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▣ BRIEF COMMUNICATION

Genetic Diversity of *Toxoplasma gondii* Strains from Different Hosts and Geographical Regions by Sequence Analysis of *GRA20* Gene

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Abstract: *Toxoplasma gondii* is a eukaryotic parasite of the phylum Apicomplexa, which infects all warm-blood animals, including humans. In the present study, we examined sequence variation in dense granule 20 (*GRA20*) genes among *T. gondii* isolates collected from different hosts and geographical regions worldwide. The complete *GRA20* genes were amplified from 16 *T. gondii* isolates using PCR, sequence were analyzed, and phylogenetic reconstruction was analyzed by maximum parsimony (MP) and maximum likelihood (ML) methods. The results showed that the complete *GRA20* gene sequence was 1,586 bp in length among all the isolates used in this study, and the sequence variations in nucleotides were 0-7.9% among all strains. However, removing the type III strains (CTG, VEG), the sequence variations became very low, only 0-0.7%. These results indicated that the *GRA20* sequence in type III was more divergence. Phylogenetic analysis of *GRA20* sequences using MP and ML methods can differentiate 2 major clonal lineage types (type I and type III) into their respective clusters, indicating the *GRA20* gene may represent a novel genetic marker for intraspecific phylogenetic analyses of *T. gondii.*

Key words: *Toxoplasma gondii*, sequence variation, dense granule 20 (GRA20), phylogenetic analysis

Toxoplasma gondii, one of the most successful intracellular protozoan parasites, can infect the majority of vertebrate spices including humans with a worldwide distribution [1-3], and approximately one-third of the population has been exposed to *T. gondii*. Normally, the infections are asymptomatic or subclinical. However, the *T. gondii* infection can cause abortion and stillbirth in pregnant women, and encephalitis, chorioretinitis, and systemic infections in immunocompromised individuals [2]. In animals, *T. gondii* can also cause abortion in livestock, especially in sheep and goats, which can spawn a great number of economic losses in livestock [3]. However, there was no effective vaccine and drugs that can help to control toxoplasmosis.

The strains of *T. gondii* that predominate in Europe and North America, classified into types I, II, and III, differ in a wide range of phenotypes, including virulence, persistence, migratory capacity, and how they interface with the immune response [4-6], Thus, the information of genetic diversity of *T. gondii* is useful for better understanding epidemiological patterns and pathogenicity, as well as exploring of new polymorphic virulence effectors.

GRA20, a novel dense granule protein, is secreted and targeted to parasitophorous vacuole membrane (PVM), which may participate in the manipulation of the host immunity [7]. Previous studies have identified the existence of polymorphisms in dense granule proteins, such as GRA15, GRA5, and GRA6 [8-10], but the sequence variation about the *GRA20* gene among different *T. gondii* isolates is still unknown. Therefore, the objective of this study was to examine sequence diversity of *GRA20* gene among *T. gondii* strains from different hosts and geographical regions worldwide.

In this study, a total 16 *T. gondii* strains from different hosts and geographic locations were used for analysis (Table 1). These *T. gondii* isolates have been genotyped and genomic DNA (gDNA) was prepared as described previously [11-13].

To acquire amplicons of *GRA20* genes concerning different *T.*

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

Isolate	Host	Geographical location	Genotype[a]
RH	Human	France	Reference, type I, ToxoDB #10
GT1	Goat	United States	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
TgCatBr64	Cat	Brazil	Reference, ToxoDB#111
TgCatBr5	Cat	Brazil	Reference, ToxoDB#19
PRU	Human	France	type II, ToxoDB #1
QHO	Sheep	Qinghai, China	type II, ToxoDB #1
PTG	Sheep	United States	Reference, type II, ToxoDB#1
TgC7	Cat	Guangzhou, China	ToxoDB #9
PYS	Pig	Panyu, China	ToxoDB #9
CTG	Cat	United States	Reference, type III, ToxoDB#2
TgWtdSc40	Deer	USA	type 12, ToxoDB#5
TgToucan	Toucan	Costa Rica	Reference, ToxoDB#52

[a]Based on the results of Zhou et al. [11,12] and Su et al. [13].

Table 2. Characteristics of *Toxoplasma gondii* GRA20 (*TgGRA20*) gene sequences

Item	DNA		CDS[a]		First Extron		First Intron		Second Extron	
	ALL[b]	Except III[c]	ALL	Except III	ALL	Except III	ALL	Except III	ALL	Except III
Length (bp)	1,586	1,586	1,242	1,242	140	140	344	344	1,102	1,102
T+A (%)	44.96-45.40	45.02-45.40	42.83-43.32	42.91-43.32	45.71-46.43	45.71-46.43	52.62-52.91	52.62-52.91	42.47-42.92	42.56-42.92
Transition	59	12	57	10	1	1	2	2	56	9
A↔G	33	7	31	5	1	1	2	2	30	4
C↔T	26	5	26	5	/	/	/	/	26	5
Transversion	62	3	61	2	0	0	1	1	61	2
A↔T	8	/	8	/	/	/	/	/	8	/
G↔C	17	1	17	1	/	/	/	/	17	1
A↔C	19	2	18	1	/	/	1	1	18	1
G↔T	18	/	18	/	/	/	/	/	18	/
loss	6	0	6	0	0	0	0	0	6	0
VN[d]	127	15	124	12	1	1	3	3	123	11
R[e]	0.95	4	0.93	5	\	\	2	2	0.92	4.5
Distance (%)	0-7.9	0-0.7	0-10.1	0-0.6	0-0.7	0-0.7	0-0.9	0-0.9	0-11.4	0-0.6

[a]CDS: coding sequence.
[b]All: all the ***T. gondii*** in this study.
[c]Except III: all the *T. gondii* except CTG and VEG.
[d]VN: variable nucleotide.
[e]R = transition/transversion.

gondii isolates, the primers GRA20-F (5′- ATGCATAGCCG-GAACTGCGTC-3′) and GRA20-R (5′- TCACGCGGGCTTTC-TACGG-3′) were designed based on *T. gondii* ME49 strain available in ToxoDB database (TGME49_200010). All the PCR products of GRA20 genes were purified by the DNA purification kit (GenStar, Beijing, China), ligated into pMD18-T vector (TaKaRa, Dalian, China), and then transformed into JM109 competent cells (Promega, Madison, Wisconsin, USA). Subsequently, the positive colonies were screened by PCR, and then sequenced by GenScript Co., Ltd. (Nanjing, China).

The acquired *GRA20* gene sequences were aligned by the Multiple Sequence Alignment Program, Clustal X 1.83 [14], and sequence variation was determined among the examined *T. gondii* strains. Phylogenetic reconstructions based on the complete sequences of *GRA20* gene among 13 *T. gondii* isolates and plus the corresponding sequences of strains TgCatBr9,

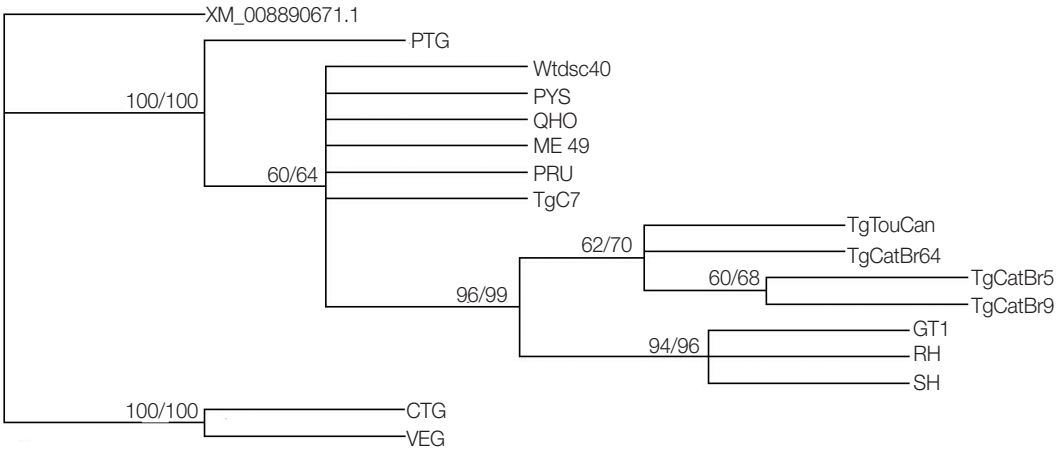

Fig. 1. Phylogram of 16 *Toxoplasma gondii* isolates determined by analysis of the entire sequences of the *GRA20* gene. The tree was reconstructed by maximum parsimony (MP) and maximum likelihood (ML) analyses. The numbers along branches indicate bootstrap values resulting from different analyses in the order: MP/ML.

VEG, and ME49 available in ToxoDB (http://toxodb.org/toxo/) were carried out by 2 inference methods, maximum likelihood (ML) and maximum parsimony (MP) methods by Paup, with the sequence of *Hammondia hammondi* (XM_008890671.1) as the out-group. Phylograms were drawn by the Tree View program version 1.65.

In the present study, the obtained entire genomic sequences of the GRA20 gene were 1,586 bp in length in all examined isolates. According to the analysis of all the 16 GRA20 complete sequences, there were 2 extrons and 1 intron in the GRA20 gene (Table 2). The A+T content ranged from 45.0% to 45.4% in the entire sequence. There were 124 nucleotide position variations with a distribution of 57 transitions (A↔G and C↔T), 61 transversions (C↔G, T↔G, A↔C, and A↔T) in CDS, and 2 transitions (A↔G and C↔T), and 1 transversion (C↔G, T↔G, A↔C, and A↔T) in the intron (Table 2). However, when we analyzed the GRA20 sequences without type III (CTG, VEG) strains, there were 12 nucleotide variations with a distribution of 10 transitions (A↔G and C↔T), 2 transversions (C↔G, T↔G, A↔C, and A↔T) in CDS, and 2 transitions (A↔G and C↔T) and 1 transversion (C↔G, T↔G, A↔C, and A↔T) in the intron. The alignment of *GRA20* gene sequences showed that sequence variation was 0-7.9% in all studied strains, while the sequence variation became 0-0.7% without the CTG and VEG strains. Phylogenetic reconstruction of all 16 *T. gondii* strains based on *GRA20* sequence data showed that the type I and type III of *T. gondii* strains were clustered into respective clusters separately (Fig. 1).

Recently, polymorphisms in the sequences of *GRA5*, *GRA6*, *GRA7*, and *GRA15* genes have been reported [8,9,15,16]. Among them, polymorphic dense granule proteins were widely used in typing *T. gondii* isolates, such as GRA6 [10]. Furthermore, polymorphic dense granule protein may have different roles in regulating the inflammatory response. For example, GRA15 in type II activate more IL-12 than type I or type III strains [8]. In this study, we found *GRA20* gene was very diverse in type III, indicating the functions may be different, too. Our results were consistent with that of some previous studies using other genetic markers, such as *GRA5*, *Rop17*, and *HSP60* for genotyping [9,17,18], but different to some previous studies, such as *Rop38* and *eIF4A* [19,20].

In conclusion, the present study examined the sequences of the *T. gondii GRA20* gene and revealed that it was more divergence in type III compared to other *T. gondii* strains, suggesting the functions of *GRA20* in type III may be different from other strains. Phylogenetic analysis indicated that the *GRA20* gene could distinguish the type I and type III strains, suggesting the *GRA20* gene may be a novel genetic marker for studying genetic variation or the population genetic structures of *T. gondii* isolates.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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