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Sequence Variation in *ROP9* Gene among *Toxoplasma gondii* Strains from Different Hosts and Geographical Locations

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Abstract: *Toxoplasma gondii* infects a wide range of animals and humans causing toxoplasmosis. *T. gondii* rhoptry protein 9 (TgROP9) is expressed only in tachyzoite stage that might be involved in the early stages of invasion and this soluble protein is constituted with putative B-cell epitope but also can trigger a T cell response and exclusively react with CD4 + T cell clone. In this study, researchers examined sequence variation in *ROP9* gene among seven *T. gondii* strains from different hosts and geographical localities by PCR amplification, sequence analysis and phylogenetic reconstruction using Maximum Parsimony (MP). The results showed that the entire genome sequence of the *TgROP9* gene was 4555 bp in length and no size variation was detected among all the strains. In addition, there were 26 (0-0.7%) variable nucleotide positions among all isolates with 9 variations of these being in the coding region and the other 11 were distributed among the 4 introns. Phylogenetic analyses revealed that the TgROP9 sequences were not a suitable genetic marker to differentiate *T. gondii* strains of different genotypes from different hosts and geographical locations. This study determined the TgROP9 genomic sequences of seven *T. gondii* strains and demonstrated the existence of low sequence variation in *TgROP9* gene, suggesting that *T. gondii* ROP9 may represent a good vaccine candidate against toxoplamosis.

Key words: *Toxoplasma gondii*, toxoplasmosis, Rhoptry Protein 9 (ROP9), sequence variation, phylogenetic analysis

INTRODUCTION

Toxoplasma gondii is a zoonotic pathogen that can invade diverse vertebrate species including humans with a worldwide distribution (Montoya and Liesenfeld, 2004; Weiss and Dubey, 2009; Dubey, 2010; Zhou *et al*., 2011; Chen *et al*., 2012a; Robert-Gangneux and Darde, 2012). In humans, *T. gondii* infection can cause severe disease in pregnant women and immunocompromised individuals although, the vast majority of infections in adults are asymptomatic (Tenter *et al*., 2000; Montoya and Liesenfeld, 2004; Weiss and Dubey, 2009; Dubey, 2010).

Infection in livestock is of great economic importance as well as a source of transmission to humans due to food-borne outbreaks (e.g., tissue cysts of *T. gondii* in the meat of infected animals) (Fayer *et al*., 2004; Dubey *et al*., 2005; Dubey, 2010).

T. gondii ROP9 (TgROP9) is a soluble rhoptry protein that is expressed only in tachyzoite stage and might be involved in the early stages of invasion (Robert-Gangneux and Darde, 2012). Significantly, this soluble protein was constituted with putative B-cell epitope and can also trigger a T cell response and exclusively react with CD4 + T cell clone

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(Robert-Gangneux and Darde, 2012). However, little is known of the sequence variation in *TgROP9* gene among different *T. gondii* strains despite its important biological functions. Therefore, the objective of this study was to examine sequence diversity in *TgROP9* gene among *T. gondii* strains from different hosts and geographical regions.

MATERIALS AND METHODS

***T. gondii* isolates:** A total of seven *T. gondii* isolates collected from different hosts and geographic locations were used for analysis in this study (Table 1). Earlier studies have genotyped these *T. gondii* strains and genomic DNA were prepared as described previously (Zhou *et al*., 2009, 2010; Su *et al*., 2010).

PCR amplification: To obtain amplicons of the genomic sequence of the *TgROP9* gene, the entire sequence of the *TgROP9* gene was amplified in two overlapping fragments with two pairs of oligonucleotide primers designed based on the *ROP9* gene sequence available in ToxoDB (accession No. TGME49_243730). Primers CJ-ROP9-1A (forward, 5'-GGCAGAAACATCTAAGCACGA-3') and CJ-ROP9-1B (reverse, 5'-AGACGAGCAAGCAACGGTGG-3') were used for amplification of the first fragment and primer CJ-ROP9-2A (forward, 5'-AGGCGAAATTCC TAGACCCT-3') and CJ-ROP9-2B (reverse, 5'-CGCG ATTCCAAAAATCTCCA-3') were used for amplification of the second fragment and 80 bp overlap between 5' termination of the first fragment and 3' termination of the second fragment of genomic sequence of the *TgROP9* gene were designed for sequence splice. The amplification reaction was carried out in a volume of 25 μL containing 10 mM Tris-HCl (pH 8.4), 50 mM Kcl, 3 mM $MgCl_2$, 250 μM each of dNTP, 0.2 μM of each primer, 100-200 ng of template DNA and 0.25 U La Taq polymerase (TaKaRa). Amplification of DNA samples from individual isolates was carried out in a thermorcycle (Biometra) under the following conditions: denaturation at 94°C for 10 min (initial denaturation), followed by 35 cycles consisting of 94°C for 30 sec (denaturation), 57.5°C for 30 sec (annealing for the first fragment) or 56°C for 30 sec (annealing for the second fragment), 72°C for 2 min (extension) and a final extension step was at 72°C for 10 min. Confirmation of the PCR amplifications was examined by electrophoresis on a 1% (w/v) agarose gel, stained with GoldenView™ and photographed using a Gel Documentation System (UVP GelDoc-It™ Imaging System, Cambridge, UK).

Table 1: Details of *Toxoplasma gondii* strains used in the present study

Strains	Host	Geographical origin	Genotype*
GT1	Goat	United States	Reference, Type I, ToxoDB #10
RH	Human	France	Reference, Type I, ToxoDB #10
PTG	Sheep	United States	Reference, Type II, ToxoDB #1
CTG	Cat	United States	Reference, Type III, ToxoDB #2
TgC7	Cat	Guangzhou, Guangdong, China	ToxoDB #9
Prugniaud (PRU)	Human	France	Type II, ToxoDB #1
QHO	Sheep	Huzhu, Qinghai, China	Type II, ToxoDB #1

*Based on genotyping results of Zhou *et al*. (2009, 2010) and Su *et al*. (2010)

Sequencing of the TgROP9 amplicons: The TgROP9 PCR products were purified using the spin columns according to the manufacturer's recommendations (Wizard™ PCR-Preps DNA Purification System, Promega, USA), ligated with pMD 18-T vector (TaKaRa) and then transformed into the JM109 competent cells (Promega, USA). Following the screening by PCR amplification and enzymatic digestion, the positive colonies were sequenced by Shanghai Songon Biological Engineering Biotechnology Company with ABI 377 automated DNA sequencer (BigDye Terminator Chemistry).

Sequence analysis and phylogenetic reconstruction: The obtained *TgROP9* gene sequences from different *T. gondii* strains were aligned using Multiple Sequence Alignment Program, Clustal X1.83 (Thompson *et al*., 1997) and sequence variation was determined among the examined *T. gondii* strains. Phylogenetic re-constructions based on the complete TgROP9 sequences among different *T. gondii* strains were performed using Maximum Parsimony (MP) (Swofford, 2002) as described earlier (Liu *et al*., 2012).

RESULTS AND DISCUSSION

The obtained amplicons of *TgROP9* gene was approximately 4500 bp in length on agarose gel for all seven *T. gondii* strains. Following sequencing of the positive colonies from all isolates, all the seven TgROP9 genomic sequences were analyzed using DNASTAR Software. The entire genomic sequence of *TgROP9* gene was 4555 bp in length and no size variation was detected among all the seven strains, four introns were distributed in the *TgROP9* gene (Table 2). Moreover, after the alignment of all seven sequences, a total of 26 nucleotide positions of the TgROP9 sequences were variable among the seven examined *T. gondii* isolates and the sequence

Table 2: Characteristics of *T. gondii* ROP9 (*TgROP9*) gene sequences including coding regions and introns among different strains

			TgROP9 introns			
Items	TgROP9 DNA	TgROP9 cDNA	First	Second	Third	Forth
Length (bp)	4555	1062	264	256	636	327
T+A (%)	49.73-50.33	44.26-45.23	56.44-57.20	51.17-51.56	51.73-52.02	56.27-56.78
Transition	21	7	2	1	4	1
Transversion	5	2	0	0	2	1
R	4	3.5	-	-	2.0	1.0
Distance (%)	0-0.7	0-0.7	0-0.7	0-0.7	0-0.7	0-0.7

R = Transition/Transversion

variations was 0-0.7%. Nine of nucleotide variations were in the coding region of the gene and the other 11 were distributed among the 4 introns. Unlike the polymorphisms in the GRA6 (Fazaeli *et al*., 2000) and *GRA5* gene (Chen *et al*., 2012a, b), the *TgROP9* gene has shown a low genetic diversity which were consistent with the earlier reports including PLP1 (Yan *et al*., 2011), MIC13 (Ren *et al*., 2011) and other genes among the clonal lineages of *T. gondii* (Khan *et al*., 2009).

In order to analyze the phylogenetic relationship among these seven *T. gondii* isolates based on the *TgROP9* gene sequence, phylogram using MP analysis was reconstructed and it indicated that *TgROP9* gene sequences failed to differentiate virulent and avirulent strains or different genotypes of *T. gondii*. Therefore, the TgROP9 sequences were not an ideal genetic marker for intra-species phylogenetic analysis and the differentiation of the examined *T. gondii* strains into their respective genotypes, although the examined number of *T. gondii* strains was small.

CONCLUSION

The present study determined and compared the TgROP9 genomic sequences of seven *T. gondii* strains and demonstrated that sequence variability in the *ROP9* gene among *T. gondii* isolates from different hosts and geographical regions is low. Phylogenetic reconstruction suggested that the TgROP9 sequence was not a suitable marker for studying genetic variation and population genetic studies of *T. gondii* isolates.

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