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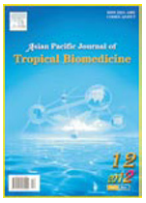

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Application of a real-time PCR method for detecting and monitoring hookworm *Necator americanus* infections in Southern China

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PEER REVIEW

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Comments

This study developed a real-time PCR method for the hookworm *N. americanus*. The method is based on the ITS sequence and proved to be specific for this parasite species. The authors have also shown that it is more sensitive than standard PCR. It can be applied to epidemiology studies to detect hookworm prevalence and infection intensities in infected individuals.

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ABSTRACT

Objective: To develop a quantitative PCR method for detecting hookworm infection and quantification. **Methods:** A real-time PCR method was designed based on the intergenic region II of ribosomal DNA of the hookworm *Necator americanus*. The detection limit of this method was compared with the microscopy-based Kato-Katz method. The real-time PCR method was used to conduct an epidemiological survey of hookworm infection in southern Fujian Province of China. **Results:** The real-time PCR method was specific for detecting *Necator americanus* infection, and was more sensitive than conventional PCR or microscopy-based method. A preliminary survey for hookworm infection in villages of Fujian Province confirmed the high prevalence of hookworm infections in the resident populations. In addition, the infection rate in women was significantly higher than that of in men. **Conclusions:** A real-time PCR method is designed, which has increased detection sensitivity for more accurate epidemiological studies of hookworm infections, especially when intensity of the infection needs to be considered.

KEYWORDS

Hookworm, Detection method, Epidemiology, Infection rate

1. Introduction

Hookworm infection, caused by the soil-transmitted helminths *Necator americanus* (*N. americanus*) and *Ancylostoma duodenale* (*A. duodenale*), is one of the most common chronic infections among the world's poorest populations living in developing countries, with up to 576 million people currently infected[1,2]. Of the two principal species of hookworm infecting humans, *N. americanus* is found throughout sub-Saharan Africa, tropical regions of the Americas, South China, and Southeast Asia[2]. Although rarely directly lethal, hookworm infection is a significant factor of morbidity, associated with chronic anemia and protein malnutrition. When measured in disability-adjusted life years, hookworm infection is the second most important parasitic infection, just behind malaria[3]. In Southern China, hookworm is one of the three major soil-transmitted helminths[4,5]. According to a report in 2003, there was an estimate of 39 million people being infected. The predominant hookworm species in Southern China like Hainan province is *N. americanus*[6,7].

Diagnosis of hookworm infection is usually based on

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detection of hookworm eggs in human fecal samples[8]. However, due to similarity in morphology, hookworm eggs cannot be reliably identified to species. Although the method of coproculture for obtaining third-stage larvae allows more accurate microscopic identification of the worms, this approach is laborious, unreliable, and requires skilled personnel[9]. Given that accurate specific diagnosis of human hookworm infections is essential for understanding the epidemiology and designing targeted control strategies, molecular methods for the identification of hookworms and other strongylid nematodes have been developed[9,10]. Interspecific differences in nuclear DNA and mitochondrial DNA (mtDNA) have been employed to design PCR-based diagnostic methods. Using differences in cox1 gene of the mtDNA, Zhan *et al.* achieved specific identification of the two major hookworm species-*N. americanus* and *A. duodenale*[11]. The differences in the cAMP-dependent protein kinase gene also allowed the development of a PCR-restriction fragment length polymorphism method for species differentiation[12]. However, methods based on the internal transcribed spacers (ITS-1 and 2) of the nuclear ribosomal DNA (rDNA) have received the widest applications because of their lower mutation rates within species and substantial differences between species[13,14]. Species-specific PCR primers have been designed to differentiate *N. americanus* from *A. duodenale*, and the method proved useful in epidemiological survey of hookworm infections[15,16]. In recent years, quantitative real-time PCR has been used in parasitology to monitor the prevalence of geohelminths[17,18]. Here, we attempt to develop a real-time PCR method to specifically identify *N. americanus* infection in humans and evaluate this method in epidemiological surveys of hookworm infection in Southern China.

2. Materials and methods

2.1. Hookworm DNA

To collect *N. americanus* hookworm samples, human stool samples were collected in a suburban district of Xiamen City, Fujian Province, China. The samples were examined using a flotation method with saturated NaCl, and helminth eggs were examined microscopically. Five grams of a positive stool sample were used for coproculture at 27 °C, and *N. americanus* third-stage larvae were identified morphologically using published keys and descriptions[19]. A total of 50 *N. americanus* third-stage larvae were collected for DNA extraction using the Wizard™ DNA isolation kit (Promega, Madison, USA). For comparison, genomic DNA of *Strongyloides stercoralis* (*S. stercoralis*) and *Ancylostoma caninum* (*A. caninum*) were extracted similarly from samples maintained in our laboratory.

2.2. PCR and sequencing of ITS-2 of *N. americanus* rDNA

For PCR amplification of the ITS-2 sequence of *N. americanus* rDNA from a Southern China isolate, two primers NAF (5′-TGT TCA GCA ATT CCC GTT TA-3′) and NAR (5′-GTC CTT CAC ATT GTC TCC GT-3′) were designed based on a GenBank sequence (accession number Y11734). A 50 μL PCR reaction was assembled with 5 μL of 10×PCR buffer, 3 μL of 25 mmol/L $MgCl_2$, 4 μL of 2.5 mmol/L dNTPs, 1.5 μL of each primer (10 μmol/L), 1 U of Taq polymerase (TaKaRa), and 10 ng of nematode DNA. PCR was performed using the following conditions: initial denaturing at 95 °C for 5 min followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, 40 seconds at 72 °C, and final extension at 72 °C for 7 min. Five microliters of the PCR reaction were electrophoresed in 15 g/L agarose gel. The band was excised from the gel and DNA was extracted using the QIAquick gel extraction kit (Qiagen, Valencia, USA). The DNA was cloned into pMD18-T and the insert was sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI3100 Genetic Analyzer. DNA sequence was BLAST searched in the GenBank.

2.3. Development of a real-time PCR method

A real-time PCR method of detection for hookworm *N. americanus* was developed based on the SYBR Green quantification kit (Tiangen Biotech, Beijing, China) on a Rotor Gene 3000 PCR machine. The real-time PCR program included 40 cycles of 15 seconds at 95 °C, 15 seconds at 56 °C, and 18 seconds at 72 °C. The reactions were set up in a final volume of 20 μL with 8 μL of 2.5×RealMasterMix, 0.8 μL of each primer (10 μmol/L), and 2 μL of the template DNA. Initially, we tested the recombinant plasmid DNA in 10-fold serial dilutions with concentrations ranging from 10^2 to 10^8. Fluorescent PCR products were detected at 72 °C. The threshold cycle, C_t, was defined as the cycle number at which the change of fluorescence in the reaction exceeds ten standard deviations above the background fluorescence. Background fluorescence was calculated as the mean fluorescence between cycle 3 and 6. To determine the sensitivity and reproducibility of the real-time PCR method, we used a dynamic range of the template concentrations. Each concentration was tested for at least six times and covariance (standard deviation/mean) of the C_t was determined.

To further determine the detection sensitivity, *N. americanus* eggs were harvested, washed three times with physiological saline and the egg number was counted under a microscope. A total of 1, 5, 10, 50, and 100 *N. americanus* eggs were added to separate microcentrifuge tubes containing 200 mg of fecal materials from a healthy person. DNA was isolated from the mixture using the QIAamp DNA Stool Kit and subjected to real-time PCR analysis.