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Characterization of the *Toxoplasma gondii hsp60* gene sequences from different hosts and geographical locations

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ABSTRACT. The intracellular protozoan *Toxoplasma gondii* is one of the most successful parasites, with the ability to invade all warm-blooded animals, including humans. *T. gondii* heat shock protein 60 (TgHSP60) plays an important role in intracellular survival and in the differentiation of the parasite, and is also recognized as being associated with its virulence. In the present study, we examined sequence variation in the *hsp60* coding region among five *T. gondii* isolates from different hosts and geographical regions, which were compared with the corresponding sequences of strains ME49, 76K, and GT1 available in the ToxoDB databases. The length of the *T. gondii hsp60* sequence was 1728 bp for all strains, and the A+T content ranged from 41.96 to 42.13%. The sequence alignment of the 8 *T. gondii* strains identified 20 variable positions (0-1.44%) and showed 1.16% overall sequence

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variation, suggesting a relatively considerable sequence diversity. Phylogenetic analysis of *hsp60* sequences using Bayesian inference and maximum parsimony differentiated the two major clonal lineage types into their respective clusters, and thus separated atypical strains from classical genotypes. The results of the present study suggested that the coding region of the *hsp60* gene may represent a novel genetic marker for intraspecies phylogenetic analyses of *T. gondii*.

Key words: *Toxoplasma gondii*; Toxoplasmosis; Sequence variation; Heat shock protein 60; Phylogenetic analysis

INTRODUCTION

As an obligate intracellular protozoan pathogen, *Toxoplasma gondii* has worldwide distribution and can invade virtually all warm-blooded animals and humans, leading to toxoplamosis, an important zoonotic parasitic disease, and thus causes serious public safety issues (Montoya and Liesenfeld, 2004; Chen et al., 2012a; Tian et al., 2012; Zhang et al., 2013). Although *T. gondii* infections in immunocompetent individuals are subclinical or usually asymptomatic, the infection can cause severe clinical problem or death in immunosuppressed and immunodeficient individuals, such as *Toxoplasma* encephalitis (TE) or neurological lesions (Montoya and Liesenfeld, 2004; Weiss and Dubey, 2009; Dubey, 2010). In animals, the parasite is responsible for considerable economic losses in the livestock industry resulting from abortion or congenital toxoplasmosis in all types of livestock, especially in sheep and goats (Fayer et al., 2004; Dubey et al., 2005).

T. gondii heat shock protein 60 (TgHSP60) is a member of the HSP60 subfamily (Toursel et al., 2000), which plays an important role in intracellular survival and in the differentiation of *T. gondii* and also mediates its virulence (Shonhai et al., 2011). In addition, TgHSP60 not only serves as a signal for the activation of antigen presenting cells by inducing the release of cytokines and stimulation of the initial immune response, but also acts as a molecular chaperone in the mitochondria (Ma et al., 2009). Regardless of the key biological roles of TgHSP60 mentioned above, no previous studies have elucidated the sequence diversity of the HSP60 gene among *T. gondii* isolates from different origins. Therefore, the objectives of the present study were to examine sequence variation in the entire coding region of the *hsp60* gene among strains from different geographical regions and hosts, and to assess whether the locus may be used as a new marker for population genetic studies of *T. gondii* isolates.

MATERIAL AND METHODS

T. gondii isolates

Five *T. gondii* isolates from different geographical locations and hosts were used for the analysis in this study (Table 1) (Zhou et al., 2009, 2010; Su et al., 2010; Huang et al., 2012). All isolates were harvested according to the method described by Yan et al. (2011), and total RNA was extracted using the Total RNA Kit I (Omega; USA) according to manufacturer recommendations. The total RNA obtained was stored at -80°C until use.

Table 1. Details of *Toxoplasma gondii* isolates used.

Isolate ID	Host	Geographical location	Genotype*
TgPLh	Human	Shanghai, China	Type I, ToxoDB 10
QHO	Sheep	Huzhu, Qinghai, China	Type II, ToxoDB 1
Prugniaud (PRU)	Human	France	Type II, ToxoDB 1
PYS	Pig	Panyu, Guangdong, China	Type #3, ToxoDB 9
TgC7	Cat	Guangzhou, Guangdong, China	Type #3, ToxoDB 9

*Based on genotyping results of Zhou et al. (2009, 2010), Su et al. (2010), and Huang et al. (2012).

Polymerase chain reaction (PCR) amplification

A pair of specific primers (forward primer HSP60F:5'-ATGCTTGCCCGCGCTTCAGC-3'; reverse primer HSP60R: 5'-CTAGTACATGCCTCCCATGCCGC-3') were designed to amplify the TgHSP60 gene based on the HSP60 gene sequence of the *T. gondii* ME49 strain available in the National Center for Biotechnology Information (NCBI; GenBank accession number XM002367081.1). Reverse transcription (RT)-PCR amplification was performed using the PrimeScript® One Step RT-PCR Kit Ver.2 (TAKARA) according to manufacturer recommendations. RT-PCR were performed in a volume of 25 µL consisting of 1 µL PrimeScript 1 step enzyme mix, 12.5 µL 2X 1 Step buffer, 2.5 µM of each primer, 100-200 ng total RNA, and 1 µL RNase-free dH_2O. The thermal cycling conditions were as follows: 1) reverse transcription at 50°C for 30 min; 2) RTase inactivation at 94°C for 2 min; 3) denaturation at 94°C for 1 min, annealing at 60.4°C for 45 s, and extension at 72°C for 2 min for 30 cycles each; and 4) additional extension for 10 min at 72°C. The amplified products were electrophoresed on 1% (w/v) agarose gels, stained with GoldenView™, and photographed using a gel documentation system (UVP GelDoc-It™ Imaging System; Cambridge, UK). The DL2000 marker (TAKARA) was utilized to estimate the sizes of the *hsp60* RT-PCR products.

Sequencing of the *hsp60* amplicons, sequence analysis, and phylogenetic reconstruction

The *hsp60* PCR products were purified using spin columns (Wizard™ PCR-Preps DNA Purification System; Promega; USA), ligated with the pMD18-T easy vector (TAKARA) according to manufacturer recommendations, and then transformed into *Escherichia coli* DH5α competent cells (Promega). The positive colonies were screened by PCR amplification and enzymatic digestion, and were sequenced by the Shanghai Songon Biological Engineering Biotechnology Company.

The sequences obtained were aligned using ClustalX 1.83 (Thompson et al., 1997), and the intraspecific sequence variation was calculated by pair-wise sequence comparison using the formula $D = 1 - (M / L)$, where M is the same number of sequence bases and L is the total number of sequence bases. Phylogenetic reconstructions based on the entire coding region of the *hsp60* gene among the different *T. gondii* strains were performed using maximum parsimony (MP) and Bayesian inference (BI), using *Neospora caninum* (Gene ID: NCLIV_065210) as an outgroup. MP analysis was performed using PAUP* 4.0b10 (Swofford, 2002), with indels being treated as missing character states. A total of 100 random addition searches using tree bisection-reconnection (TBR) branch swapping were performed for

each MP analysis. Bootstrap probability (BP) was calculated from 1000 bootstrap replicates with 10 random additions per replicate in PAUP. BI analyses were conducted with four independent Markov chains run for 1,000,000 metropolis-coupled Markov chain Monte Carlo (MCMC) generations, sampling a tree every 1000 generations in MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003).

RESULTS AND DISCUSSION

The length of the coding sequence of the *hsp60* gene was 1728 bp for all of the examined *T. gondii* strains, and the A+T contents varied from 41.96 to 42.13%. Sequence variations in the entire coding region of *hsp60* among the examined *T. gondii* strains were 0-1.44%, which is similar to that observed in the ROP7, ROP13, and MIC13 genes (Ren et al., 2012; Wang et al., 2012; Zhou et al., 2012). The alignment of all the 8 sequences revealed 20 variable positions, showing 1.16% overall sequence variation, which suggested a relatively considerable sequence diversity, but a lower genetic variation than that of the coding regions of the GRA5, GRA6, and AK69 genes (Fazaeli et al., 2000; Boughattas et al., 2011; Chen et al., 2012b). Of these variable positions, there were 15 transitions (A↔G, and C↔T) and 5 transversions (A↔C, A↔T, G↔C, and G↔T) in the coding region of the *T. gondii hsp60* gene (R = transition/transversion = 3).

Phylogenetic analysis using BI and MP based on the *hsp60* coding sequences showed that *T. gondii* strains representing genotype I and genotype II, the two major clonal lineage types, were grouped into their respective clusters separately, and the ToxoDB #9 strains could be readily distinguished from the classical genotypes (Figure 1). This result is consistent with that of some previous studies using other genetic markers such as GRA5, GRA6, and AK69 for genotyping (Fazaeli et al., 2000; Boughattas et al., 2011; Chen et al., 2012b).

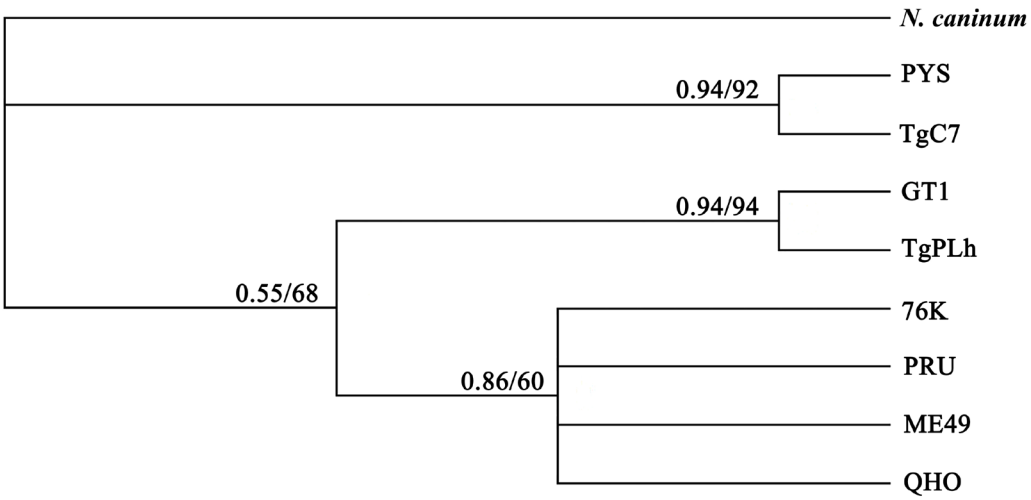

Figure 1. Phylogram of 8 *Toxoplasma gondii* strains determined by analysis of the entire sequences of the *hsp60* gene sequences. The high genetic divergence of the *hsp60* gene revealed two major (denoted by I and II) and atypical ToxoDB 9. The tree was built by Bayesian inference (BI) and maximum parsimony (MP) analysis. The numbers along branches indicate bootstrap values resulting from different analysis in the order: BI/MP.

In conclusion, the present study determined the entire coding sequences of the *T. gondii hsp60* gene and revealed relatively considerable sequence variability in this locus among *T. gondii* isolates from different hosts and geographical regions. Phylogenetic analysis suggested that the *T. gondii hsp60* coding sequence may represent a novel genetic marker for studying the population diversity and molecular epidemiology of *T. gondii*.

Conflicts of interest

The authors declare no conflict of interest.

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