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Sequence Variation in *Perforin-Like Protein 1* Gene among Six *Toxoplasma gondii* Strains

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Abstract: Perforin-like Protein 1 (PLP1) plays an important role in the invasion process of *T. gondii*. In this study, we examined sequence variation in the *PLP1* gene among six *T. gondii* strains from Guangzhou and Panyu in Guangdong, Suhe in Henan, Huzhu in Qinghai provinces of China, France and the USA, representing different genotypes. The *PLP1* gene was amplified from the >6 strains by Polymerase Chain Reaction (PCR) and the amplicons were cloned and sequenced. The length of all of the PLP1 sequences was 3453 bp, consistent with that available in GenBank (EF 102772.2). In total, there were 42 (1.22%) variable nucleotide positions among the six *PLP1* gene sequences and the 22 of which represented transversions. The A+T contents of the sequences was 48.3~48.9%. Intra-specific nucleotide variation was related mainly to changes at the 2nd and 3rd codon positions while fewer changes were detected at the 1st codon position. These results demonstrated that sequence variation in *PLP1* gene among the six *T. gondii* strains was low and the *PLP1* gene may not be an appropriate marker for the studies of genetic variation among *T. gondii* strains.

Key words: *Toxoplasma gondii*, toxoplasmosis, Perforin-like Protein 1 (*PLP1*) gene, genetic variation, sequence, China

INTRODUCTION

Toxoplasmosis is a parasitic disease caused by the intracellular protozoan parasite *Toxoplasma gondii*. It is one of the most epidemic parasitic diseases in human beings and warm-blooded animals. It is an opportunistic disease which is often lethal for patients with AIDS organ transplant recipients and those with neoplastic diseases such as birth defects, retinitis and brain damage if acutely infected via vertical transmission (Montoya and Liesenfeld, 2004). Furthermore, toxoplasmosis can result in serious economic losses to livestock industries (Buxton, 1998; Dubey *et al*., 2005; Zou *et al*., 2009).

Perforin (PF) and members of the Membrane Attack Complex (MAC) (complement proteins C6-C9) are pore-forming proteins of the innate and adaptive immune response that constitute the founding members of the MACPF domain family (Voskoboinik *et al*., 2006). Perforin-like Proteins (PLPs) are expressed by many bacterial and protozoan pathogens including *T. gondii* (Kaiser *et al*., 2004; Rosado *et al*., 2008). *T. gondii* Perforin-like Protein 1 (TgPLP1) deficient parasites failed to exit normally after intracellular growth, resulting in entrapment within host cells (Kafsack *et al*., 2009). Perforin-dependent killing of infected dendritic cells led to active egress of infectious parasites that rapidly infected adjacent effector NK cells (Persson *et al*., 2009).

Upon acting on infected cells via perforin-dependent pathways, T cells induce rapid egress of infectious parasites able to infect surrounding cells (Persson *et al*., 2007). All these data shows that PLPs play important roles in toxoplasmosis. However, sequence diversity in *TgPLP1* gene is still unknown. Therefore, the objective of the present study was to examine sequence variation in *TgPLP1* gene among six different *T. gondii* strains from different hosts and geographical locations which represents different *T. gondii* genotypes.

MATERIALS AND METHODS

Parasites, RNA extraction and RT-PCR: Tachyzoites of six *T. gondii* strains, namely RH, TgC7, PYS, TgPLh,

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Prugniaud (PRU) and QHO representing different genotypes (Zhou *et al*., 2009, 2010) were conserved in liquid nitrogen in the Laboratory of Parasitology, College of Veterinary Medicine, South China Agricultural University (Table 1). Tachyzoites were dissolved in 38°C water-bath and were used to infect Kunming mice intraperitoneally. Tachyzoites were harvested 72 h after infection from the peritoneal fluid of mice and stored at -80°C. Total RNA was isolated from the tachyzoites, column-purified (MagaZorb® Total RNA Mini-Prep kit, QIAGEN) and then eluted into 20 μL RNase-free water according to the manufacturer's recommendations. RNA samples were stored at -70°C until use.

RT-PCR reactions (20 μL) were performed in 12 μL thermal denatured RNA (65°C for 5 min with 9 μL RNase-free H_2O, 1 μL random primer, 2 μL total RNA), 4 μL 5×RT buffer, 2 μL dNTP mixture (10 mM), 1 μL RNase inhibitor (10 U μL^{-1}) and 1 μL ReverTra Ace in a thermocycler (biometra) under the following conditions after an incubation at 30°C for 10 min then 42°C for 50 min; 99°C for 5 min and finally at 4°C for 5 min.

Enzymatic amplification: The *PLP1* gene was amplified by Polymerase Chain Reaction (PCR) using a pair of primers (forward primer: 5'-ATGAGGTCACTCACAC ATGG (A/T/C)-3' and reverse primer: 5'-TTACAGGTCT AACAG CTTGACG(A/T/C)-3'). PCR reactions (25 μL) were performed in 3 mM of $MgCl_2$, 0.5 μM of each primer, 2.5 μL LA-Buffer, 0.2 mM of each dNTPs, 1.25 U of LA Taq polymerase (TaKaRa) and 2 μL of cDNA sample in a thermocycler (biometra) under the following conditions after an initial denaturation at 94°C for 5 min then 94°C for 30 sec (denaturation); 62°C for 30 sec (annealing); 72°C for 30 sec (extension) for 32 cycles followed by a final extension at 72°C for 5 min.

Samples without *T. gondii* cDNA were included in each PCR run as negative controls. Also, host (mouse) control-DNA samples were subjected to the same amplification procedures as for parasite DNA. Each amplicon (5 μL) was examined on 1% agarose gels, stained with ethidium bromide and photographed using a gel documentation system (UVItec). The DL5000 marker (TaKaRa) was utilized to estimate the sizes of the PLP1 PCR products.

Purification, cloning, sequencing and analysis of *PLP1* gene: Representative PCR products were purified using spin columns (Agarose Gel DNA Purification kit Ver. 2.0 TaKaRa) and the purified PCR products were ligated into the pGEM-T easy plasmid vector (Promega) according to manufacturer's recommendations. The recombinant plasmid was then transformed into JM109 competent cells (Promega) and positive transformants containing recombinant plasmids were selected by PCR amplification. Cell cultures with confirmed recombinant plasmid were sent to Shanghai Songon Biological Engineering, Biotechnology Company for sequencing.

Table 1: Geographical origins, hosts of *Toxoplasma gondii* strains and the GenBank accession number of their Perforin-like Protein 1 (*PLP1*) genes

Sample code	Genotype[a]	Geographical origin	Host	Accession no.
TgC7	#3	Guangzhou, Guangdong	Cat	JF792807
TgPLh	Type I	Suhe, henan	Pig	JF792808
PRU	Type 2	France	Human	JF792809
PYS	#3	Panyu, Guangdong	Pig	JF792810
QHO	Type 2	Huzhu, Qinghai	Sheep	JF792811
RH	Type I	USA	Human	JF792812

[a]Zhou *et al*. (2009, 2010)

Three colonies from each sample were sequenced from both directions. Sequences of the *PLP1* gene were aligned using the computer program ClustalX 1.81 (Thompson *et al*., 1997), genetic distance calculation was performed using PUZZLE 4.1 (Strimmer and von Haeseler, 1996), sequence homology analysis was performed and the nucleotide composition, transition and transversion were counted using the Megalign program in the software DNA Star Version 5.0.

RESULTS AND DISCUSSION

The total RNA was prepared from six *T. gondii* strains from different hosts and geographical locations, representing different *T. gondii* genotypes (Table 1). As expected, a fragment of approximately 3450 bp in length was amplified from cDNA sample representing each *T. gondii* strain. On agarose gels, no size variation was detected among all of the amplicons and no products were amplified from the host or no-DNA control samples (Fig. 1). Representative PCR products were purified and cloned into pGEM-T easy plasmid vector. The positive recombinant plasmids were selected by bacterial PCR amplification and sequenced.

Six PLP1 sequences of 3453 bp were obtained. Sequence comparison among the six PLP1 sequences revealed 42 (1.22%) variable nucleotide positions of which 20 were transitions and 22 were transversions. The A+T contents of the sequences was 48.3~48.9% which was lower than that of C+G contents (51.1~51.7%). Intra-specific nucleotide variation in PLP1 sequences was related mainly to changes at the 2nd and 3rd codon positions while fewer changes being detected at the 1st codon position.

Although, only 42 (1.22%) nucleotides in PLP1 sequences were different among the six *T. gondii* strains (Table 2) indicating a low sequence divergence in

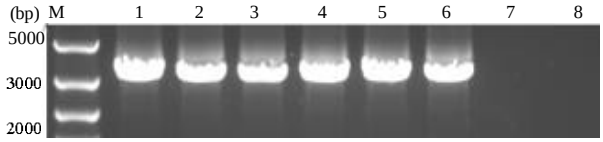

Fig. 1: Analysis of the PCR products of the Perforin-like Protein 1 (*PLP1*) gene among six *Toxoplasma gondii* strains by 1% agarose gel electrophoresis. Lanes 1-8 represent strains RH, TgC7, PYS, TgPLh, PRU, QHO, host (mouse) control and negative (no-DNA) control, respectively. M represents a DNA size marker (ordinate values in bp)

Table 2: Pairwise comparison of sequence percent identity (%) and divergence in the Perforin-like Protein 1 (*PLP1*) gene among six *Toxoplasma gondii* strains from China, France and USA

Strains 1	RH	TgC7	PYS	TgPLh	PRU	QHO
RH	-	98.5	98.2	98.1	98.4	98.4
TgC7	1.5	-	99.4	99.4	99.6	99.8
PYS	1.8	0.6	-	99.0	99.2	99.4
TgPLh	1.9	0.6	1.0	-	99.1	99.3
PRU	1.6	0.4	0.8	0.9	-	99.4
QHO	1.6	0.2	0.6	0.7	0.6	-

T. gondii PLP1 gene, 13 nucleotides were different in the PLP1 sequences between the RH stain stored in the laboratory and the corresponding sequence of the RH strain available in GenBank (EF 102772.2) which may due to gene mutations induced by survival pressure in different experimental hosts.

CONCLUSION

The present study examined sequence variation in the *PLP1* gene among six *T. gondii* strains from different hosts and geographical locations, representing different *T. gondii* genotypes. The results revealed a low sequence divergence in *T. gondii PLP1* gene and the *PLP1* gene may not be an appropriate marker for the studies of genetic variation and virulence among *T. gondii* strains.

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