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Full Length Research Paper

Sequence variation in *Toxoplasma gondii* MIC13 gene among isolates from different hosts and geographical locations

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***Toxoplasma gondii*, a eukaryotic parasite of the phylum Apicomplexa, can infect almost any nucleated mammalian and avian cells. Micronemes are sub-cellular organelles of apicomplexan parasites which can secrete microneme proteins (MICs) playing a key role in the invasion process of *T. gondii* and are potential vaccine candidate molecules against toxoplasmosis. In this work, we examined sequence variation in *T. gondii* microneme protein 13 (TgMIC13) gene sequences among 18 isolates from different hosts and geographical locations by polymerase chain reaction (PCR) amplification and subsequent sequence analysis. The complete TgMIC13 DNA and cDNA sequences were 2506-2507 and 1407 bp, respectively, and they were quite conserved among the *T. gondii* isolates, with intra-specific variation of only up to 0.84% (21/2507) for genomic DNA and up to 0.71% (10/1407) for cDNA sequences. Phylogenetic analysis using maximum parsimony (MP) revealed that the TgMIC13 DNA sequences were not able to provide an effective molecular marker for intra-species phylogenetic analysis and differential identification of *T. gondii* isolates from different hosts and geographical locations. However, the results demonstrated that sequence variation in TgMIC13 gene was quite low among different *T. gondii* isolates, which may be a useful feature as an anti-toxoplasmosis vaccine candidate molecule in further studies.**

Key words: *Toxoplasma gondii*, toxoplasmosis, microneme protein 13 (MIC13), sequence variation, phylogenetic analysis.

INTRODUCTION

Toxoplasma gondii can infect almost any nucleated mammalian and avian cell types, and causes several clinical syndromes including encephalitis, chorioretinitis, systemic infections and abortion in its hosts, particularly seriously in individuals with HIV/AIDS (Montoya and Liesenfeld, 2004; Weiss and Dubey, 2009; Dubey, 2010; Zhou et al., 2011). The main measures for controlling toxoplasmosis in humans and animals are based on chemotherapy. However, the currently used chemotherapeutic agents have some disadvantages such as inadequacy, high cost or toxicity. Obviously an effective vaccine would be ideal for the prevention and control of toxoplasmosis in humans and animals.

As sub-cellular organelles of *T. gondii*, micronemes, rhoptries and dense granules play key roles during the active invasion process of *T. gondii* into host cells (Carruthers and Sibley, 1997). Micronemal proteins (MICs) have been shown to contribute critically to host cell invasion, although their precise roles remained to be

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Table 1. Details of *Toxoplasma gondii* isolates used in this study and the GenBank accession numbers of their MIC13 gene sequences.

Isolate ID	Host	Geographical location	Genotype*	GenBank accession No.
GT1	Goat	United States	Reference, Type I, ToxoDB 10	JN995546
RH	Human	France	Reference, Type I, ToxoDB 10	JN995554
PTG	Sheep	United States	Reference, Type II, ToxoDB 1	JN995559
CTG	Cat	United States	Reference, Type III, ToxoDB 2	JN995545
MAS	Human	France	Reference, ToxoDB 17	JN995547
TgCgCa1	Cougar	Canada	Reference, ToxoDB 66	JN995557
TgCatBr5	Cat	Brazil	Reference, ToxoDB 19	JN995556
SH	Human	Shanghai, China	Type I, ToxoDB 10	JN995555
TgPXD	Pig	Xiangfan Hubei, China	Type I, ToxoDB 10	JN995560
TgPNY	Pig	Luying, Henan, China	Type I, ToxoDB 10	JN995558
NT	Pig	Tanshan, Nanjing, China	Type I, ToxoDB 10	JN995548
NTA	Pig	Tanshan, Nanjing, China	Type I, ToxoDB 10	JN995549
NY11	Pig	Nanyang, Henan, China	Type II, ToxoDB 1	JN995550
QHO	Sheep	Huzhu, Qinghai, China	Type II, ToxoDB 1	JN995553
PRU	Human	France	Type II, ToxoDB 1	JN995551
PYS	Pig	Panyu, Guangdong, China	Type #3, ToxoDB 9	JN995552
ZS	Human	Guangzhou, Guangdong, China	Type #3, ToxoDB 9	JN995561
TgC7	Cat	Guangzhou, Guangdong, China	Type #3, ToxoDB 9	JN995544

***** based on genotyping results of Zhou et al. (2009, 2010).

fully understood (Soldati et al., 2001; Dowse and Soldati, 2004; Friedrich et al., 2010). Previous studies have also indicated that *T. gondii* MICs, such as MIC1, 3, 4 and 6, are potential vaccine candidate molecules against toxoplasmosis (Lourenço et al., 2006; Peng et al., 2009; Wang et al., 2009; Xiang et al., 2009).

Like the *T. gondii* MIC1 (TgMIC1) having the microneme adhesive repeat (MAR) domain which recognizes sialic acid (Sia, a key determinant on the host cell surface for invasion by *T. gondii*), *T. gondii* MIC13 (TgMIC13), a novel Sia-binding lectin, is also involved in Sia-dependent invasion of host cells (Friedrich et al., 2010). However, it is yet to know whether there are sequence variations in TgMIC13 gene among *T. gondii* isolates from different hosts and geographical regions, representing different genotypes. The objective of the present study was to examine sequence variability in TgMIC13 gene among different *Toxoplasma gondii* isolates from different host and geographical origins.

MATERIALS AND METHODS

T. gondii isolates and extraction of RNA and genomic DNA

Tachyzoites of the highly virulent RH strain of *T. gondii* (Type I) were prepared and collected according to the method described previously (Yan et al., 2011). Total RNA and genomic DNA (gDNA) were extracted by using Tissue RNA Kit (Biomiga) and Wizard SV Genomic DNA Purification System (Promega), respectively, according to the manufacturer's recommendations. The extracted total RNA and DNA samples were stored at -80 and -20ºC, respectively, until use. The cDNA was reverse-transcribed by using Rever Tra Ace-α Enzyme (First Strand cDNA Synthesis Kit, TOYOBO) according to the manufacturer's recommendations. All the other 17 *T. gondii* isolates were from different geographical locations and hosts (Table 1). These isolates have been genotyped previously (Zhou et al., 2009, 2010).

Enzymatic amplification and sequencing

A pair of primers (Forward primer MIC13F: 5'-CGAATTCCCTTTTTCGACAAAATGGTGGGTGTTTTCCAGCTG-3', and reverse primer MIC13R: 5'-CCCTGCAGGCTAGCACTCTGTCGAGGCGCTTTTCATAGC-3') were designed to amplify the TgMIC13 gene based on *T. gondii* RH strain MIC13 gene sequence (GenBank accession no. EU374172.1). PCR reactions (25 µL) were performed in 2.5 µL Ex*Taq* buffer, 0.2 mM of each dNTPs, 2 mM of $MgCl_2$, 2.5 µM of each primer, 1.25 U of *ExTaq* DNA polymerase (TAKARA) and 2 µL of DNA sample in a thermocycler (Biometra) under the optimized cycling conditions: initial denaturation at 94 ºC for 5 min, then 94ºC for 1 min (denaturation), 60ºC for 1 min (annealing), 72ºC for 2.5 min (extension) for 30 cycles followed by a final extension of 72ºC for 7 min. A negative control sample without genomic DNA was included in each amplification run.

Each amplicon (5 µL) was examined by agarose gel electrophoresis to validate amplification efficiency. The size of MIC13 amplicons was estimated using the DL5000 DNA marker (TAKARA). Positive amplicons were purified using spin columns (Wizard PCR-Preps DNA Purification System, Promega), ligated with pGEM-T Easy vector (Promega) and sequenced as reported previously (Huang et al., 2004).

Table 2. Characteristics of *Toxoplasma gondii* MIC13 (TgMIC13) gene sequences including their expressed regions and introns.

Item	TgMIC13 DNA	TgMIC13 cDNA	TgMIC13 introns				
			First	Second	Third	Forth	Fifth
Length (bp)	2507	1407	322	216-217	323	101	137
T+A (%)	49.2-49.6	47.9-48.3	48.9-50.3	47.9-48.8	50.5-51.6	54.5-55.5	54.1-56.2
Transition	16	7	3	1	2	1	2
Transversion	5	3	1	0	0	0	1
R	3.2	2.3	3	/	/	/	2
Distance (%)	0-1.5	0-1.4	0-1.3	0-1.4	0-1.6	0-1.0	0-2.1

R=transition/transversion.

Sequence analysis and re-construction of phylogenetic relationships

The TgMIC13 gene sequences were aligned using the computer program ClustalX 1.81 (Thompson et al., 1997). Nucleotide variations in the TgMIC13 gene sequences among the examined *T. gondii* isolates were examined by pair-wise sequence comparison using the formula $D = 1-(M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton et al., 1995).

Phylogenetic reconstruction among the examined *T. gondii* isolates was conducted using maximum parsimony (MP) based on the TgMIC13 gene sequences using *Neospora caninum* (GenBank accession No. AF421187.1) as the out-group. MP analysis was performed using PAUP* 4.0b10 (Swofford, 2002), with indels treated as missing character states. A total of 1,000 random addition searches using TBR were performed for each MP analysis. Bootstrap probability (BP) was calculated from 1,000 bootstrap replicates with 10 random additions per replicate in PAUP. Phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

RESULTS AND DISCUSSION

The genomic TgMIC13 sequences were 2506-2507 bp in length for all the examined *T. gondii* isolates. Among them, the TgMIC13 sequences of *T. gondii* NT and SH isolates were 2506 bp in length, whereas those of the other isolates were 2507 bp. The TgMIC13 cDNA sequence of *T. gondii* RH strain was 1407 bp in length, consistent with previous report (Friedrich et al., 2010). By comparing the TgMIC13 genomic sequences and cDNA sequences, five introns were identified in the TgMIC13 gene (Table 2). A total of 21 variable nucleotide positions were identified in the TgMIC13 gene sequences, 10 of them were in the expressed regions and the other 11 were distributed among the 5 introns, with intra-specific variation of up to 0.84% (21/2507) for genomic DNA sequences and 0.71% (10/1407) for cDNA sequences.

There were 16 transitions (C<–>T, T<–>G, A<–>C and A<–>G) and 5 transversions (A<–>T and C<–>G) in (R=transition/transversion=3.2), 7 transitions (A<–>G and TgMIC13 DNA sequences C<–>T) and 3 transversion (A<–>T and C<–>G) in TgMIC13 coding regions (R=transition/transversion=2.3), and 9 transitions (A<–>G and C<–>T) and 2 transversions (A<–>T and C<–>G) in introns regions (R=transition/transversion=4.5) (Table 2). More nucleotide changes were at the first and second codon positions, while fewer changes were detected at the third codon position. These results revealed that variation in TgMIC13 sequences among the examined *T. gondii* isolates was low.

Phylogenetic reconstruction of the examined 18 *T. gondii* isolates using MP analysis revealed that *T. gondii* isolates of the same genotypes were clustered in different clades (Figure 1), suggesting that the TgMIC13 DNA sequences were not an ideal genetic marker for intra-species phylogenetic analysis and differential identification of *T. gondii* isolates of different genotypes from different hosts and geographical locations. This result was not consistent with that of previous studies using other genetic markers (e.g. PK1 and SAG2) (Su et al., 2006).

Conclusions

The present study revealed low level variability in MIC13 gene sequences among *T. gondii* isolates of different genotypes from different geographical regions and hosts. TgMIC13 gene sequence was not an effective genetic marker for intra-species phylogenetic and population genetic studies of *T. gondii* isolates of different host and geographical origins.

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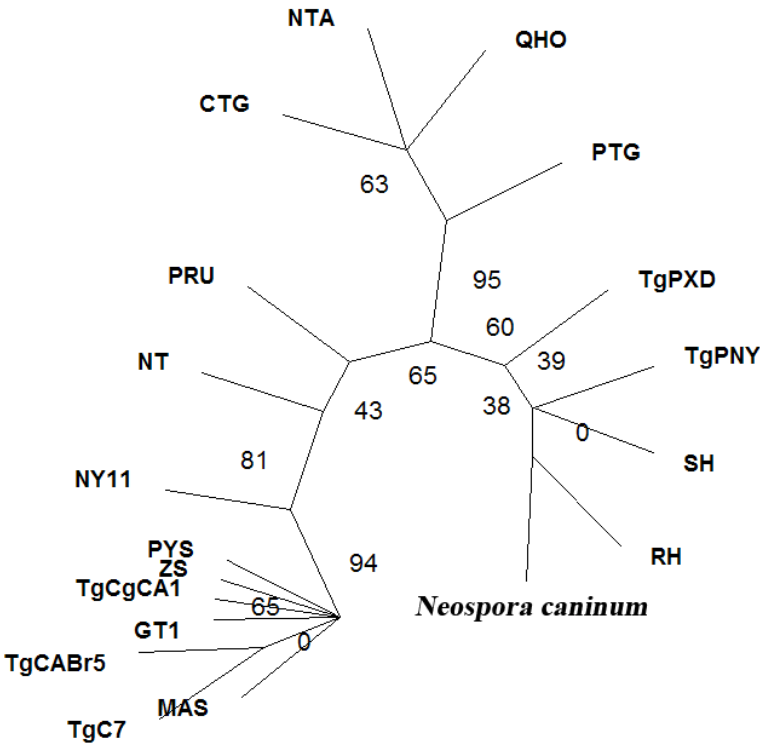

Figure 1. Phylogenetic relationships of *Toxoplasma gondii* isolates from different hosts and geographical locations inferred by maximum parsimony (MP) analyses based on the TgMIC13 gene sequences using *Neospora caninum* (GenBank accession number AF421187.1) as the outgroup. Numbers at nodes indicate bootstrap values (%).

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