ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 52, No. 2: 205-209, April 2014
http://dx.doi.org/10.3347/kjp.2014.52.2.205

▣ BRIEF COMMUNICATION

Sequence Analysis of *cytb* Gene in *Echinococcus granulosus* from Western China

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Abstract: *Echinococcus granulosus* is the causative agent of cystic echinococcosis with medical and veterinary importance in China. Our main objective was to discuss the genotypes and genetic diversity of *E. granulosus* present in domestic animals and humans in western China. A total of 45 hydatid cyst samples were collected from sheep, humans, and a yak and subjected to an analysis of the sequences of mitochondrial cytochrome *b* (*cytb*) gene. The amplified PCR product for all samples was a 1,068 bp band. The phylogenetic analysis showed that all 45 samples were identified as *E. granulosus* (genotype G1). Ten haplotypes were detected among the samples, with the main haplotype being H1. The haplotype diversity was 0.626, while the nucleotide diversity was 0.001. These results suggested that genetic diversity was low among our samples collected from the west of China based on *cytb* gene analysis. These findings may provide more information on molecular characteristics of *E. granulosus* from this Chinese region.

Key words: *Echinococcus granulosus*, cytochrome *b*, sequence analysis, western China

Cystic echinococcosis (CE, also known as hydatid disease), a globally important and pathogenic zoonoses, is caused by the atiological agent, *Echinococcus granulosus*. China is one of the most important endemic regions of CE [1]. *E. granulosus* is now considered as a complex consisting of at least 4 species including *E. granulosus* sensu stricto (G1-G3 genotypes), *E. equinus* (G4), and *E. ortleppi* (G5). There are controversies for considering G6 to G10 as 1 or 2 species, namely *E. canadensis* and *E.intermedius* [2-4]. Among them, *E. granulosus* s.s. is known to have a broad geographical distribution and a wide host range [5]. G1-G3 genotypes are also named the sheep (G1), the Tasmanian sheep (G2), and the buffalo (G3) strains, respectively. In China, the sheep strain is the predominant epidemic strain, while G3 and G6 strains also exist [6-14].

Increased knowledge of the genetic structure of a parasite could provide more information about its epidemiology and transmission, since genetic variants may affect infectivity and pathogenicity [15]. In China, some mitochondrial and nuclear genes have been applied in the analysis of genetic information in *E. granulosus*, including cytochrome *c* oxidase subunit 1 (*cox1*), NADH dehydrogenase 1 (*nad1*), ATP synthase F0 subunit 6 (*atp6*), cytochrome *b* (*cytb*), elongation factor-1 alpha (*ef1*α), 12S, and 16S [2,13,14,16,17]. However, the genetic diversity of *E. granulosus* in China has never been characterized based on the complete *cytb* gene, and only the partial *cytb* gene have been reported [17,18]. Previous studies demonstrated that both the complete *cytb* and *cox1* sequences showed 1 causative agent of CE in a cat from Russsia, i.e., *E. granulosus* s.s. (G1) [19]. Therefore, it is feasible that we use the complete *cytb* gene as a genetic marker to analyse genetic information of *E. granulosus*. The main purpose of this study was to figure out the genotypes and genetic diversity of *E. granulosus* present in western China.

A total of 45 hydatid cysts were obtained from several hosts (sheep, yak, and humans) in Tibet Autonomous Region, Qinghai, and Sichuan Province, China. Table 1 summarizes the number and origin of isolates examined in the 3 localities. All samples were stored at -70˚C. Genomic DNA was extracted from each cyst by proteinase K treatment and phenol-chloroform extraction. All DNA samples were stored at -20˚C.

• Received 19 October 2013, revised 4 January 2014, accepted 10 January 2014.
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Table 1. Geographic origin and host of China isolates of *Echinococcus granulosus* identified by DNA sequencing of mitochondrial *cytb* gene

Province	Host			Total
	Sheep	Human	Yak	
Qinghai	28	0	0	28
Sichuan	0	9	1	10
Tibet	7	0	0	7
Total	35	9	1	45

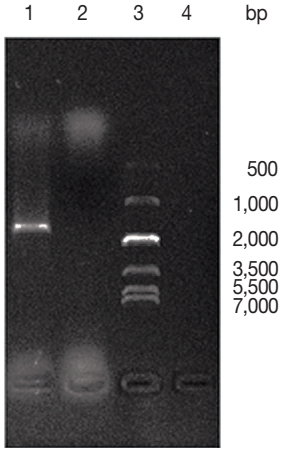

Fig. 1. Electrophoresis results of amplification product of *cytb* gene. Lane 1, *E. granulosus* DNA; Lane 2, template free; Lane 3, marker; Lane 4, primers free.

Based on the published complete mitochondrial genome of *E. granulosus* (GenBank accession no. AF297617), the *cytb* gene was amplified using the following primer pairs: forward 5′-AGATATTAGAGATGGAGGAG-3′ and reverse 5′-ACCCGA GTTAATACAGTCAG-3′. The primers were designed by Primer 5.0 software, and the product was 1,609 bp that contained the complete sequence of *cytb* gene. PCR was performed in a 25 µl reaction mixture containing 1 µl of template (the genomic DNA of each sample was used as a template for PCR), 1 µl of each primer (10 pmol/µl), 12.5 µl of 2× Taq PCR MasterMix and 9.5 µl of ddH_2O. Negative controls were always included in PCR reactions to assess possible contamination. The PCR protocol consisted of 30 thermal cycles with a denaturation step (90 sec at 94℃), a hybridization step (90 sec at 46℃) and an elongation step (90 sec at 72℃) for each cycle. Afterwards, 5 µl of the PCR product was run in a 1% agarose gel and stained with ethidium bromide to detect the PCR amplicons. Amplification products were purified by using the Universal DNA Purification Kit (Cowin Biotech, Beijing, China) and subjected to automated sequencing following subcloning into the pMD19-T vector (TaKaRa, Beijing, China). All amplicons were sequenced at least 3 times.

Multiple alignments of nucleotide sequences were performed using the Clustal X 1.83 software, with variation sites deleted from the alignments. Population diversity indices (nucleotide diversity, number of haplotypes, and haplotype diversity) were calculated using DnaSP 5.0 software [20]. Phylogenetic trees were constructed from the alignments with the neighbour-joining method in MEGA 5.0 software, with *Echinococcus multilocularis* as the outgroup. Confidence intervals were obtained via 1,000 bootstrap replications for each branch of the tree. The identification of haplotypes and construction of the haplotype network was undertaken by TCS 1.21 software using statistical parsimony [21]. The network estimation was run at a 95% connection limit. Several sequences were obtained from GenBank for similarity comparison and phylogenetic analysis. The complete mitochondrial genome of *E. granulosus* G1 (AF297617), G4 (AF346403), G5 (AB208063), G6 (AB235846) G7 (AB23587), and G8 genotype (AB235848) were served as reference sequences [22,23], and *E. multilocularis* (AB018440) as the outgroup [24]. Published mtDNA sequences of *cytb* (AY278067 and AB622276) [17,19] were used for comparison.

PCR amplification products of *cytb* gene were analyzed by using 1% agarose gel electrophoresis, and the results showed that a 1,609 bp band was amplified between 1,000-2,000 bp, which was consistent with the expected length of the target fragment (Fig. 1). DNA sequencing revealed that the *cytb* gene sequences of all 45 samples contained 1,068 nucleotides, and sequences were deposited in GenBank with accession numbers KC709522 to KC709566. The average contents of A, T, C, and G were 19.4%, 47.7%, 8.9%, and 24.0%, respectively, revealing that AT content was greater than GC content. There were 11 mutation sites among these sequences of *cytb* genes (Table 2). Only nucleotide substitutions were detected, while deletions or insertion mutations were not observed. High similarity was shown among all the sequences obtained in this study (99.5% to 100%). In addition, all sequences in this study showed more than 88.0% identity with the previously published *cytb* sequences for *E. granulosus* genotypes.

We detected 10 haplotypes within the 45 isolates obtained from the west of China (Table 2). It was indicated that each of the haplotypes possessed intermediate host specificity. Haplotype diversity (Hd) was 0.626, while nucleotide diversity (Pi) was 0.001. All the H1 haplotype sequences shared 100% iden-

Table 2. Variation sites of *cytb* gene of *Echinococcus granulosus* from the west of China

Haplotype	Locality	Host	N[a]	97	120	180	285	471	680	768	850	888	984	1,038
H1	Qinghai, Sichuan, and Tibet	Sheep Human	27	A	A	A	T	T	C	T	C	G	C	G
H2	Qinghai, Tibet	Sheep	5	-	G	-	-	-	-	-	-	-	-	-
H3	Qinghai	Sheep	3	G	-	G	-	-	-	-	T	-	T	-
H4	Qinghai, Tibet	Sheep	4	-	-	G	-	-	-	-	-	-	-	-
H5	Qinghai	Sheep	1	-	-	-	-	-	-	-	-	-	-	A
H6	Qinghai	Sheep	1	-	-	-	-	C	-	-	-	-	-	-
H7	Qinghai	Sheep	1	-	-	-	C	-	-	-	-	-	-	-
H8	Qinghai	Sheep	1	-	-	-	-	-	-	-	-	A	-	-
H9	Sichuan	Human	1	-	-	-	-	-	T	-	-	-	-	-
H10	Tibet	Sheep	1	-	-	-	-	-	-	C	-	-	-	-

[a]Number of isolates.

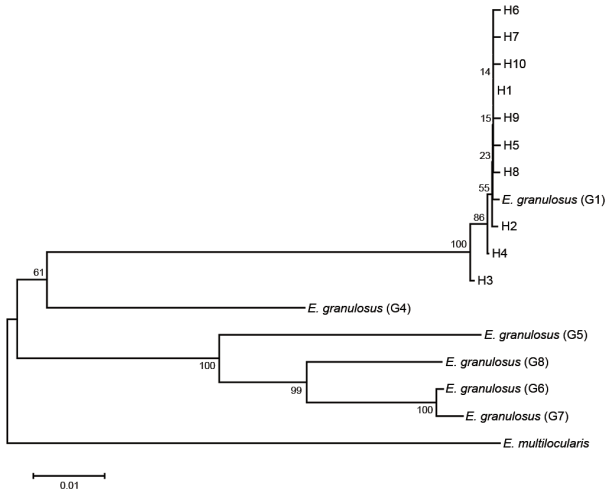

Fig. 2. Neighbour-Joining phylogenetic tree of complete mitochondrial *Echinococcus granulosus cytb* sequences showing the phylogenetic relationship of haplotypes, H1 to H10.

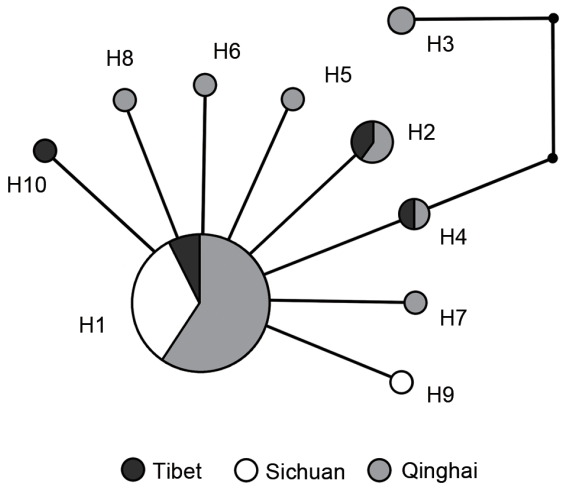

Fig. 3. Haplotype network for the 10 haplotypes (H1 to H10) identified among 45 isolates of *Echinococcus granulosus* collected from western China.

tity with a partial *cytb* gene sequence of a yak *E. granulosus* strain from China (AY278067) [17], and differed by only 1 nucleotide (a C/T substitution at the 263th site) from a cat *E. granulosus* strain found in Japan (AB622276) [19].

The phylogenetic tree showed that all isolates from the west of China were located on a single branch of the phylogenetic tree corresponding to *E. granulosus* genotype G1, and all haplotypes identified in this study were distinguished from each other (Fig. 2). The haplotype network of 45 isolates of *E. granulosus* revealed that H1 was the predominant haplotype (Fig. 3). H1 emitted 7 additional haplotypes and a branch containing 2 haplotypes (H3 and H4).

To date, a few studies have explored in detail genetic diversity of *E. granulosus* based on *cytb* gene. The partial nucleotide sequences of *cox1* (795 bp) and *cytb* (568 bp) have amplified from a presumptive echinococcal liver lesion of a yak in Sichuan Province, and the results demonstrated that the yak was infected with *E. granulosus* G1 [17]. Based on partial sequences of *cytb* gene (549 bp) and *rrnL* (570 bp), Li et al. [18] reported that 33 of 53 isolates obtained from humans in Sichuan and Qinghai were *E. granulosus* G1, while the remaining 20 isolates were *E. multilocularis*. In addition, *cytb* (1,068 bp) and *cox1* (1,069 bp) gene sequences revealed that the causative agent of CE in a cat from Russia was *E. granulosus* s.s. (G1) [19]. In our study, using complete sequences of *cytb* gene (1,068 bp) we identified all 45 isolates obtained from western China as belonging to the strain of *E. granulosus* genotype G1. The data obtained in this study are more representative and comprehensive than the partial sequence. The complete sequence may provide a more effective evidence to verify the validity of the genotypes of *E. granulosus*.

In recent years, studies based on nuclear and mitochondrial

genes indicated that the G1 genotype is the predominant epidemic strain in the west of China, while the G3 and G6 strains also exist. Based on *cox1* (789 bp) and *ef1*α (656 bp), the isolates of *E. granulosus* s.s. (n = 181) and *E. canadensis* G6 (n = 1) were identified successfully in Qinghai, Sichuan, and Xingjiang Province [2]. Phylogenetic analysis based on *nad1* (332 bp) and *cox1* (450 bp) revealed that 28 isolates of *E. granulosus* belonged to G1-G3 complex (*E. granulosus* s.s.), and 2 isolates were placed in G6-G10 complex (*E. canadensis*) in Qinghai [11]. Yan et al. [13] reported that 84 isolates of *E. granulosus* from the Tibetan plateau in China were sequenced for the complete *nad1* (894 bp) and *atp6* (513 bp) gene, and the results showed that 82 isolates were identified as G1 genotype, while 2 as G3 genotype. Our findings are similar to previous results that G1 genotype of *E. granulosus* is the main epidemic genotype in the west of China, but we did not detect G3 or G6 genotype in 45 samples of *E. granulosus*.

Our results showed low genetic diversity among the 45 isolates of *E. granulosus* from the west of China. In addition, the similarities of *cytb* genes of 45 isolates was very high (99.5-100%), indicating that the genetic diversity among the 45 isolates was small.

In conclusion, G1 genotype was identified to be the only genotype in all 45 samples of *E. granulosus* from Tibet, Sichuan, and Qinghai provinces. The complete *cytb* gene investigated in this study enriched the genetic information among *E. granulosus* genotypes from the west of China.

ACKNOWLEDGMENTS

The authors thank Zhongrong Jiang, Aiguo Yang, Shijin Deng, Li Guo, Dawa Tsering, Weishu Kong for their helpful assistance in collecting samples. This study was supported by grants from the National Key Technology R & D Program of China (no. 2006BAI06B09).

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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