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Sequence variation in the *Toxoplasma gondii ROP20* gene among strains from different hosts and geographical locations

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ABSTRACT. *Toxoplasma gondii*, an opportunistic protozoan parasite, infects almost all warm-blooded animals. In this study, we examined the sequence variation in rhoptry protein 20 (*ROP20*) genes among 18 *T. gondii* isolates collected from different hosts and geographical regions. Full length *ROP20* genes were amplified and sequenced. The results showed that the genes were 1659 bp in length and contained only a single exon, and that the A+T content varied from 46.68 to 47.20%

among the 18 strains. The results of sequence alignment indicated that there were 30 variable nucleotide positions (0-1.40%) in the 18 *T. gondii* strains containing 18 transitions and 11 transversions, representing 1.81% overall sequence variation. Phylogenetic analysis of the *ROP20* sequences showed that *ROP20* variation could differentiate between the clonal lineage genotypes I and ToxoDB #9, indicating that *ROP20* exhibits a relatively marked degree of sequence diversity and might represent a novel genetic marker for intraspecies phylogenetic analyses of *T. gondii*.

Key words: *Toxoplasma gondii*; Toxoplasmosis; Sequence variation; Rhoptry protein 20; Phylogenetic analysis

INTRODUCTION

Toxoplasma gondii is an important zoonotic protozoan with worldwide distribution, and can infect virtually all warm-blooded animals including humans (Dubey, 2010; Huang et al., 2012; Tian et al., 2012). *T. gondii* has been considered to be one of the most successful parasites because nearly one third of the world population has been exposed to this protozoan (Tenter et al., 2000; Hill and Dubey, 2002; Montoya and Liesenfeld, 2004; Zhou et al., 2011). Although infections in humans are asymptomatic, *T. gondii* can cause severe disease in pregnant women or in immunocompromised individuals (Fuentes et al., 2001; Djurkovic-Djakovic, 2002; Petersen, 2007; Dubey, 2010; Robert-Gangneux and Dardé, 2012). In animals, this parasite is responsible for considerable economic losses in the livestock industry because of abortion or congenital toxoplasmosis in all types of livestock, and especially in sheep and goats (Fayer et al., 2004; Dubey et al., 2005). The complicated biological and epidemiological features have led to *T. gondii* genetic diversity. Accordingly, the majority of *T. gondii* isolates in North America and Europe have been clustered into four major clonal lineage types (I, II, III, and 12) utilizing 11 genetic markers (Su et al., 2006; Khan et al., 2011).

The rhoptry is a unique secretory organelle that is located in all Apicomplexan parasites and plays an important role in parasite invasion; it is also a master regulator that manipulates the host inflammatory responses (Dubremetz, 2007; Peixoto et al., 2010; Melo et al., 2011; Reese and Boyle, 2012; Kemp et al., 2013). *T. gondii* ROP20, a novel rhoptry protein, is localized on rhoptries and in the parasitophorous vacuoles, and might participate in the manipulation of the host signaling pathways; however, its physiological function is still unknown **(**Peixoto et al., 2010**)**. Our previous studies have demonstrated the existence of sequence variation in certain rhoptry proteins such as ROP7, ROP9, ROP13, and ROP38 (Chen et al., 2012a; Wang et al., 2012; Zhou et al., 2012; Xu et al., 2014). However, no information about the sequence variation in the *ROP20* gene across different *T. gondii* isolates is available. Therefore, the aim of this study was to examine the sequence variation in the *ROP20* gene among *T. gondii* strains from different hosts and geographical regions.

MATERIAL AND METHODS

T. gondii isolates

In the present study, 18 *T. gondii* strains previously collected from different hosts and

geographic locations were used for analysis (Table 1). These *T. gondii* isolates were genotyped and genomic DNA was prepared as described previously (Su et al., 2010; Zhou et al., 2009, 2010).

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

Strain	Host	Geographical origin	Genotype
TgCatBr5	Cat	Brazil	Reference, ToxoDB #19
TgCatBr64	Cat	Brazil	Reference, ToxoDB #111
MAS	Human	France	Reference, ToxoDB #17
TgCgCa1	Cougar	Canada	Reference, ToxoDB #66
TgWtdSc40	White-tailed Deer	United States	Type 12, ToxoDB #5
TgToucan (TgRsCr1)	Toucan	Costa Rica	Reference, ToxoDB #52
TgC7	Cat	Guangzhou,Guangdong, China	ToxoDB #9
PYS	Pig	Panyu, Guangdong, China	ToxoDB #9
Prugniaud (PRU)	Human	France	Type II, ToxoDB #1
QHO	Sheep	China	Type II, ToxoDB number 1
PTG	Sheep	United States	Reference, Type II, ToxoDB #1
CTG	Cat	United States	Reference, Type III, ToxoDB #2
GT1	Goat	United States	Reference, Type I, ToxoDB #10
RH	Human	France	Reference, Type I, ToxoDB #10
SH	Human	Shanghai, China	Type I, ToxoDB #10
TgCatBr9	Cat	Brazil	ToxoDB #42
VEG	Human	United States	Reference, ToxoDB #2
ME49	Sheep	United States	Type II, ToxoDB #1

Amplification and sequencing of the *ROP20* genes

The primers ROP20-F (5'-CAG CAA GGA AAC CAT AAC CAC-3') and ROP20-R (5'-CGA ATA AAA TAA ATC TCA AAC-3') were designed to amplify the *ROP20* gene based on *T. gondii* ME49 strain sequence available in the ToxoDB database (TGME49_258230). The amplification reaction was carried out in a 25μL total volume, including 12.5 μL Premix Ex Taq (TaKaRa Bio; Otsu, Shiga, Japan), 0.2 μM of each primer, and 100-200 ng template DNA. Amplification of DNA samples from individual strains was carried out in a thermocycler (Bio-Rad; Hercules, CA, USA) under the following conditions: denaturation at 94°C for 5 min (initial denaturation), followed by 35 cycles of 94°C for 40 s (denaturation), 57°C for 40 s (annealing), 72°C for 2 min 40 s (extension), and a final extension step at 72°C for 10 min. Successful polymerase chain reaction (PCR) amplifications were confirmed by electrophoresis on a 1% (w/v) agarose gel subsequently stained with GoldenView™; the DL 5000 marker (TaKaRa) was used to estimate the sizes of the *ROP20* PCR products. All PCR products were purified using a DNA purification kit (GenStar Biosolutions, Beijing, China) according to the manufacturer recommendations, and then ligated to the pMD18-T vector (TaKaRa). The recombinant vectors were transformed into JM109 competent cells (Promega, Madison, WI, USA). Positive recombinant vectors were identified directly by bacterial PCR amplification using the primers described previously, and sent to GenScript Co., Ltd. (Nanjing, China) for sequencing.

Sequence analysis and phylogenetic reconstruction

The *ROP20* gene sequences of the VEG, ME49, and TgCatBr9 strains were obtained from the ToxoDB database (http://toxodb.org). All *ROP20* gene sequences were aligned using the computer program Clustal X 1.81 (Thompson et al., 1997) and then the sequence variation

among the examined 18 *T. gondii* strains was determined. Phylogenetic reconstructions based on the complete *ROP20* gene sequences of different *T. gondii* strains were performed using maximum parsimony (MP) and neighbor joining (NJ) with the outgroup consisting of the corresponding sequence from *Neospora caninum* (GenBank accession No. FR823391.1). MP and NJ analyses were performed using PAUP* 4.0 with indels treated as missing character states. A total of 1000 random addition searches using tree bisection-reconnection branch swapping were performed for each MP and NJ analysis. The bootstrap probability was calculated from 1000 bootstrap replicates with 10 random additions per replicate in PAUP (Wang et al., 2015).

RESULTS and DISCUSSION

The full length *ROP20* gene sequence was found to be 1659 bp and to contain only a single exon for all of the examined *T. gondii* strains, and the A+T content varied from 46.68 to 47.20%. Sequence variation in the *ROP20* genes among the 18 examined *T. gondii* isolates ranged from 0 to 1.4%, which is higher than the values reported for the *ROP38*, *eIF4A*, and *ROP9* genes in our previous studies (Chen et al., 2012a, 2014; Xu et al., 2014). Alignment of all 18 sequences revealed 30 variable positions, representing 1.81% overall sequence variation, which indicated a relatively considerable degree of sequence diversity, but a lower level of genetic variation than that reported for the coding regions of the *GRA5* and *GRA6* genes (Fazaeli et al., 2000; Chen et al., 2012b). Of these variable positions, 18 were transitions (A↔G and C↔T) and 11 were transversions (A↔C, A↔T, G↔C, and G↔T) in the coding region of the *ROP20* gene (R = transition/transversion = 1.6). These results were similar to those found in previous studies for *Hsp60* and *ROP17* (Lu et al., 2014; Zhang et al., 2014).

Phylogenetic analysis using NJ and MP based on the *ROP20* coding sequences showed that the *T. gondii* strains representing genotype I were grouped into a separate cluster and that the ToxoDB #9 strains could be readily distinguished from other genotypes (Figure 1). Based

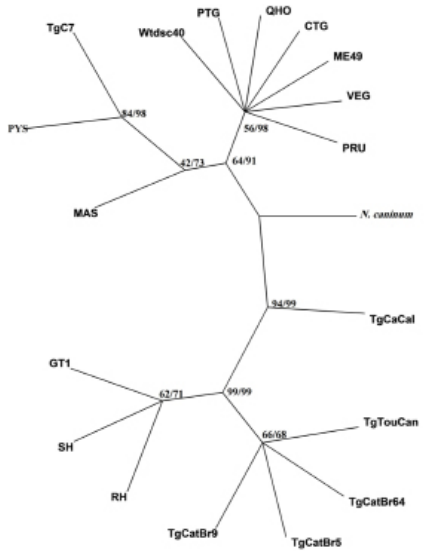

Figure 1. Phylogram of 15 *Toxoplasma gondii* strains determined by analysis of the *ROP20* gene. The phylogenetic relationships were inferred by maximum parsimony (MP) and neighbor joining (NJ) using *Neospora caninum* as the outgroup.

on the 18 *T. gondii* strains examined in the present study, the respective *ROP20* gene sequences could distinguish the genotype I and ToxoDB #9 strains illustrating the differentiation between the *T. gondii* genotypes. However, as compared to the *GRA5*, *GRA6*, and *Hsp60* genes, *ROP20* is not as good as them, because they could distinguish the genotype I and other genotypes (Fazaeli et al., 2000; Chen et al., 2012b; Lu et al., 2014).

In conclusion, the present study determined the full-length coding sequences of the *T. gondii ROP20* gene and revealed relatively considerable sequence variability within this locus among *T. gondii* isolates from different hosts and geographical regions. Phylogenetic analysis showed that the *ROP20* gene could be used to distinguish between the genotype I and ToxoDB #9 strains, indicating that *ROP20* might serve as a novel genetic marker for population genetic studies of *T. gondii* isolates.

Conflicts of interest

The authors declare no conflict of interest.

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