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ORIGINAL PAPER

Analysis of cytochrome *c*-oxidase (*COI*) gene of mitochondrial DNA from the *Trichinella* spp. in China

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Abstract *Trichinella* species are parasitic nematodes of great economical and health importance. In this study, approximately 400 bp of the mitochondrial cytochrome *c*-oxidase (*COI*) gene from six species of *Trichinella* parasites from China and two reference species (T3 and T5) was sequenced and compared. The results showed that parasites from Yunnan, Hubei, and Dongbei (north-eastern) of China were very similar to the reference T1 isolated from Dongbei. The T2 parasite was, however, more closely related to the T5 (*T. murrelli*); and a parasite from Henan was very different from the rest of the parasites. These results suggested that genetic differences among most of the geographic strains in China existed. The Henan isolate may represent a new strain or species that requires further investigation.

Introduction

Trichinella is a parasitic nematode with its adult worm living in the intestine and its larvae parasitizing muscle tissues of a wide range of animals. *Trichinella* infects more than 150 species of animals, including human beings, swine, rodent, and canine. The newborn larvae L1 migrate, after emerging from female adults in the host intestine, to the striated skeletal muscle where they grow, encapsulating within muscle cells, and develop into an infective larvae stage L3 (or muscle larvae). They stay within transformed muscle cells until ingested by another host. The parasite causes a zoonosis called Trichinellosis. Eating raw or not fully cooked meat infected with *Trichinella* is the main cause of Trichinellosis outbreaks. Outbreaks of Trichinellosis have been reported in many countries (Gomez-Garcia et al. 2003; Gamble et al. 2005; Haim et al. 1997; Lopez Hernandez et al. 2001; Pozio 2007; Pozio and La Rosa 2000; Pozio et al. 1999, 2002, 2005; Barennes et al. 2008; Moller 2007; Ranque et al. 2000; Reiterova et al. 2007), including China (Wang and Cui 2001a, b; Wang et al. 1998, 2006, 2007; Chen et al. 1997; Cui and Wang 2001, 2005; Cui et al. 2006; Liu and Boireau 2002). Therefore, *Trichinella* not only can inflict heavy economic losses to the livestock husbandry, food industry, and meat exportation but is also a threat to human health. *Trichinella* T1 and T2 species as well as various geographic strains have been reported in China (Gasser et al. 1998; Liu et al. 2001; Wang et al. 1995; Liu et al. 2001). It is difficult to control the disease because of its diverse hosts and complex factors involved in parasite transmission.

Many molecular methods have been developed for genotyping and species identification of *Trichinella* parasites, including polymerase chain reaction (PCR) (Comes et al. 1996; Gasser et al. 1998; Zarlenga et al. 1999, 2001), microsatellite (Zarlenga et al. 1996), restriction fragment length polymorphism (RFLP) of internal transcribed spacer (ITS) (Kwon et al. 2001; De Bruyne et al. 2005; Rombout et al. 2001a) and RFLP of *COI* (Nagano et al.

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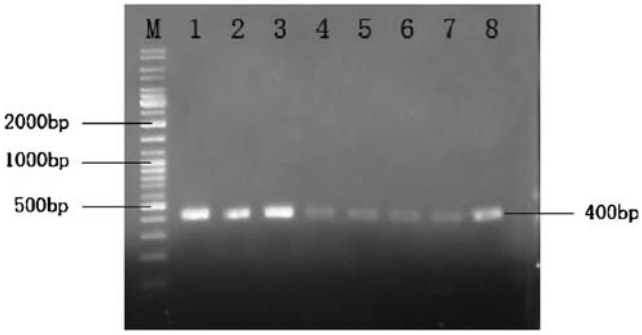

Fig. 1 Band of approximately 400 bp fragment obtained from each of parasite species or strain

1999), 5SrRNA (Van der Giessen et al. 2005; De Bruyne et al. 2005) and amplified fragment length polymorphism (AFLP) (Mikkonen et al. 2005), reverse line blot hybridization (Rombout et al. 2001b) and single-strand conformation polymorphism analysis (SSCP) (Gasser et al. 1998, 2005). The mitochondrial DNA has some important characteristics such as maternal inheritance and relatively higher substitution rate in some parts of the genome which can be useful for studying parasite relationship and evolution.

The mitochondrial *COI* gene is a subunit of cytochrome *c*-oxidase (subunit I to III) that exists in the cytoplasm.

Fig. 2 DNA sequences aligned using Bioedit and Clustal X

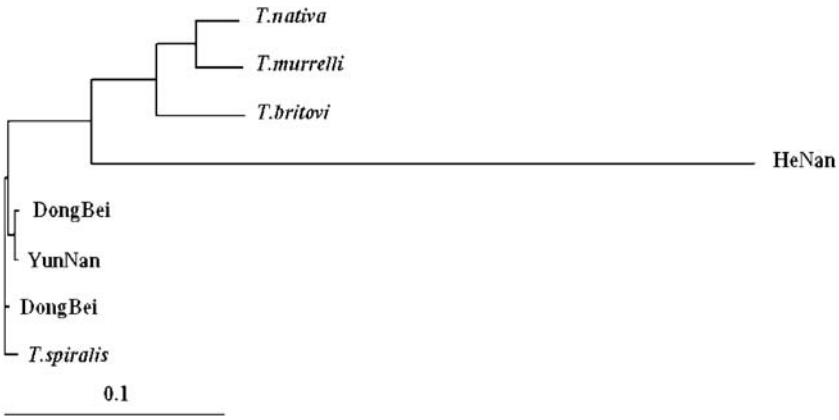

Fig. 3 Phylogenetic tree displayed using Treeview32

There have been no studies on mitochondrial cytochrome *c*-oxidase subunit I (*COI*) gene of *Trichinella* in China. In this study, we analyzed a fragment of *COI* gene from eight *Trichinella* species and geographical strains, including four strains from different endemic regions in China. Our data showed that the majority of *Trichinella* parasites were very similar to T1 species. One strain from Henan had very unique *COI* sequence that suggested a potential new species or strains.

Materials and methods

Trichinella isolates

A total of eight isolates were used in this study, including four reference species [*T. spiralis* (T1) and *T. native* (T2) were gifts from Prof. Mingyuan Liu (Jinlin University, China), *T. britovi* (T3), and *T. murrelli* (T5)] and four strains isolated from China [Henan (HN), Hubei (HB), Yunban (YN), Dongbei (DB)]. All isolates and T1 and T2 were maintained in laboratory mice (Kunming mouse) by serial passages, except T3 and T5 that were obtained from the International *Trichinella* Reference Center in Rome, Italy.

Collection of muscle larvae

Muscle larvae were isolated by digestion of infected animal carcasses in pepsin solution (1 g of pepsin and 1 mL of 36% HCl in 100 mL of distilled water) for 37°C overnight. The proportion of muscle and digestion solution was 1:10 (*v*/*v*). After the muscle was completely digested, the undigested debris were removed by passing the solution through a 150-μm mesh and then a 100-μm mesh filters. Physiological saline was used to wash the parasites settled at the bottom of the container several times until the solution became clear. Clean muscle larvae were collected after centrifugation.

Total DNA extraction

DNA was extracted from approximately 2,000 muscle larvae. Briefly, parasites were homogenized in 600 μL extraction buffer [100 mmol/L NaCl, 10 mmol/L Tris–Cl (pH 8.0), 25 mmol/L EDTA (pH 8.0), 0.5% (*w*/*v*) SDS] and incubated with 3 μL 20 μg/mL proteinase K at 50°C overnight. After phenol–chloroform extraction, DNA was precipitated with ethanol. The qualities of the genomic DNA samples were estimated using 1% agarose gel electrophoresis and OD_{260} measurement.

Polymerase chain reaction amplification

The mitochondrial cytochrome *c*-oxidase subunit I (*COI*) gene was amplified using two primers: cytcF (5′-GTTCT TTGGTCATCCAGAAGT-3′) and cytcR (5′-ACGACGTA GTAGGTGTCATGF-3′). The PCR mixture (total volume of 20 μL) consisted of 1.5 μL (concentration) of *Trichinella* DNA, 2 μL of 10x PCR buffer (Takara Biotechnology), 2 μL of 2 mM each deoxynucleotide triphosphate (MBI Fermentas), 0.1 μL *Taq* polymerase (5 U/μL; Takara Biotechnology), 1 μL of 20 μM for each primer, and 12.4 μL ddH_2O. Reactions were run in a Bio-Rad Thermocycler under the following conditions: 95°C for

Table 1 Sequence identity of matrix of *Trichinella* mitochondrial partial cytochrome *c*-oxidase subunit 1 gene from eight *Trichinella* strains/species

	HB	YN	DB	T1	T2	T5	T3	HN
HB	–							
YN	0.869	–						
DB	0.886	0.970	–					
T1	0.889	0.954	0.978	–				
T2	0.806	0.858	0.880	0.888	–			
T5	0.812	0.854	0.870	0.880	0.949	–		
T3	0.783	0.880	0.873	0.867	0.898	0.896	–	
HN	0.475	0.539	0.531	0.519	0.503	0.496	0.515	–

5 min for one cycle; 95°C for 30 s, 48°C for 30 s, and 72°C for 2 min for 39 cycles; followed by an 8-min extension step at 72°C. The PCR products were electrophoresed on 1% agarose gel and visualized under an ultraviolet transilluminator after staining with ethidium bromide.

DNA sequencing

The PCR products were purified using a Gel Extraction Mini Kit (Watson Biotechnologies) according to the manufacturer's instructions and were directly sequenced commercially. The DNA sequences were assembled and analyzed using the software Bioedit, Clustal X, and Treeview.

Results

Polymerase chain reaction amplification of cytochrome *c*-oxidase subunit I gene

We run a gradient PCR program to optimize the annealing temperature, annealing time, and extending time. A band of approximately 400 bp fragment was obtained from each of parasite species or strain (Fig. 1).

The PCR products were sequenced after purification. The length of the *COI* gene from each parasite was quite different with T1 having 376 bp, T2 having 374 bp, HN having 322 bp, HB having 415 bp, DB having 371 bp, YN having 362 bp, T3 having 367 bp, and T5 having 378 bp (Fig. 2).

Sequence analysis of cytochrome *c*-oxidase subunit I gene

The DNA sequences were aligned using Bioedit and Clustal X (Fig. 2), and a phylogenetic tree was displayed using Treeview32 (Fig. 3).

As shown in the Fig. 3 and Table 1, parasites from YN, DB, and HB were very similar to the T1 (*T. spiralis*) strain isolated from Dongbei, China. These parasites can be considered as one type. The T2 (*T. native*) strain was close to T5 (*T. murrelli*). Interestingly, the HN strain was quite different from the rest of the strains, suggesting a new species.

Discussion

This study investigated the relationship of six *Trichinella* isolates from different provinces in China using DNA sequences encoding cytochrome *c*-oxidase (*COI*). The *COI* gene is a mitochondrial gene encoding an important component in the parasite respiration chain. The evolution rate of the *COI* gene is generally more than ten times higher than a nuclear gene; and the gene is often used in species and strain identification. Because it is maternally inherited, recombination within the gene can be ignored, making the *COI* an ideal gene for studying parasite relationship and evolution.

Our results suggested that *Trichinella* parasites in China could be grouped into three types: one was the typical T1 species; a second one was closely related to T5; and a parasite from Henan was unique, possibly representing a new species or new strain, which requires more studies.

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References

Barennes H, Sayasone S, Odermatt P, De Bruyne A, Hongsakhone S, Newton PN, Vongphrachanh P, Martinez-Aussel B, Strobel M, Dupouy-Camet J (2008) A major trichinellosis outbreak suggesting a high endemicity of *Trichinella* infection in northern Laos. Am J Trop Med Hyg 78:40–44

Chen S, Li L, Wu C, Ye J (1997) Serological investigation on human *Trichinellosis spiralis* in Hubei Province of PR China. Southeast Asian J Trop Med Public Health 28(Suppl 1):107–109

Comes AM, Humbert JF, Cabaret J, Elard L (1996) Using molecular tools for diagnosis in veterinary parasitology. Vet Res 27(4–5):333–42 (Review)

Cui J, Wang ZQ (2001) Outbreaks of human trichinellosis caused by consumption of dog meat in China. Parasite 8:S74–S77

Cui J, Wang ZQ (2005) Epidemiological trends and control measures of trichinellosis in China. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 23(5 Suppl):344–8, 354

Cui J, Wang ZQ, Kennedy MW (2006) The re-emergence of trichinellosis in China? Trends in Parasitology 22:54–55

De Bruyne A, Yera H, Le Guerhier F, Boireau P, Dupouy-Camet J (2005) Simple species identification of *Trichinella* isolates by amplification and sequencing of the 5S ribosomal DNA intergenic spacer region. Vet Parasitol 132(1–2):57–61

Gamble HR, Pozio E, Lichtenfels JR, Zarlenga DS, Hill DE (2005) *Trichinella seudospiralis* from a wild pig in Texas. Vet Parasitol 132(1–2):147–150

Gasser RB, Zhu XQ, Monti JR, Dou L, Cai X, Pozio E (1998) PCR-SSCP of rDNA for the identification of *Trichinella* isolates from mainland china. Mol Cell Probes 12:27–34

Gasser RB, Hu M, El-Osta YA, Zarlenga DS, Pozio E (2005) Genetic analysis of *Trichinella* populations by 'cold' single-strand conformation polymorphism analysis. Vet Parasitol 132(1–2):23–26

Gomez-Garcia V, Hernandez-Quero J, Rodriguez-Osorio M (2003) Short report: human infection with *Trichinella britovi* in Granada, Spain. Am J Trop Med Hyg 68:463–464

Haim M, Efrat M, Wilson M, Schantz PM, Cohen D, Shemer J (1997) An outbreak of *Trichinella spiralis* infection in southern Lebanon. Epidemiol Infect 119:357–362

Kwon HS, Chung MS, Joo KH (2001) PCR-RFLP patterns of four isolates of *Trichinella* for rDNA ITS1 region. Korean Journal of Parasitology 39(1):43–48

La Rosa G, Marucci G, Zarlenga DS, Pozio E (2001) *Trichinella pseudospiralis* populations of the Palearctic region and their relationship with populations of the Nearctic and Australian regions. Int J Parasitol 31(3):297–305

Liu M, Boireau P (2002) Trichinellosis in China: epidemiology and control. Trends in Parasitology 18:553–556

Liu MY, Zhu XP, Xu KC, Lu Q, Boireau P (2001) Biological and genetic characteristics of two *Trichinella* isolates in China; comparison with European species. Parasite 8:S34–S38

Lopez Hernandez B, Velazquez de Castro MT, Galicia Garcia MD, Sabonet JC (2001) Outbreak of *Trichinella britovi* infection in Granada in the spring of 2000. Rev Esp Salud Publica 75:467–473

Mikkonen T, Koort JM, Bjorkroth KJ, Sukura A (2005) Testing of amplified fragment length polymorphism (AFLP) technique as a tool for molecular epidemiology of *Trichinella nativa*. Vet Parasitol 132(1–2):19–22

Moller LN (2007) Epidemiology of *Trichinella* in Greenland—occurrence in animals and man. Int J Circumpolar Health 66: 77–79

Nagano I, Wu Z, Matsuo A, Pozio E, Takahashi Y (1999) Identification of *Trichinella* isolates by polymerase chain reaction—restriction fragment length polymorphism of the mitochondrial cytochrome c-oxidase subunit I gene. Int J Parasitol 29:1113–1120

Pozio E (2007) World distribution of *Trichinella* spp. infections in animals and humans. Vet Parasitol 149:3–21

Pozio E, La Rosa G (2000) *Trichinella murrelli* n. sp: etiological agent of sylvatic trichinellosis in temperate areas of North America. J Parasitol 86(1):134–139

Pozio E, Owen IL, La Rosa G, Sacchi L, Rossi P, Corona S (1999) *Trichinella papuae* n.sp. (Nematoda), a new non-encapsulated species from domestic and sylvatic swine of Papua New Guinea. Int J Parasitol 29(11):1825–1839

Pozio E, Foggin CM, Marucci G, La Rosa G, Sacchi L, Corona S, Rossi P, Mukaratirwa S (2002) *Trichinella zimbabwensis* n.sp. (Nematoda), a new non-encapsulated species from crocodiles (*Crocodylus niloticus*) in Zimbabwe also infecting mammals. Int J Parasitol 32(14):1787–1799

Pozio E, Owen IL, Marucci G, La Rosa G (2005) Inappropriate feeding practice favors the transmission of *Trichinella papuae* from wild pigs to saltwater crocodiles in Papua New Guinea. Vet Parasitol 127(3–4):245–251

Ranque S, Faugere B, Pozio E, La Rosa G, Tamburrini A, Pellissier JF, Brouqui P (2000) *Trichinella pseudospiralis* outbreak in France. Emerg Infect Dis 6:543–547

Reiterova K, Kincekova J, Snabel V, Marucci G, Pozio E, Dubinsky P (2007) *Trichinella spiralis*—outbreak in the Slovak Republic. Infection 35:89–93

Rombout YB, Bosch S, Mandjes B, Homan W, Van der Giessen JW (2001a) Genetic diversity within the genus *Trichinella* as shown by cleavage fragment length polymorphism analysis. J Helminthol 75(1):67–72

Rombout YB, Bosch S, Van Der Giessen JW (2001b) Detection and identification of eight *Trichinella* genotypes by reverse line blot hybridization. J Clin Microbiol 39(2):642–646

Van der Giessen JW, Fonville M, Briels I, Pozio E (2005) Phylogenetic analysis of encapsulated and non-encapsulated *Trichinella* species by studying the 5S rDNA tandemly repeated intergenic region. Vet Parasitol 5 132(1–2):51–55

Wang ZQ, Cui J (2001a) The epidemiology of human trichinellosis in China during 1964–1999. Parasite 8:S63–S66

Wang ZQ, Cui J (2001b) Epidemiology of swine trichinellosis in China. Parasite 8:S67–S70

Wang H, Zhang Y, Lao W, Wu Z (1995) Restriction fragment length polymorphism (RFLP) analysis of genomic DNA of 5 strains of *Trichinella spiralis* in China. Chin Med Sci J 10:131–135

Wang ZQ, Cui J, Wu F, Mao FR, Jin XX (1998) Epidemiological, clinical and serological studies on trichinellosis in Henan Province, China. Acta Trop 71:255–268

Wang ZQ, Cui J, Xu BL (2006) The epidemiology of human trichinellosis in China during 2000–2003. Acta Trop 97:247–251

Wang ZQ, Cui J, Shen LJ (2007) The epidemiology of animal trichinellosis in China. Vet J 173:391–398

Zarlenga DS, Aschenbrenner RA, Lichtenfels JR (1996) Variations in microsatellite sequences provide evidence for population differences and multiple ribosomal gene repeats within *Trichinella pseudospiralis*. J Parasitol 82(4):534–538

Zarlenga DS, Chute MB, Martin A, Kapel CM (1999) A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. Int J Parasitol 29(11): 1859–1867

Zarlenga DS, Chute MB, Martin A, Kapel CM (2001) A single, multiplex PCR for differentiating all species of *Trichinella*. Parasite 8(2 Suppl):S24–S26