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Molecular Identification of a *Trichinella* Isolate from a Naturally Infected Pig in Tibet, China

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Abstract: The first human case with trichinellosis was reported in 1964 in Tibet, China. However, up to the present, the etiological agent of trichinellosis has been unclear. The aim of this study was to identify a Tibet *Trichinella* isolate at a species level by PCR-based methods. Multiplex PCR revealed amplicon of the expected size (173 bp) for *Trichinella spiralis* in assays containing larval DNA from Tibet *Trichinella* isolate from a naturally infected pig. The Tibet *Trichinella* isolate was also identified by PCR amplification of the 5S ribosomal DNA intergenic spacer region (5S ISR) and mitochondrial large-subunit ribosomal RNA (mt-lsrDNA) gene sequences. The results showed that 2 DNA fragments (749 bp and 445 bp) of the Tibet *Trichinella* isolate were identical to that of the reference isolates of *T. spiralis*. The Tibet *Trichinella* isolate might be classifiable to *T. spiralis*. This is the first report on *T. spiralis* in southwestern China.

Key words: *Trichinella spiralis*, taxonomy, molecular identification, Tibet, China

INTRODUCTION

Trichinellosis is a cosmopolitan zoonotic disease caused by the ingestion of raw or undercooked meat containing *Trichinella* larvae. Currently, taxonomy of the genus *Trichinella* encompasses 8 species (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli*, *T. nelsoni*, *T. papuae*, and *T. zimbabwensis*) and 4 additional genotypes ("*Trichinella* T6", "T8", "T9" and "T12") [1,2]. Of all the species and genotypes, *T. spiralis* is the most widely distributed, often found in domestic pigs, and the most common cause of human trichinellosis [3].

In China, the first human case with trichinellosis was reported in 1964 in Tibet [4]. From this observation until 2009, a total of 15 outbreaks, consisting of 187 cases and 12 deaths, were recorded in Tibet [5,6]. Although trichinellosis is an important zoonotic diseases and a serious public health concern in Tibet, the etiological agent of trichinellosis has been unclear. All *Trichinella* isolates from the patients' biopsy muscle tissues or pork have not been identified at the species level although it was assumed to be *T. spiralis*. The species identification of *Trichinella* isolates is of great importance for etiological, epidemiological, and phylogenetic studies.

The aim of this study was to identify the species of Tibet *Trichinella* isolate by multiplex PCR, then to analyze its genetic variation within *Trichinella* species by PCR-based methods using 5S ribosomal DNA intergenic spacer region (5S ISR) and mitochondrial large-subunit ribosomal DNA (mt-lsrDNA) as genetic markers.

MATERIALS AND METHODS

Collection of Tibet *Trichinella* isolates and experimental infection

In February 2009, a small familial outbreak of trichinellosis due to consumption of raw pork occurred in a village of Linzhi prefecture of Tibet, southwestern China. The raw pork samples of the residue posterior leg during this outbreak were collected, and examined by the compression method and histological sections; the encapsulated *Trichinella* larvae were found. The pork was cut into pieces and digested with 0.33% pepsin (1:31,000) - 1% HCl for 4 hr at 43°C [7,8]. The muscle larvae were recovered after artificially digesting the pork. After washing, individual muscle larvae were stored at -80°C until used. This Tibet *Trichinella* isolate was also maintained by serial passage in 6-week-old, SPF male Kunming mice (Experimental Animal Center of Henan Province, China) at 6- to 8-month

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intervals in our department (Department of Parasitology, Medical College, Zhengzhou University). The reference *Trichinella* isolates used in this study was *T. nativa* (ISS10) and *T. nelsoni* (ISS29), which were obtained from the *Trichinella* Reference Center (IRC; Rome, Italy), and Henan isolate of *T. spiralis* (ISS-534) from domestic pigs in Nanyang city of Henan Province, China.

Species identification of Tibet *Trichinella* isolate by multiplex PCR

For multiplex PCR, about 20 muscle larvae recovered from the naturally infected pork were each washed 3 times in Hank's Balanced Salt Solution, pelleted, and subjected to genomic DNA extraction utilizing a DNeasy Tissue Kit (Qiagen Inc., Valencia, California, USA). *Trichinella* larvae were identified at the species level by multiplex PCR, as previously described [9]. The PCR-amplified fragments were visualized by agarose gel electrophoresis (2% standard agarose).

PCR and sequencing of 5S ISR and mt-lsrDNA of Tibet *Trichinella* isolate

For further confirmation of molecular identification of the Tibet *Trichinella* isolate, the 5S ISR and mt-lsrDNA fragments of the Tibet isolate and reference *T. spiralis* Henan isolate were amplified using the following primers: 5′ GCGAATTCTTG-GATCGGAGACGGCCTG and 5′ GCTCTAGACGAGATGTCGT-GCTTTCAACG for 5SISR, 5′ WACAATGGTCCTTTCGT ACT and 5′ TGAGGACATTAAGGTAGC for mt-lsrDNA [10,11]. PCR products of the Tibet *Trichinella* isolate and reference *T. spiralis* Henan isolate were delivered to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China), which were purified and directly sequenced using 15 *p*mol of the PCR primers with an ABI PRISM 3730 Genetic Analyzer. The sequencing reagent was BigDye terminator v3.1. The primers used for sequencing were the same as those used for PCR amplification. The products of 2 independent amplification tubes for the 2 genes were completely sequenced on both strands to confirm the nucleotide sequences.

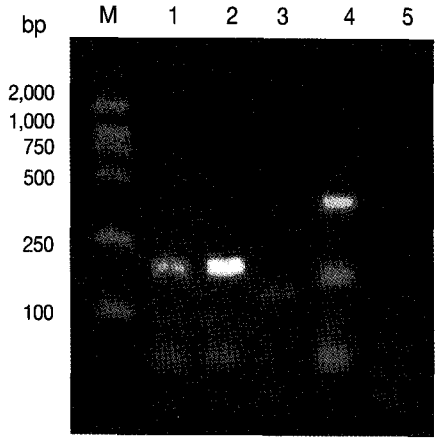

Fig. 1. Agarose gel separation of multiplex PCR products using DNA from the Tibet *Trichinella* isolate from a naturally infected pig. M, molecular weight markers. Lane 1, Tibet *Trichinella* isolate from a naturally infected pig. Lane 2, *T. spiralis* isolate (ISS-534) control. Lane 3, *T. nativa* isolate (ISS10) control. Lane 4, *T. nelsoni* isolate (ISS29) control. Lane 5, Double-distilled water control.

RESULTS

Morphological features

Examination of pork samples obtained from the posterior legs of the pig showed infection with the coiled intracellular *Trichinella* larvae. These pork samples were subsequently examined by the artificial digestion method, and the coiled and motile muscle larvae were collected with an intensity of infection of 12 larvae per gram of muscle tissues.

Multiplex PCR

Multiplex PCR revealed amplicon of the expected size (173 bp) for *T. spiralis* in assays containing larval DNA from the Tibet *Trichinella* isolate from a naturally infected pig (Fig. 1).

PCR analysis of 5S-ISR DNA and mt-lsrDNA

Results of agarose gel electrophoresis with primers derived from 5S ISR and mt-lsrDNA using genomic DNA from different *Trichinella* isolates are shown in Fig. 2. Two DNA fragments (749 bp and 445 bp) of the Tibet *Trichinella* isolate were iden-

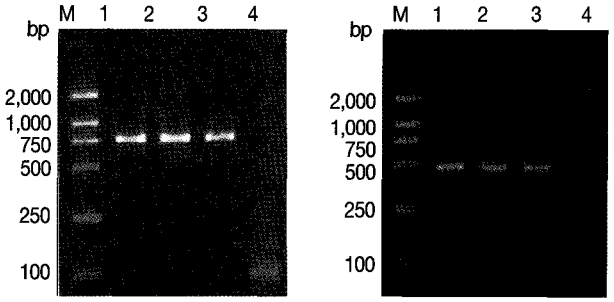

Fig. 2. Agarose gel separation of PCR products of 5S ISR (left) and mt-lsrDNA (right) of the Tibet *Trichinella* isolate. *M, molecular* weight markers. Lanes 1-2, Tibet *Trichinella* isolate. Lane 3, *T. spiralis* isolate (ISS534) control. Lane 4, Double-distilled water control.

tical to that of the reference isolate of *T. spiralis*. The BLAST analysis with DNA sequences of the Tibet *Trichinella* isolate showed that 5S ISR DNA and mt-lsrDNA regions of the Tibet isolate most closely resembled those of *T. spiralis* sequences available in GenBank. Homologous analysis showed that the consistence of 5S ISR sequences of the Tibet *Trichinella* isolate and *T. spiralis* (accession number AY009946) was 100%; the consistency of mt-lsrDNA sequences of the Tibet *Trichinella* isolate and *T. spiralis* (accession number AY851277) was 99.75%, with just 1 nucleotide difference (53 A-G).

DISCUSSION

Two species of *Trichinella* (*T. spiralis* from domestic pigs in central and northeastern areas and *T. nativa* from dogs in northeastern areas) have been described in China [12,13]. However, the *Trichinella* isolates in southwestern China were not identified at the species level. Our results showed that the *Trichinella* isolate from a naturally infected pig in Tibet was identified as *T. spiralis* by multiplex PCR and PCR using 5S ISR as well as mt-lsrDNA as genetic markers. This is the first species identification of *Trichinella* isolates in southwestern China.

At present, *Trichinella* is a serious food safety risk to consumers of porks in southwestern China [6]. In Tibet, pigs are often raised in backyards under poor hygienic condition or in open areas where pigs are pastured freely and feed on raw waste products or animal carcasses. The local minor ethnic groups slaughter their self-raised pigs at home without veterinary inspection [14]. In addition, because of the high altitude with 3,000 m above the sea level and low air pressure, the meat is often insufficiently cooked; the ethnic groups enjoy eating raw meat. So, outbreaks of trichinellosis occurred often in Tibet. From 2004 to 2009, all of 4 outbreaks occurred in Tibet were caused by eating raw or undercooked porks [6]. Our results showed that the etiological agent of human trichinellosis in Tibet is *T. spiralis*, and suggested that domestic pigs play an important role in the maintenance of the life cycle of *T. spiralis*. Hence, implementation of a *Trichinella* control programme, together with general health education, will be necessary for the local ethnic villagers [15].

In conclusion, the *Trichinella* isolate from a naturally infected pig in Tibet was identified as *T. spiralis*. This is the first report of species identification of *Trichinella* isolates in southwestern China.

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