatBr64	Cat	Brazil	Reference, ToxoDB#111
7	TgCatBr5	Cat	Brazil	Reference, ToxoDB#19
8	PRU	Human	France	Type II, ToxoDB#1
9	QHO	Sheep	Qinghai, China	Type II, ToxoDB#1
10	PTG	Sheep	USA	Reference, Type II, ToxoDB#1
11	TgC7	Cat	Guangzhou, China	ToxoDB#9
12	PYS	Pig	Panyu, China	ToxoDB#9
13	GJS	Pig	Jingyuan, Gansu, China	Type #3, ToxoDB#9
14	CTG	Cat	USA	Reference, Type III, ToxoDB#2
15	TgWtdSc40	Deer	USA	Type 12, ToxoDB#5
16	TgToucan	Toucan	Costa Rica	Reference, ToxoDB#52

*based on the results of Zhou et al. (2009, 2010) and Su et al. (2010).

Polymerase chain reaction (PCR) amplification

Based on the ROP8 gene sequence of the *T. gondii* ME49 strain available in ToxoDB database (http://toxodb.org/toxo/), a pair of specific primers (forward primer, 5'-ATGTTTTCTG TGTTACGTAACCG-3'; reverse primer, 5'-TCATGCCGGTTCTCCATC-3') was designed to amplify the ROP8 gene from the individual strains. The amplification reaction was carried out in a volume of 25 μL containing 2.5 μL 10X Ex Taq Buffer (Mg^{2+} plus), 2 μL 2.5 mM each dNTP, 0.5 μL 0.2 μM of each primer, 100-200 ng gDNA, and 0.125 μL 5 U/μL Ex Taq polymerase (TaKaRa, Dalian, China). Amplification of the DNA samples was carried out in a thermocycler (BioRad, USA). The PCR regimen was: 94.0°C for 4 min (initial denaturation), followed by 35 cycles of 94.0°C for 45 s (denaturation), 62.1°C for 45 s (annealing), 72.0°C for 2 min (extension), and a final extension step at 72.0°C for 7 min. Confirmation of the PCR amplification products was carried out by electrophoresis on 1% (w/v) agarose gel containing 0.05% (v/v) GoldView™ (Solarbio, Beijing, China), which was photographed using a gel documentation system (GelDoc-It™ Imaging System, UVP, Cambridge, UK).

Sequencing of ROP8 transformants

To ensure the accuracy and integrity of the TgROP8 sequence from individual isolates, all the PCR products were purified using spin columns according to the manufacturer recommendations (Wizard™ PCR-Preps DNA Purification System, Promega, USA), and ligated with a pMD18-T vector (TaKaRa) followed by transformation into JM109 competent cells (Promega). The positive colony identified by PCR amplification was sequenced in triplicate by the Shanghai Sangon Biological Engineering Biotechnology Company on an ABI377 automated DNA sequencer (BigDye Terminator Chemistry).

Sequence analysis and phylogenetic reconstruction

The sequences obtained were compared with each other using Clustal X 2.11 (Thompson et al., 1997), and evolutionary analysis was conducted using MEGA 5.2 (Tamura et al.,

2011). The intra-specific sequence variation was shown by the percent of the different sequence bases. Phylogenetic reconstructions based on the TgROP8 gene sequences among the different *T. gondii* isolates including TgME49 were performed using Bayesian inference (BI), maximum parsimony (MP), and maximum likelihood (ML). BI analysis was performed with four independent Markov chains run for 100,000 metropolis-coupled MCMC generations, sampling a tree every 1000 generations in MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003); MP and ML analyses were conducted using PAUP 4.0b10 with indels treated as missing character states (Swofford, 2002). Bootstrap probability was calculated from 1000 bootstrap replicates with 10 random additions per replicate in PAUP. A total of 100 random addition searches were performed for each analysis using tree bisection-reconnection branch swapping (Chen et al., 2012).

Characterization of *T. gondii* isolates by PCR-restriction fragment length polymorphism (PCR-RFLP)

The *T. gondii* isolates have been characterized by PCR-RFLP, and three dominant genotypes (types I, II, and III) were identified (Su et al., 2006, 2010). To determine whether the TgROP8 gene sequence was suitable for genotyping *T. gondii* isolates, the PCR-RFLP method was used in this study. Briefly, all the TgROP8 PCR products were digested using the three restriction enzymes *Bst*BI, *Bsa*I, and *Xho*I by incubating at 37°C for 2 h followed by 65°C for 4 h, according to the manufacturer's instructions (New England Biolabs, Beijing, China). The restriction fragments were separated on 1% agarose gel containing 0.05% (v/v) GoldenView™ and photographed using a gel documentation system (GelDoc-It™ Imaging System, UVP).

RESULTS AND DISCUSSION

The transformants of TgROP8 PCR products of all the *T. gondii* isolates examined produced a single band of approximately 1700 bp in length on agarose gel (not shown). All the sequences of the TgROP8 gene were 1728 bp in length, and their A+T contents varied from 45.37 to 45.95%. The alignment of all the 16 sequences plus the corresponding sequence of TgME49 (ToxoDB: TGME49_215775) revealed nucleotide polymorphisms at 140 positions (0.06-5.56%), higher than in the GRA5, GRA6, and ROP38 genes (Fazaeli et al., 2000; Chen et al., 2012; Xu et al., 2014). Interestingly, the mutation rate in the TgROP8 gene sequence of TgWtdSc40 was the highest with 5.56% at 96 positions, followed by that of TgToucan with 3.41% at 59 positions (Figure 1A). Moreover, there were 18 transitions (C↔T and A↔G) and 20 transversions (A↔C, A↔T, G↔T, and G↔C) (R = transition/transversion = 0.9) among all the sequences obtained. In addition, estimates of evolutionary divergence revealed that the distance was 0-6.8%, and 96 positions (0-10.78%) were variable in amino acid sequences (Figure 1B), suggesting that TgROP8 may not be a vaccine candidate against toxoplasmosis.

Analysis of nucleotide polymorphisms in the TgROP8 gene among all the sequences obtained revealed the presence of polymorphic restriction sites for endonucleases *Bst*BI, *Bsa*I, and *Xho*I, which can differentiate the three classical genotypes (type I: RH, TgPLH, and GT1; type II: PRU, QHO, and PTG; type III: CTG) (Su et al., 2006, 2010). The results of PCR-RFLP analyses showed that strains representing the three classical genotypes (types I, II, and

III) could be separated readily using restriction enzymes (*Bst*BI, *Bsa*I, and *Xho*I) (Figure 2). Consistent with type-I strains RH, TgPLH, and GT1, the PCR products of strains MAS, TgCatBr64, TgCatBr5, and TgCgCa1 were fragmented into three segments (136, 352, and 1240 bp). ToxoDB#9 strains (TgC7, PYS, and GJS) were fragmented into four segments (175, 177, 535, and 841 bp), which were the same as those in type-II strains. Type-III strain CTG was separated into two fragments of 488 and 1240 bp. The type-12 strain TgWtdSc40 was digested into five fragments of 136, 175, 177, 399, and 841 bp, while the unique reference strain TgToucan was digested into four fragments of 9, 127, 352, and 1240 bp, which were different from all the other isolates (Figure 2).

Phylogenetic analyses showed that strains representing the three classical genotypes could not be grouped consistently into their respective genotypes (Figure 3), which is different from that using GRA5 gene sequences (Chen et al., 2012).

In conclusion, the present study revealed slightly higher sequence variation in the TgROP8 gene among strains from different hosts and geographical localities, but the TgROP8 gene sequence may not a suitable marker for studying the population genetics of *T. gondii*.

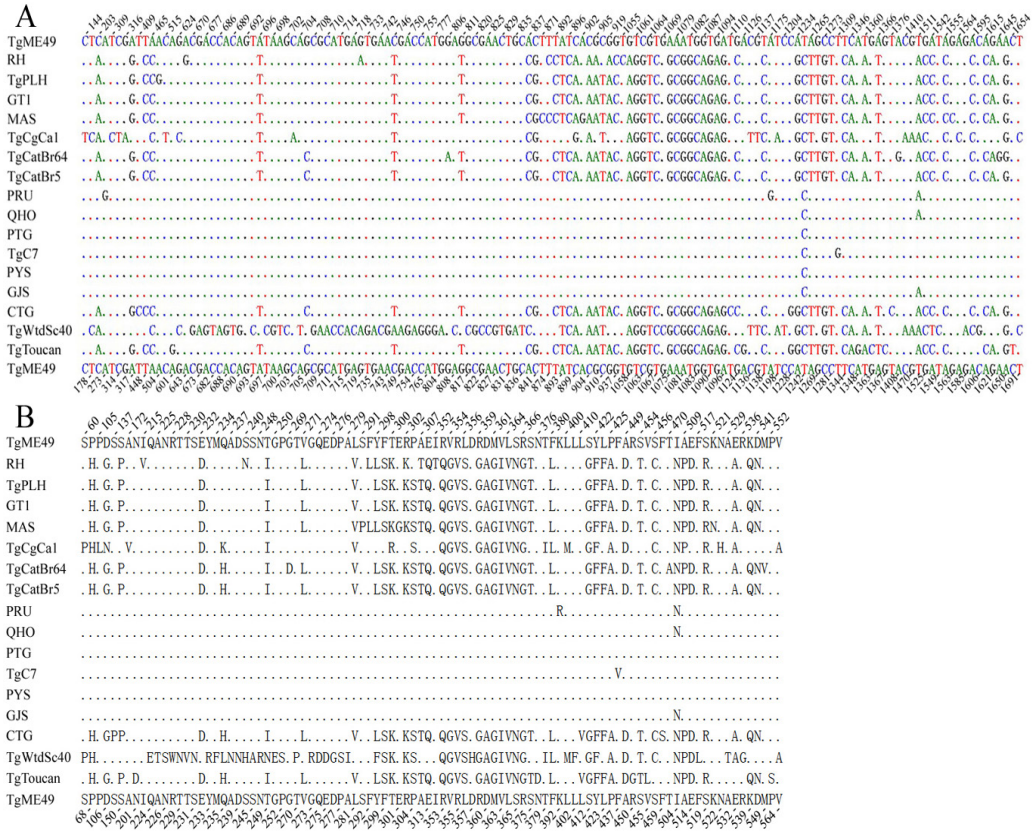

Figure 1. Multiple alignment analyses of nucleotides (**A**) or amino acid sequences (**B**) of *Toxoplasma gondii* ROP8. Dots indicate identical nucleotides or amino acids compared with theat of TgME49 isolate (ToxoDB: TGME49_215775) (top and bottom lines), and the numbers indicate the variable sequence positions for nucleotides (A) or amino acids (B).

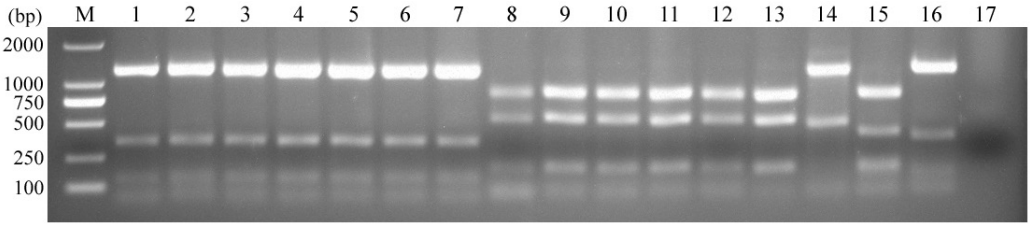

Figure 2. Agarose gel electrophoresis of *Toxoplasma gondii* ROP8 PCR products after digestion with endonucleases *Bst*BI, *Bsa*I, and *Xho*I. *Lane M* = DNA size marker 2000. *Lanes 1-17 = T. gondii* type-I strains RH, TgPLH, and GT1; reference strains MAS, TgCgCa1, TgCatBr64, and TgCatBr5; type-II strains PRU, QHO, and PTG; strains TgC7, PYS, and GJS; type-III strain CTG; type-12 strain TgWtdSc40; strain TgToucan; and the blank control, respectively.

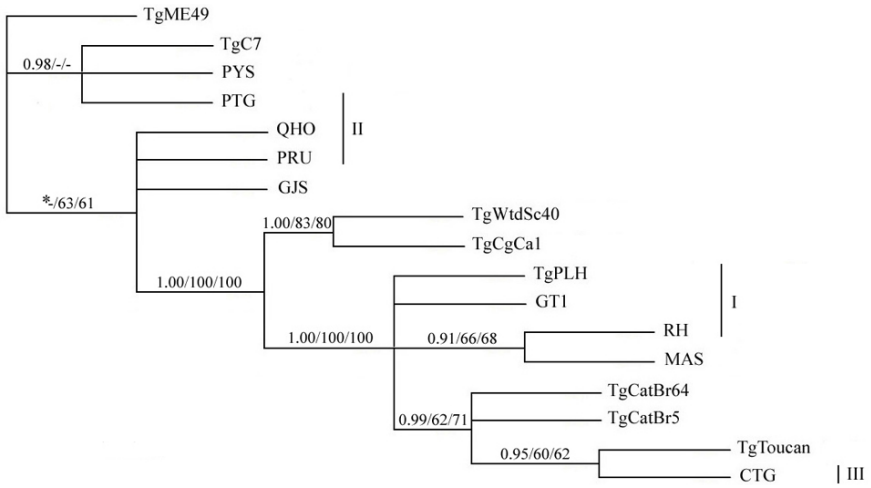

Figure 3. Phylogram of 17 *Toxoplasma gondii* strains based on ROP8 gene sequences using Bayesian inference (BI), maximum parsimony (MP), and maximum likelihood (ML) methods. Numbers near the branches stand for bootstrap values from different analyses in the order: BI, MP, and ML. Clusters of three classical genotypes are denoted by I, II, and III, and the asterisk indicates no data.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Boothroyd JC and Dubremetz JF (2008). Kiss and spit: the dual roles of *Toxoplasma* rhoptries. *Nat. Rev. Microbiol.* 6: 79-88.

Bradley PJ and Sibley LD (2007). Rhoptries: an arsenal of secreted virulence factors. *Curr. Opin. Microbiol.* 10: 582-587.
Chen J, Li ZY, Zhou DH, Liu GH, et al. (2012). Genetic diversity among *Toxoplasma gondii* strains from different hosts and geographical regions revealed by sequence analysis of GRA5 gene. *Parasit. Vectors* 5: 279.
Dubey JP, Hill DE, Jones JL, Hightower AW, et al. (2005). 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.
Fayer R, Dubey JP and Lindsay DS (2004). Zoonotic protozoa: from land to sea. *Trends Parasitol.* 20: 531-536.
Fazaeli A, Carter PE, Darde ML, Pennington TH (2000). Molecular typing of *Toxoplasma gondii* strains by GRA6 gene sequence analysis. *Int. J. Parasitol.* 30: 637-642.
Hunter CA and Sibley LD (2012). Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nat. Rev. Microbiol.* 10: 766-778.
Innes EA (2010). Vaccination against *Toxoplasma gondii*: an increasing priority for collaborative research? *Expert Rev. Vaccines* 9: 1117-1119.
Kim K and Weiss LM (2008). *Toxoplasma*: the next 100years. *Microbes Infect.* 10: 978-984.
McAuley JB (2014). Congenital toxoplasmosis. *J. Pediatric. Infect. Dis. Soc.* 3: S30-S35.
Montoya JG and Liesenfeld O (2004). Toxoplasmosis. *Lancet* 363: 1965-1976.
Morrissette NS and Sibley LD (2002). Cytoskeleton of apicomplexan parasites. *Microbiol. Mol. Biol. Rev.* 66: 21-38.
Nardoni S, Angelici MC, Mugnaini L and Mancianti F (2011). Prevalence of *Toxoplasma gondii* infection in *Myocastor coypus* in a protected Italian wetland. *Parasit. Vectors* 4: 240.
Parthasarathy S, Fong MY, Ramaswamy K and Lau YL (2013). Protective immune response in BALB/c mice induced by DNA vaccine of the ROP8 gene of *Toxoplasma gondii*. *Am. J. Trop. Med. Hyg.* 88: 883-887.
Peixoto L, Chen F, Harb OS, Davis PH, et al. (2010). Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. *Cell Host Microbe* 8: 208-218.
Qiu W, Wernimont A, Tang K, Taylor S, et al. (2009). Novel structural and regulatory features of rhoptry secretory kinases in *Toxoplasma gondii*. *EMBO J.* 28: 969-979.
Robert-Gangneux F and Dardé ML (2012). Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin. Microbiol. Rev.* 25: 264-296.
Ronquist F and Huelsenbeck JP (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.
Schlüter D, Däubener W, Schares G, Groß U, et al. (2014). Animals are key to human toxoplasmosis. *Int. J. Med. Microbiol.* 304: 917-929.
Sibley LD and Ajioka JW (2008). Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. *Annu. Rev. Microbiol.* 62: 329-351.
Silva MG, Câmara JT, Vinaud MC and Castro AM (2014). Epidemiological factors associated with seropositivity for toxoplasmosis in pregnant women from Gurupi, State of Tocantins, Brazil. *Rev. Soc. Bras. Med. Trop.* 47: 469-475.
Su C, Zhang X and Dubey JP (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.
Su C, Shwab EK, Zhou P, Zhu XQ, et al. (2010). Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitology* 137: 1-11.
Swofford DL (2002). PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland.
Talevich E and Kannan N (2013). Structural and evolutionary adaptation of rhoptry kinases and pseudokinases, a family of coccidian virulence factors. *BMC Evol. Biol.* 13: 117.
Tamura K, Peterson D, Peterson N, Stecher G, et al. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731-2739.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, et al. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876-4882.
Weiss LM and Dubey JP (2009). Toxoplasmosis: A history of clinical observations. *Int. J. Parasitol.* 39: 895-901.
Xu Y, Zhang NZ, Chen J, Liu GH, et al. (2014). *Toxoplasma gondii* rhoptry protein 38 gene: sequence variation among isolates from different hosts and geographical locations. *Genet. Mol. Res.* 13: 4839-4844.
Yuan ZG, Zhang XX, Lin RQ, Petersen E, et al. (2011). Protective effect against toxoplasmosis in mice induced by DNA immunization with gene encoding *Toxoplasma gondii* ROP18. *Vaccine* 29: 6614-6619.
Zhou P, Zhang H, Lin RQ, Zhang DL, et al. (2009). Genetic characterization of *Toxoplasma gondii* isolates from China. *Parasitol. Int.* 58: 193-195.
Zhou P, Nie H, Zhang LX, Wang HY, et al. (2010). Genetic characterization of *Toxoplasma gondii* isolates from pigs in China. *J. Parasitol.* 96: 1027-1029.