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Early detection of *Trichinella spiralis* DNA in the feces of experimentally infected mice by using PCR

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ABSTRACT

The aim of this study was to detect *Trichinella spiralis* DNA in mouse feces during the early stages of infection using PCR. The target gene fragment, a 1.6 kb repetitive sequence of *T. spiralis* genome, was amplified by PCR from feces of mice infected with 100 or 300 larvae at 3–24 h post infection (hpi) and 2–28 dpi. The sensitivity of PCR was 0.016 larvae in feces. The primers used were highly specific for *T. spiralis*. No cross-reactivity was observed with the DNA of other intestinal helminths. *T. spiralis* DNA was detected in 100% (12/12) of feces of mice infected with 100 or 300 larvae as early as 3 hpi, with the peak detection lasting to 12–24 hpi, and then fluctuating before declining gradually. By 28 dpi, the detection rate of *T. spiralis* DNA in feces of the two groups of infected mice decreased to 8.33% and 25%, respectively. PCR detection of *T. spiralis* DNA in feces is simple and specific; it might be useful for the early diagnosis of *Trichinella* infection.

1. Introduction

Trichinellosis is a serious foodborne parasitic zoonosis acquired by eating raw or undercooked meat contaminated with infective larvae of the nematode genus *Trichinella* (Murrell and Pozio, 2011). From 2004 to 2009 in China, 15 outbreaks of human trichinellosis affected 1387 people and caused 4 deaths (Cui et al., 2011). Trichinellosis is not only a public health hazard that affects patients but also represents an economic problem for the pork industry (Cui et al., 2013). Unfortunately, early diagnosis of trichinellosis is difficult because its clinical symptoms are nonspecific (Dupouy-Camet et al., 2002; Cui et al., 2015). Definitive diagnosis of trichinellosis requires either larval detection in a biopsy or serum specific anti-*Trichinella* antibodies (Gottstein et al., 2009). Muscle biopsies are not sensitive enough for diagnosis of trichinellosis during the early stages of infection. Instead, the most commonly used serological method for diagnosis of trichinellosis is an ELISA with excretory-secretory (ES) antigens of *Trichinella spiralis* muscle larvae (ML) (Gamble et al., 2004). However, the problem with detection of anti-*Trichinella* antibodies is the occurrence of a high rate of false negative results during the early stage of infection (Liu et al., 2013a). Several studies have shown that 100% detection of IgG antibodies is not possible for at least 1–3 months after initial infection with the parasite (Bruschi et al., 1990; Wang et al., 1998). There is an obvious lag time (window period) between clinical symptoms and positive serology during the intestinal stages of trichinellosis. Additionally, the ML ES antigens also have cross-reaction with sera from patients with other helminthiases (e.g., schistosomiasis, paragonimiasis, cysticercosis, or anisakiasis) (Cui et al., 2015; Dea-Ayuela et al., 2001; Yera et al., 2003), and the cross-reaction depends of the antigen quality (Escalante et al., 2004; Gamble et al., 1988).

Trichinella circulating antigen (CAg) is an excretory secretory antigen produced by live worms, which directly enters the peripheral blood circulation and appears earlier than anti-*Trichinella* antibodies. Detecting CAg could be a possible diagnostic marker during early *Trichinella* infection. However, serum levels of *Trichinella* CAg are usually quite low (Liu et al., 2013a; Wang et al., 2012), and the detection rate of CAg in sera of patients with clinical trichinellosis was only 30–50% (Nishiyama et al., 1992). Hence, current serological methods for detecting CAg cannot be used to diagnose early stages of trichinellosis (Gamble et al., 2004). PCR has been widely used to amplify *T. spiralis* DNA for detection of circulating larvae in host blood samples (Caballero-Garcia and Jimenez-Cardoso, 2001; Robert et al., 1996; Uparanukraw and Morakote, 1997), but larval detection rates are low and are associated with infection intensity and the examination time after infection (Li et al., 2010b).

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After ingestion, *T. spiralis* ML are released from the nurse-cells in the stomach and then, develop into intestinal infective larvae (IIL) at 0.9 h post-infection (hpi). After that, the larvae invade the intestinal epithelium cells and develop into adults after undergoing four molts within 31 hpi (Campbell, 1983; Liu et al., 2013b). Adult worms live in the intestinal mucosa and persist for 10–20 days in mice and rats or 4–6 weeks in humans (Bell, 1998). The female adults produce newborn larvae at 5 days post-infection (dpi), and one female can produce approximately 1500 larvae during 5–10 days. The intestinal stage is terminated by a sudden or gradual loss of worms. The larval cuticles are shed during molting of the larvae. The ES products of larvae and adults along with newborn larvae and dead adults are passed in the feces during the intestinal stage of the *Trichinella* infection. Additionally, about 75% of an oral challenge dose of larvae leave the intestine within 24 hpi in hosts primarily infected with *T. spiralis* (Alizadeh and Wakelin, 1982; Carlisle et al., 1990). Hence, *T. spiralis* DNA may be present and detectable in the feces of hosts during early *Trichinella* infection. The aim of this study was to detect *Trichinella* DNA in mouse feces in the early stage of infection by using PCR.

2. Materials and methods

2.1. Parasite and experimental animals

Trichinella spiralis isolate (ISS534) was obtained from a domestic pig in Nanyang city of Henan province, China. The isolate was maintained by serial passage in Kunming mice in the laboratory of Department of Parasitology, Medical College, Zhengzhou University. Specific pathogen-free (SPF) female BALB/c mice, six weeks old, were purchased from the Experimental Animal Centre of Henan Province, China. Experimental procedures in this study for *T. spiralis* infection were approved by the Life Science Ethics Committee of Zhengzhou University (no. 2011–016).

2.2. Experimental infection and collection of feces sample

T. spiralis ML were recovered from infected mice at 42 dpi by digesting carcasses using 1% pepsin (1:3000) and 1% hydrochloric acid as previously described (Gamble et al., 2000; Li et al., 2010a). The recovered ML were enumerated under microscope. Twenty-four BALB/c mice were divided into two groups of 12 animals per group; each group of mice was infected orally with 100 or 300 ML. Each infected mouse was kept in a single cage under specific-pathogen-free conditions with sterilized food and water. The cage floors were bare and without sawdust. Fecal samples from each mouse were collected at 3, 6, 12, 18, and 24 hpi, and then daily from 2 dpi to 28 dpi. Feces from uninfected mice were used as negative controls. The fecal material was frozen at −80 °C until needed. At 35 dpi, the carcass of each infected mouse was individually digested by artificial digestion, and the larvae were collected and counted from each mouse. The results are expressed as the mean larvae ± SD of each infected group.

2.3. DNA extraction

Total genomic DNA was extracted from individual fecal samples of infected mice using the *EasyPure*® Genomic DNA Kit according to manufacturer instructions (TransGen Biotech Co., Beijing China). Briefly, 20 mg of feces were homogenized with a glass tissue grinder, then added to a 1.5 ml sterile microcentrifuge tube containing 100 μl of lysis buffer 2 and 20 μl of Proteinase K. These tubes were incubated at 55 °C for 24 h, and then centrifuged at 12,000 × *g* for 5 min. The supernatant was transferred to a microcentrifuge tube containing 500 μl of binding buffer 2, vortexed for 5 s, and incubated at room temperature for 10 min. After centrifugation, the lysate was transferred to a spin column, centrifuged at 12,000 × *g* for 30 s, and the flow-through discarded. The spin column was put in a sterile microcentrifuge tube, 100 μl of elution buffer (preheated to 65 °C) was added to the column matrix, incubated at room temperature for 1 min, and centrifuged at 12,000 × *g* for 1 min to elute the genomic DNA. DNA was stored at −20 °C for subsequent analysis by PCR.

2.4. Positive and negative control samples

Two sets of positive control samples were prepared. One control contained DNA of 1 larva together with 20 mg of feces from uninfected mice, and the other control had DNA of only 1 larva of *T. spiralis*. Negative control samples were the DNA isolated from feces of uninfected normal mice.

2.5. PCR amplification

Primers were derived from a 1.6 kb repetitive sequence of the *T. spiralis* genome (de Vos et al., 1988) and had the following sequences: 5′-CTTGTAAAGCGGTGGTGCGTA-3′ and 5′-CATAGAGAGGCAACATTACCT-3′ (Dick et al., 1992; Li et al., 2012). The repetitive element sequence (accession number: X06625.1) was downloaded from NCBI GenBank (http://www.ncbi.nlm.nih.gov/nuccore/X06625.1) for primer design using Primer Premier V5.0 software (http://www.premierbiosoft.com/primerdesign/index.html). The PCR was performed in a 20 μl volume with 7 μl distilled water, 1 μl DNA, 10 μl 2 × *EasyTaq*® PCR SuperMIx (TransGen Biotech Co., Beijing, China), 1 μl forward primer and 1 μl reverse primer. The cycling protocol was as follows: 94 °C for 3 min, 35 cycles at 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, followed by an extra extension step at 72 °C for 5 min. All fecal samples were tested in duplicate.

2.6. Determination of sensitivity of PCR detection

Total genomic DNA of 300, 10, 5, 2 and 1 larvae of *T. spiralis* were extracted as templates for PCR amplification. The DNA of 1 larva is diluted from 1:2 to 1:512. The DNA of 1 larva added to 20 mg of uninfected mouse feces and the larval DNA-feces combination was extracted again. The larval DNA-feces combination was also diluted from 1:2 to 1:512. Every dilution of the DNA samples was used for PCR amplification. PCR products were analyzed by agarose gel 2% (w/v) electrophoresis with ethidium bromide in the gel for subsequent UV visualization.

2.7. Determination of specificity of PCR detection

Genomic DNA of one intestinal worm of *Enterobius vermicularis, Trichuris trichiura, Hymenolepis nana,* and *Spirometra erinaceieuropaei* were extracted as templates for PCR amplification. The PCR products were analyzed as previously described above.

2.8. Statistical analysis

Data were expressed as the mean ± standard deviation. All statistical analyses of the data were performed using SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL). The PCR detection rate between two groups of infected mice was determined by a paired-sample *t*-test. The difference between PCR detection rates at different days post-infection were compared using the Fisher's exact test. The independent-samples *t*-test was used to compare the differences of the number of recovered muscle larval burden