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▣ ORIGINAL ARTICLE

Molecular Characterization of *Taenia multiceps* Isolates from Gansu Province, China by Sequencing of Mitochondrial Cytochrome *C* Oxidase Subunit 1

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Abstract: A total of 16 *Taenia multiceps* isolates collected from naturally infected sheep or goats in Gansu Province, China were characterized by sequences of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. The complete *cox1* gene was amplified for individual *T. multiceps* isolates by PCR, ligated to pMD18T vector, and sequenced. Sequence analysis indicated that out of 16 *T. multiceps* isolates 10 unique *cox1* gene sequences of 1,623 bp were obtained with sequence variation of 0.12-0.68%. The results showed that the *cox1* gene sequences were highly conserved among the examined *T. multiceps* isolates. However, they were quite different from those of the other *Taenia* species. Phylogenetic analysis based on complete *cox1* gene sequences revealed that *T. multiceps* isolates were composed of 3 genotypes and distinguished from the other *Taenia* species.

Key words: *Taenia multiceps*, cytochrome *c* oxidase subunit 1 (*cox1*), genotype, phylogeny, China

INTRODUCTION

Coenurosis, caused by the larval form of *Taenia multiceps*, is a parasitic disease of various livestock species, especially ruminants. Coenurosis is common in sheep and goats worldwide and also has public health concerns because of its infection in humans [1,2]. *T. multiceps* larval cysts are usually found in nervous system, including brain and spinal cord of many herbivores. The presence of cysts typically leads to neurological symptoms that cause the infected animals to die after some weeks from starvation. However, animals in most cases remain normal without clinical symptoms and the disease is diagnosed only after the death of the animals. *T. multiceps* is a taeniid cestode whose adult stage lives in the small intestine of definitive hosts such as dogs and other canids [3]. *T. multiceps* larvae infection was reported in countries where sheep and goats herding remain important components of local economies, including some areas of Europe, Africa, Americas, and Asia. In China, *T. multiceps* infection was reported in dogs in several provinces with infection rate between 2.3% and 14.6% [4,5]. Although coenurosis in animals was reported in more than 20 provinces of China, molecular identification of *T. multiceps* isolates was rarely recorded. Moreover, the molecular identification of *T. multiceps* isolates is of great importance for etiological, epidemiological, and phylogenetic studies.

Mitochondrial (mt) DNA is known to have a faster evolutionary rate than nuclear DNA and is generally inherited maternally in almost all metazoans [6,7]. The mt genome is considered to be clonal and rarely or never undergoes recombination. Sequences generated from the mt genome provide excellent molecular markers for defining population groups, for tracing the genetic history of an individual or a particular group of related individuals, and for reconstructing deep-branch taxonomic phylogenies. With respect to the molecular identification of cestodes, early studies using partial cytochrome c oxidase subunit 1 gene (*cox1*) and NADH dehydrogenase-1 gene helped to establish the population and genetic relationship of *T. multiceps* and other members of the Taeni-

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idae [8-10]. However, to our knowledge, there have been rare reports on molecular characterization of *T. multiceps* isolates in China. For this reason, we described in this paper an investigation of genetic variability among 16 *T. multiceps* isolates from Gansu Province, China by using sequences of *cox1* gene as genetic marker.

MATERIALS AND METHODS

Collection of *T. multiceps* isolates

Between May 2008 and June 2010, a total of 16 isolates of *T. multiceps* larval stage were obtained from naturally infected sheep or goats in Gansu Province which is situated in the northwestern part of China between the northern latitudes of 32° to 40° and eastern longitudes of 94° to 108°. Sample code, geographical origin, and host are shown in detail in Table 1. The protoscolices were removed from individual larval cysts, washed 3 times with saline and stored at -70℃ before extraction of genomic DNA.

Genomic DNA extraction and amplification by PCR

Genomic DNA was extracted from protoscolices using Genomic Tissue DNA Kit (Omega Biotek, Norcross, Georgia, USA) according to the manufacturer's recommendations. Genomic DNA concentrations were determined spectrophotometrically by GeneQuant 100 and the DNA samples were stored at -20℃ until used. The primers to amplify the complete *cox1* gene were designed according to the mitochondrial genome sequence of *T. multiceps* [11] as follows: PF 5′-GACTTTGTTTGAGTCATCTTATGTAA-3′ and PR 5′-GATTTACAAAATCTACATTTTAAA-3′. PCR (50 μl) was performed in 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer and 2 U *Taq* DNA polymerase (Takara, Tokyo, Japan) under the following cycling conditions: after an initial denaturation at 94℃ for 5 min, then 94℃ for 1 min (denaturation), 55℃ for 1 min (annealing), 72℃ for 1min (extension) for 35 cycles, followed by a final extension at 72℃ for 10 min. Samples without genomic DNA were included in each amplification run as negative controls. PCR products were separated on a 1% agarose gel and detected by ethidium bromide staining.

Table 1. *Taenia multiceps* isolates used in the present study

Sample code	Geographical origin	Host	Accession number of *cox1* gene
JT080526	Jingtai, Gansu	Sheep	JX535567
JT081008	Jingtai, Gansu	Sheep	JX535568
JT081204	Jingtai, Gansu	Sheep	JX535569
JT090318	Jingtai, Gansu	Sheep	JX535569
JT090104	Jingtai, Gansu	Sheep	JX535570
PL090603	Pingliang, Gansu	Sheep	JX535570
JT090115-1	Jingtai, Gansu	Sheep	JX535571
JT090115-2	Jingtai, Gansu	Sheep	JX535572
JT090115-3	Jingtai, Gansu	Sheep	JX535573
JT090603	Jingtai, Gansu	Sheep	JX535573
JT090331	Jingtai, Gansu	Sheep	JX535574
JT100124-1	Jingtai, Gansu	Sheep	JX535575
JT100124-2	Jingtai, Gansu	Sheep	JX535575
JT100202	Jingtai, Gansu	Sheep	JX535575
YJ100610	Yongjing, Gansu	Sheep	JX535576
ZJCH100610	Zhangjiachuan, Gansu	Goat	JX535576

DNA sequencing and analysis

All of the amplicons were ligated into pMD-18 T vector (Takara) and transformed to *Escherichia coli* DH5α competent cells. The positive colonies were sequenced with ABI PRISM 3730 Genetic Analyzer, and sequences were analyzed with DNAtools software. Further comparison with taeniid *cox1* gene sequences available in GenBank was made using the BLAST and Clustal W software. Phylogenetic analyses were performed using the neighbor joining (NJ), maximum parsimony (MP), and minimum evolution (ME) methods, and the Mega 5.05 program. The bootstrap value was set as 1,000 replications with a cutoff value of 50%.

RESULTS

PCR amplification of *cox1* gene

In the present study, 1 to 3 larval cysts were observed in the brain of naturally infected sheep, while 7 cysts were found in the brain of the infected goat. The characteristics and morphology of *T. multiceps* larvae were seen in all of the cysts containing clear fluid with numerous fertile protoscolices (Fig. 1). Using 16 isolates of *T. multiceps* protoscolices DNA as templates, a band of approximately 1.7 kb fragment was generated after PCR amplification of *cox1* genes. Each of the PCR products was ligated to pMD-18 T vector, and 3 colonies for each sample were sequenced on both direction and the consensus sequences were obtained.

Sequence analysis of *cox1* gene

After removal of the primer sequences, the *cox1* gene from each isolate was 1,623 bp in length encoding 540 amino acids. Sequencing results indicated that out of 16 *T. multiceps* isolates

10 unique *cox1* gene sequences were obtained with sequence variation of 0.12-0.68% and a total of 19 nucleotide variation sites. Some of the isolates had identical *cox1* gene sequences, such as isolates JT081204 and JT090318, isolates JT090104 and PL090603, isolates JT09115-3 and JT090603, isolates YJ100610 and ZJCH100610, and isolates JT100124-1, JT100124-2 and JT100202. The G+C contents of the *cox1* gene sequences of *T. multiceps* isolates were 29.9-30.4%. The percent identities among *T. multiceps* isolates from Gansu Province were more than 99% and showed a lesser degree of variability. Jingtai isolates JT081204 and JT090318 were identical with the corresponding sequence available in GenBank (GQ228818). When the 10 unique *cox1* gene sequences were translated into amino acids sequence, 7 unique protein sequences were obtained with sequence variation of 0.19-0.56% and a total of 6 amino acid variation sites. Amino acid sequences of isolates JT080526, JT090104, JT090115-2, and YJ100610 were identical to the corresponding sequence available in GenBank (ACK55903) and that of isolate JT081204 was identical to *T. multiceps cox1* available in GenBank (ACS37254).

When Blast analysis with *cox1* gene sequences was performed, more than 20 *T. multiceps* partial *cox1* gene sequences from 346 to 445 bp were also available in GenBank. Partial *cox1* gene sequences from the 10 unique sequences in this study could be further grouped into 4 unique sequences with variation rate of 0.25-0.75%. The first group included JT080526, JT090104, and JT100124-1, and was identical to the Turkish *T. multiceps* isolate tmtr01 (EF393620). The second group consisted of JT081204, JT090115-3, and JT090331, and was identical to the Italian *T. multiceps* isolate Tm2 (DQ309768). The third group included JT081008, JT090115-2, and YJ100610, and the last group was JT090115-1. Comparison of all the partial *cox1* gene sequences revealed 10 groups with variation rate of 0.25-4.55%.

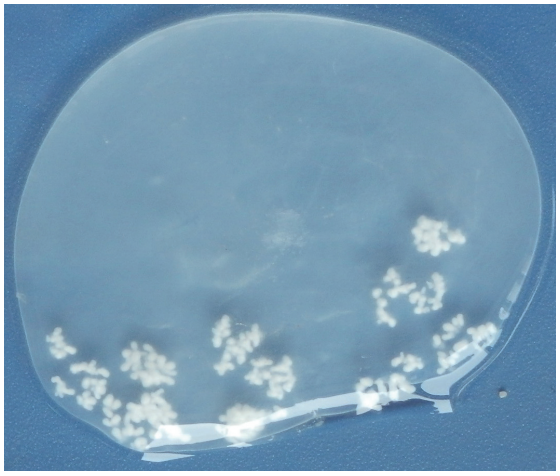

Fig. 1. *T. multiceps* larval cyst from the brain of a naturally infected sheep.

Phylogenetic analysis of taeniid *cox1* gene

The complete *cox1* gene sequences from the other *Taenia* mt genomes available in GenBank were selected and aligned. Accordingly, a phylogenetic tree was constructed with *cox1* gene sequences from this study and previously published mt sequences of related *Taenia* species, including *T. multiceps* (GQ228818 and FJ495086), *T. saginata* (AY684274), *T. asiatica* (AF445798), *T. solium* (AB086256), *T. hydatigena* (GQ228819 and FJ518620), *T. crassiceps* (AF216699), *T. pisiformis* (GU569096 and NC013844), and *T. taeniaeformis* (JQ663994 and FJ597547), with *E. granulosus* (AF297617) as the outgroup. The *cox1* gene sequences were 1,623 bp in length for all *T. multiceps* isolates, but it was 1,620 bp for *T. saginata, T. asiatica, T. solium, T. hydatigena,* and *T. pisiformis*. However, it was 1,614 bp and 1,635 bp for *T. crassiceps* and *T. taeniaeformis*, respectively.

Phylogenetic reconstruction of *cox1* gene from *Taenia* species using the NJ, MP, and ME analysis showed similar topology; 3 genotypes were revealed in the examined *T. multiceps* isolates

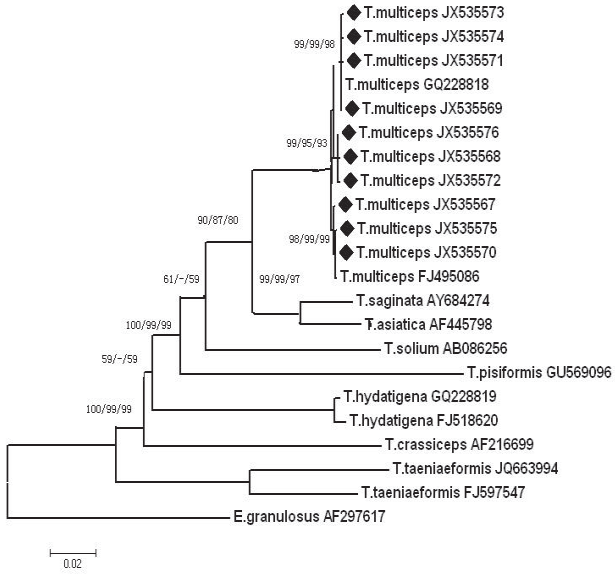

Fig. 2. Phylogenetic relationship among the examined *Taenia* species inferred by neighbor joining (NJ), maximum-parsimony (MP), and minimum-evolution (ME) analyses based on mitochondrial *cox1* sequences, using *Echinococcus granulosus* as the outgroup. The numbers along branches indicate bootstrap values resulting from different analyses in the order: NJ/MP/ME. Values less than 50 are given as '-'.

(Fig. 2). The first genotype consisted of JT090115-3, JT090115-1, *T. multiceps* GQ228818, JT081204, and JT090331. The second genotype included JT081008, JT090115-2, and YJ100610. The third genotype comprised of JT090104, JT101124-1, JT080526, and *T. multiceps* FJ495086. The *cox1* gene sequences of *T. multiceps* were more similar to those of *T. saginata* and *T. asiatica*, suggesting that the *cox1* gene could be a good genetic marker for molecular identification of *Taenia* species.

DISCUSSION

Cestodes of the family Taeniidae (taeniids) occur as adult tapeworms in the small intestine of carnivorous definitive hosts, and are transmitted to specific intermediate mammalian hosts where they develop as fluid-filled metacestodes in tissues. A number of *Taenia* species including *T. multiceps* are of medical and/or economic significance [12]. For the molecular characterization and phylogeny study of taeniid cestodes, many genetic markers have been used, including 28S rDNA, *cox1*, *nad1*, *nad4*, ITS rDNA, and nuclear protein coding genes such as *rpbz*, *pepck*, and *pold* [8-10,13-18]. The *cox1* gene is one of the most used markers for *Taenia* identification. Varcasia et al. [19] analyzed genetic variation within 40 *T. multiceps* isolates obtained from various locations of Sardinia. Partial sequences of *nad1* and *cox1* showed differences ranging from 1.27 to 2.54% and from 0.22 to 0.67%, respectively, and a conclusion was made that Sardinian sheep samples had at least 3 specific genetic variants [19]. However, comparison between the partial *cox1* sequences of the *T. multiceps* isolates from infected cattle from Erzurum Province, Turkey and other *T. multiceps* isolates available in GenBank showed nucleotide differences ranging from 0.2 to 2.6% [20].

Although coenurosis in sheep and goats were reported in China, the molecular identification of *T. multiceps* was rarely conducted. Recently, the complete mt genome of *T. multiceps* from Chinese isolates was reported and provided useful markers for studying the systematics, population genetics, and molecular epidemiology of *Taenia* [13,21]. Partial *cox1* genese of 3 larval isolates from goats in Hunan Province were sequenced and showed 97.9% identity to the *T. multiceps cox1* gene (EF393620), indicating that these parasites in goats belong to the larvae of *T. multiceps* [22]. The partial *cox1* gene sequences in this study could be divided into 3 types with variation rates of 0.25 to 0.75%, which is similar to the previous report [19]. The phylogenetic tree based on *cox1* gene sequences showed that all *T. multiceps* isolates constitute a branch consisting of 3 genotypes. *T. multiceps*, *T. saginata*, and *T. asiatica* showed the closest relationships, followed by *T. solium*, *T. pisiformis*, *T. hydatigena*, *T. crassiceps*, and the other *Taenia* species. Therefore, the *cox1* gene sequence can be used as a molecular marker for the interspecific identification of *Taenia* species.

When partial sequences of the *cox1* gene between *T. asiatica* and *T. saginata* were compared, the sequence differences varied from 2% in the conserved region (nucleotide position of 742-1,107) to 7% in the variable region (positions 25-340) [23]. It was suggested that partial *cox1* sequences might give a bias to analysis, but the complete sequence data should provide a more reliable result. In this study, *T. multiceps* metacestode (larval form) from naturally infected sheep or goat in 4 geographical origins in Gansu Province were genetically characterized for the first time with complete *cox1* gene sequences. We have detected some polymorphisms within the common 'sheep' isolates. It is evident that intraspecific variation existed in *T. multiceps*, whether such minor genetic variation has biological or epidemiologic significance warrants further study. In addition, for studying the population genetics of *T. multiceps* from sheep and goats in China, more isolates should be collected and analyzed. Further investigations will require more complete *cox1* gene sequences from *T. multiceps* isolates from different endemic regions in China and will determine whether there are morphological, developmental, or pathological differences between the genotypes.

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REFERENCES

1. Sharma DK, Chauhan PPS. Coenurosis status in Afro-Asian region: A review. Small Ruminant Res 2006; 64: 197-202.
2. Collomb J, Machouart M, Biava MF, Brizion M, Montagne K, Plé-

nat F, Fortier B. Contribution of NADH dehydrogenase subunit I and cytochrome C oxidase subunit I sequences toward identifying a case of human coenuriasis in France. J Parasitol 2007; 93: 934-937.
3. Scala A, Varcasia A. Updates on morphobiology, epidemiology and molecular characterization of coenurosis in sheep. Parassitologia 2006; 48: 61-63.
4. Wang CR, Qiu JH, Zhao JP, Xu LM, Yu WC, Zhu XQ. Prevalence of helminthes in adult dogs in Heilongjiang Province, the People's Republic of China. Parasitol Res 2006; 99: 627-630.
5. Dai RS, Li ZY, Li F, Liu DX, Liu W, Liu GH, He SW, Tan MY, Lin RQ, Liu Y, Zhu XQ. Severe infection of adult dogs with helminths in Hunan Province, China poses significant public health concerns. Vet Parasitol 2009; 160: 348-350.
6. Ballard JWO, Rand DM. The population biology of mitochondrial DNA and its phylogenetic implications. Annu Rev Ecol Evol Syst 2005; 36: 621-642.
7. Le TH, Blair D, McManus DP. Mitochondrial genomes of parasitic flatworms. Trends Parasitol 2002; 18: 206-213.
8. Gasser RB, Zhu X, McManus DP. NADH dehydrogenase subunit 1 and cytochrome *c* oxidase subunit I sequences compared for members of the genus *Taenia* (Cestoda). Int J Parasitol 1999; 29: 1965-1970.
9. Zhang L, Hu M, Jones A, Allsopp BA, Beveridge I, Schindler AR, Gasser RB. Characterization of *Taenia madoquae* and *Taenia regis* from carnivores in Kenya using genetic markers in nuclear and mitochondrial DNA, and their relationships with other selected taeniids. Mol Cell Probes 2007; 21: 379-385.
10. Lavikainen A, Haukisalmi V, Lehtinen MJ, Henttonen H, Oksanen A, Meri S. A phylogeny of members of the family Taeniidae based on the mitochondrial *cox1* and *nad1* gene data. Parasitology 2008; 135: 1457-1467.
11. Jia WZ, Yan HB, Guo AJ, Zhu XQ, Wang YC, Shi WG, Chen HT, Zhan F, Zhang SH, Fu BQ, Littlewood DT, Cai XP. Complete mitochondrial genomes of *Taenia multiceps*, *T. hydatigena* and *T. pisiformis:* additional molecular markers for a tapeworm genus of human and animal health significance. BMC Genomics 2010; 11: 447.
12. Hoberg EP. *Taenia* tapeworms: their biology, evolution and socioeconomic significance. Microbes Infect 2002; 4: 859-866.
13. Bowles J, McManus DP. Genetic characterization of the Asian *Taenia*, a newly described taeniid cestode of humans. Am J Trop Med Hyg 1994; 50: 33-44.
14. Gasser RB, Chilton NB. Characterisation of taeniid cestode species by PCR-RFLP of ITS2 ribosomal DNA. Acta Trop 1995; 59: 31-40.
15. de Queiroz A, Alkire NL. The phylogenetic placement of *Taenia* cestodes that parasitize humans. J Parasitol 1998; 84: 379-383.
16. Eom KS, Chai JY, Yong TS, Min DY, Rim HJ, Kihamia C, Jeon HK. Morphologic and genetic identification of *Taenia* tapeworms in Tanzania and DNA genotyping of *Taenia solium*. Korean J Parasitol 2011; 49: 399-403.
17. Knapp J, Nakao M, Yanagida T, Okamoto M, Saarma U, Lavikainen A, Ito A. Phylogenetic relationships within *Echinococcus* and *Taenia* tapeworms (Cestoda: Taeniidae): an inference from nuclear protein-coding genes. Mol Phylogenet Evol 2011; 61: 628-638.
18. Dai RS, Liu GH, Song HQ, Lin RQ, Yuan ZG, Li MW, Huang SY, Liu W, Zhu XQ. Sequence variability in two mitochondrial DNA regions and internal transcribed spacer among three cestodes infecting animals and humans from China. J Helminthol. 2012; 86: 245-251.
19. Varcasia A, Lightowlers MW, Cattoli G, Cancedda GM, Canu S, Garippa G, Scala A. Genetic variation within Taenia multiceps in Sardinia, Western Mediterranean (Italy). Parasitol Res 2006; 99: 622-626.
20. Avcioglu H, Yildirim A, Duzlu O, Inci A, Terim KA, Balkaya I. Prevalence and molecular characterization of bovine coenurosis from Eastern Anatolian region of Turkey. Vet Parasitol 2011; 176: 59-64.
21. Liu GH, Lin RQ, Li MW, Liu W, Liu Y, Yuan ZG, Song HQ, Zhao GH, Zhang KX, Zhu XQ. The complete mitochondrial genomes of three cestode species of *Taenia* infecting animals and humans. Mol Biol Rep 2011; 38: 2249-2256.
22. Dai RS, Li F, Liu W, Lin RQ, He SW, Liu Y. Prevalence and identification of *Coenurus* on goats in Xiang-xi region. Chin J Zoonoses 2009; 25: 391-394 (in Chinese).
23. Jeon HK, Eom KS. *Taenia asiatica* and *Taenia saginata*: genetic divergence estimated from their mitochondrial genomes. Exp Parasitol 2006; 113: 58-61.