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A specific PCR assay for the diagnosis of *Clonorchis sinensis* infection in humans, cats and fishes

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ABSTRACT

Clonorchiasis caused by *Clonorchis sinensis* is a fish-borne parasitic disease which is endemic in a number of countries. Using the sequences of the internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) of *C. sinensis* as genetic markers, a pair of *C. sinensis*-specific primers was designed and used to establish a specific PCR assay for the diagnosis of *C. sinensis* infection in humans, cats and fish. This approach allowed the specific identification of *C. sinensis* after optimizing amplification conditions, with no amplicons being amplified from related heterogeneous DNA samples, and sequencing of amplicons confirmed the identity of the sequences amplified. The detection limit of this assay was 1.03 pg of adult *C. sinensis*, 1.1 metacercariae per gram of fish filet, and a single egg in human and cat feces. The PCR assay should provide a useful tool for the diagnosis and molecular epidemiological investigation of clonorchiasis in humans and animals.

1. Introduction

Clonorchiasis caused by *Clonorchis sinensis* is a fish-borne trematode infection which is endemic in China, Korea, Japan, and other Southeast Asian countries, with approximately 35 million people being infected globally, of whom approximately 13 million were in China [1]. Snails, freshwater fishes or shrimps are intermediate hosts of *C. sinensis*, and humans, cats, dogs and pigs could serve as definitive hosts for *C. sinensis*. In mainland China, approximately 140 species of freshwater fish and four species of shrimp have been recognized as second intermediate hosts for *C. sinensis*. Recent analyses of epidemiological data in China suggest that clonorchiasis is having an increased human health impact due to the increased consumption of raw freshwater fish [2].

The specific identification of *C. sinensis* is a prerequisite for studying its epidemiology, population biology and genetic variability, and is also crucial for the diagnosis and control of clonorchiasis in humans and animals. Microscopic morphological examination of *C. sinensis* adults, eggs and metacercariae is the traditional approach for the identification of *C. sinensis*. However, this method is time-consuming, labor intensive and has limitation in its capacity to differentiate between closely taxa of the families Opisthorchiidae, Heterophyidae and Lecithodendriidae [1,3,4].

To overcome the diagnostic limitations associated with conventional parasitological methods, previous studies have shown that PCR-based techniques provide useful alternatives for the specific identification and diagnosis of *C. sinensis* [3–9]. However, prior to the present study, there had been no reports of specific PCR assay for the identification and diagnosis of *C. sinensis* infection in humans, cats and fishes in China. A number of previous studies have indicated that the first and/or second internal transcribed spacers (ITS-1, ITS-2) of nuclear ribosomal DNA (rDNA) provide accurate genetic markers for the identification of many parasites [9–12]. Hence, the objective of the present study was to establish, using genetic markers in the ITS-1 and ITS-2, a PCR tool for the specific diagnosis of *C. sinensis* infection in humans, cats, and fishes.

2. Materials and methods

2.1. Samples

Adult trematodes of *C. sinensis* were collected from naturally infected cats from Guangdong province, China. Fish (*Pseudorasbora*

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Table 1
Clonorchis sinensis samples and heterologous reference samples used in the present study.

Species	Sample code	Stage	Host	Geographical origin	No. of samples
Clonorchis sinensis	CsGZ4-10	Adult	Cat	Guangdong, China	7
	CsM1-12	Metacercaria	Fish	Guangdong, China	12
	CsFH1-16	Egg	Human	Guangdong, China	16
	CsFC1-26	Egg	Cat	Guangdong, China	26
Schistosoma japonicum	SJ	Adult	Rabbit	Hunan, China	1
Fasciola hepatica	FH	Adult	Cattle	Guizhou, China	1
Fasciola gigantica	FG	Adult	Cattle	Guangxi, China	1
Orientobilharzia turkestanicum	OT	Adult	Cattle	Heilongjiang, China	1
Toxocara canis	TC	Adult	Dog	Guangdong, China	1
Ancylostoma caninum	AC	Adult	Dog	Guangdong, China	1
Toxocara cati	TCA	Adult	Cat	Guangdong, China	1
Trichuris suis	TS	Adult	Pig	Guangdong, China	1
Oesophagostomum dentatum	OD	Adult	Pig	Guangdong, China	1
Opisthorchis viverrini	OV	Adult	Hamster	Laos	1

parve) samples confirmed to be infected with *C. sinensis* metacercariae by microscopy, and human and cat fecal samples from infected humans and cats (the presence of *C. sinensis* eggs were confirmed by fecal flotation and microscopic examination) were also used. The samples used in the present study, together with their sample codes, developmental stages, hosts and geographical origins are listed in Table 1. Also, some common trematodes and nematodes infecting humans and animals, namely *Opisthorchis viverrini*, *Schistosoma japonicum*, *Fasciola gigantica*, *Fasciola hepatica*, *Orientobilharzia turkestanicum*, *Toxocara canis*, *Ancylostoma caninum*, *Toxocara cati*, *Trichuris suis* and *Oesophagostomum dentatum* were included as 'heterologous control' samples (Table 1).

2.2. Extraction of genomic DNA and quality evaluation

Total genomic DNA from individual adult worms of *C. sinensis*, those 'heterologous control' samples, as well as infected (with *C. sinensis* metacercariae) and non-infected (without *C. sinensis* metacercariae) fish muscle was extracted by sodium dodecyl sulfate/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega), and eluted into 60 μl H_2O according to the manufacturer's recommendations [7]. The extraction of genomic DNA from human and cat fecal samples followed essentially a previous description [13].

In order to evaluate the quality of the DNA samples, for *S. japonicum*, *F. gigantica* and *F. hepatica*, the mitochondrial cytochrome *c* oxidase subunit 1 fragment was amplified by PCR using oligonucleotide primers JB3 (forward: 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (reverse: 5′-TAAAGAAAGAACATAATGAAAATG-3′) [14]. For *O. turkestanicum* and *T. cati*, the ITS fragments were amplified using primes BD1 (forward: 5′-GTCGTAACAAGGTTTCCGTA-3′) and BD2 (reverse: 5′-TTATGCTTAAATTCAGCGGGT-3′) [15], while for *C. sinensis*, *T. canis*, *A. caninum*, *T. suis*, *O. dentatum* and *O. viverrini*, the ITS fragments were amplified using primers NC5 (forward: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse: 5′-TTAGTTTCTTTTCCTCCGCT-3′) [16].

2.3. Design of species-specific primers and optimization of specific PCR assays

Based on comparison of the ITS-1 and ITS-2 sequences of *C. sinensis*, *O. viverrini* and *O. felineus* (GenBank™ accession numbers EU038125, AF408147 and DQ456828), a pair of primers CStjd1 (5′-TGGCCTGACTGGCTGGCCGG-3′) and CStjd2 (5′-CGGCACCCCACACACATACA-3′) was designed to amplify the partial ITS of *C. sinensis*. The PCR conditions were optimized for the specific amplification of *C. sinensis* ITS fragments by varying the annealing temperatures and magnesium concentrations. Eventually, PCR reactions (in a volume of 25 μl) were performed in 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each of dNTP, 50 pmol of each primer, and 0.625 U *Taq* polymerase (Takara) in a thermocycler (Biometra) under the following conditions: 94 °C for 5 min (initial denaturation), followed by 30 cycles of 94 °C for 30 s (denaturation), 61 °C for 30 s (annealing), 72 °C for 30 s (extension) and a final extension of 72 °C for 5 min. Samples with non-infected fish muscle DNA or without any genomic DNA were included in each PCR run as negative controls. The amplicons (4 μl) produced in each PCR were separated by electrophoresis in 1.0% (w/v) agarose gel, stained with ethidium bromide, and photographed using a gel documentation system (UVItec).

2.4. Assessment of specificity and sensitivity of the specific PCR assay

The *C. sinensis*-specific primers were also evaluated for specificity using DNA samples representing those 'heterologous control' samples: *O. viverrini*, *S. japonicum*, *F. gigantica*, *F. hepatica*, *O. turkestanicum*, *T. canis*, *A. caninum*, *T. cati*, *T. suis* and *O. dentatum* (Table 1). For evaluation of sensitivity, the smallest amount of *C. sinensis* genomic DNA detectable by the specific PCR assay was estimated by amplification of 10-fold serial dilutions of one genomic DNA sample (103 ng/μl) from an adult *C. sinensis* specimen. To determine the minimum number of *C. sinensis* metacercariae in infected fish detectable by the specific PCR assay, 30 mg of non-infected (without *C. sinensis* metacercariae) fish filet was spiked with 1 *C. sinensis*

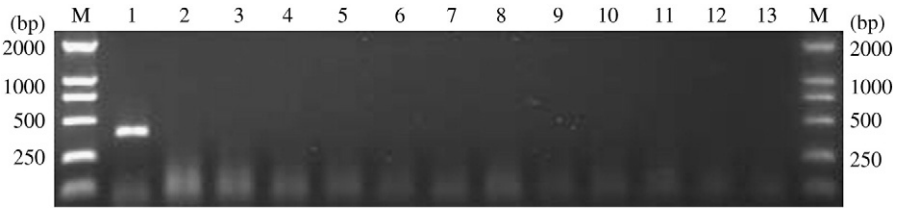

Fig. 1. Agarose gel electrophoresis of specific amplification of the internal transcribed spacers (ITS) using *Clonorchis sinensis*-specific primers. Lanes 1–11 represent PCR products amplified from genomic DNA extracted from *C. sinensis*, *Opisthorchis viverrini*, *Schistosoma japonicum*, *Fasciola gigantica*, *Fasciola hepatica*, *Orientobilharzia turkestanicum*, *Toxocara canis*, *Ancylostoma caninum*, *Toxocara cati*, *Trichuris suis* and *Oesophagostomum dentatum*, respectively. Lane 12 represents PCR products amplified from genomic DNA extracted from uninfected fish, and lane 13 represents no-DNA control. M represents a DNA size marker (ordinate values in bp).