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SHORT COMMUNICATION

Sensitive and species-specific detection of *Clonorchis sinensis* by PCR in infected snails and fishes

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Abstract In the present study, PCR procedures have been established for a rapid and easy preparation of DNA of parasite stages from the intermediate hosts, i.e. sporocysts and rediae in snails and metacercariae in fishes. Primers have been developed, which enable a highly sensitive and species-specific detection of *Clonorchis sinensis*.

Introduction

Liver flukes of the family Opisthorchiidae with the human pathogenic species *Clonorchis sinensis*, *Opisthorchis viverrini* and *O. felineus* cause severe fish-borne diseases in Southeast Asia and eastern parts of the countries of the former Soviet Union (Mehlhorn et al. 1983; Rim 1986). The human infections occur by eating raw or undercooked cyprinoid fishes harbouring infectious metacercariae. The worms inhabit the bile ducts and provoke hepatobiliary damages like cholangitis, which may lead to cholangiocarcinomas (Schwartz 1980; Rim 1986; Mehlhorn 2001). The opisthorchiid flukes have an aquatic life cycle, using freshwater snails as first and cyprinid fishes as second intermediate hosts, and final hosts are fish-eating mammals including humans.

To date, it remains difficult to record infection intensities in snails and fishes, and it is difficult to determine the parasite stages to the species level since they appear morphologically very similar to other digenea. Furthermore, only a very small percentage of snails can be found to be infected when testing the shedding of cercariae (Adams et al. 1993; Haas et al. 1990). Fish that harbour only a few metacercariae cannot reliably be recognized as infected.

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Preparation of snails with rediae

The method for the detection of sporocysts and rediae in snails has the advantage that very few steps have to be carried out, and that only inexpensive chemicals are required.

Each snail is collected in a 2-ml Eppendorf tube, and the shell is crushed by using clean sticks. An amount of 1.5 ml of 25 mM potassium hydroxide is added to the tube and the snail's soft body is lysed by incubation at about 95°C for 15 min. After a brief centrifugation to sediment the shell particles, about 10 µl of a saturated aqueous solution, cresol red, is added, and the supernatant is neutralized by the addition of 1 N HCl. The indicator dye appears violet at alkaline pH and becomes yellow when an appropriate pH $\leq$7.5 is reached. Of this sample, 1 µl is added to a PCR assay volume of 50 µl. The muscular food of the snail may not be entirely lysed during the short incubation. However, the rediae reside in the hepatopancreas and are easily freed from this soft tissue. The rediae are completely lysed by alkaline treatment, and the parasite DNA is efficiently liberated. It has been amply evaluated in the present study that it is not necessary to apply methods for further purification of the DNA, e.g. by using commercial kits.

PCR has been carried out with samples from *Bithynia fuchsiana* infected with *C. sinensis* rediae, and it was found that the snail lysate does not inhibit the amplification reaction. The procedure enables a reliable detection of

infected snails. To determine the inhibitory potential of the lysates, 1 μl of lysate from uninfected snails was added to the PCR assay, together with serial diluted genomic DNA from *C. sinensis*. The results reveal a detection limit of the primer pair CS1/CS2 of 10^{-13} g DNA. Obviously, the reported technique removes inhibitory substances of the snail tissue because the detection limit with parasite DNA without the presence of snail lysate was identical (Fig. 1). This means that there is a detection limit of 1.5×10^{-10} g DNA of rediae from an infected snail using a preparation volume of 1.5 ml. Thus, it appears that practically any infected snail can be recognized as being positive.

Detection of metacercariae in fish

Conventionally, metacercariae isolated from fish are detected by light microscopy. However, it is often difficult to differentiate unambiguously metacercariae of *C. sinensis* from those of related human pathogenic species of the genera *Opisthorchis* and *Heterophyes* and also from other less known and less pathogenic digenean species. All mentioned digenean metacercariae form ovoid cysts and have thick-walled, ovoid excretory vesicles filled with dark corpuscles. To determine the specimens reliably, it may be necessary to infect susceptible mammals with the metacercariae to obtain adult worms.

The preparation procedure of metacercariae from fishes for PCR is rapid and simple. It is not required to carry out further procedures for the extraction of DNA, e.g. by use of phenol-chloroform or even more complex purification methods. Inhibitors contained in fish samples are removed by simple washing steps, whereby they are diluted to a value that enables the PCR to reliably work. The aim of the new method was to develop an easy test to find *C. sinensis* metacercariae in fish without microscopical inspection.

Two- to three-gram filets were taken from *Pseudorasbora parva*, finely minced, and then given in a 50-ml Falcon tube, which contains artificial gastric juice consisting of 0.9% pepsin, 0.7% HCl, and 1% Triton X-100. Detergent Triton X-100 was shown to improve the dissolution of the fish muscles and did not destroy the metacercarial cysts. This mixture was incubated at 38–40°C and occasionally shaken by hand. Digestion was complete within 45–60 min. Then, the samples were briefly centrifuged to sediment the metacercariae. The sediment was washed twice by removing the supernatant and filling it up with water. Subsequently, the supernatant was removed but left a residual volume of 1–1.5 ml. The disruption of the metacercariae and the liberation of the DNA were achieved by homogenization with a tissue homogenizer (Omni TH220, Omni International, Marietta, GA, USA) at 10,000 rpm for 1 min. After a brief centrifugation, the supernatant was boiled in a water bath for 5 min to inactivate the pepsin and, subsequently, was neutralized by the addition of 1 N potassium hydroxide. Neutralization was carried out with the aid of cresol red as indicator dye. Cresol red appears yellow at acid pH and becomes violet when an appropriate pH of around ≥7.5 is reached. Two microliters of this sample was added to 50 μl PCR assay volume.

To demonstrate the validity and reliability of the reported preparation of metacercariae DNA from fish for PCR, the procedure was repeated 10 times. Fresh fish filet from 10 uninfested *P. parva*, bred parasite-free in the laboratory, were inoculated with one metacercaria of *C. sinensis* (Fig. 2). The results indicate that the developed PCR-based method is a suitable tool for the detection of *C. sinensis* in infected fish. This method is very sensitive and suitable to detect only one metacercaria per fish sample. PCR-based methods have, in contrast to conventional techniques, a higher potential for the differentiation of digenea species, which is essential for epidemiological studies.

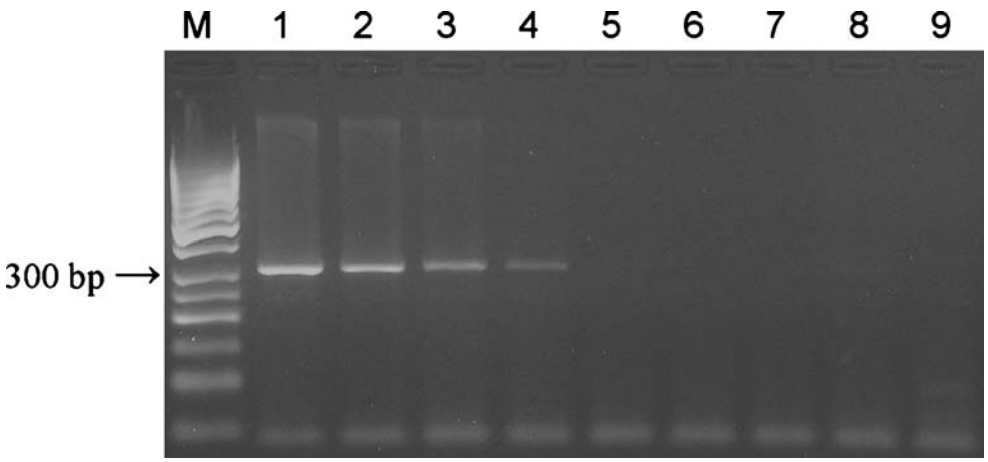

Fig. 1 Sensitivity of the CS1/CS2 primer system with purified DNA from *C. sinensis*. *Lane M*, DNA size marker; *lane 1*, 10^{-10} g; *lane 2*, 10^{-11} g; *lane 3*, 10^{-12} g; *lane 4*, 10^{-13} g; *lane 5*, 10^{-14} g; *lane 6*, 10^{-15} g; *lane 7*, 10^{-16} g; *lane 8*, 10^{-17} g genomic DNA; *lane 9*, negative control

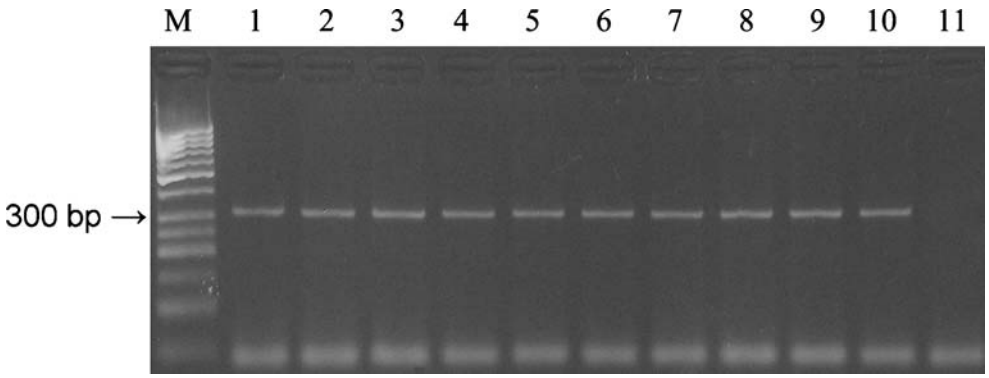

Fig. 2 Results of the detection of metacercariae of *C. sinensis* from fish by the present PCR method. One metacercaria was artificially inoculated into each 3 g fish meat prior to processing the samples. *Lane M*, DNA size marker; *lanes1–10*, samples from infected fish; *lane 11*, negative control containing no metacercaria

Primers specific for *C. sinensis*

A suitable primer pair for the specific detection of *C. sinensis* has been developed from the internal transcribed spacer 2 (ITS2) of the ribosomal DNA. For this, sequences of several related opisthorchiid species have been examined for differences Müller et al. (2006). The primer pair consists of the CS1 primer 5′-CGAGGGTCGGCTTATAAAC-3′ as a forward primer and the CS2 primer 5′-GGAAAGT TAAGCACCGACC-3′ as a reverse primer.

For establishing the PCR for the detection of rediae in snails or metacercariae in fishes, adult *C. sinensis* (stored in 70% ethanol, originating from Korea) were used as source material. To this, the DNA extraction of the liver flukes was carried out with the commercially available QIAamp DNA mini kit (Qiagen, Hilden, Germany), and the extracted DNA was stored at 4°C.

In order to examine the PCR system for cross-reactivity, DNA from the closely related liver flukes *Opisthorchis viverrini*, *O. felineus*, *Pseudamphistomum truncatum*, and *Metorchis xanthosomus* were extracted in the same way. In addition, DNA from the Digenea *Echinostoma caproni*, *Fasciola hepatica*, *Schistosoma mansoni*, *Holostephanus dubenini*, and *Paracoenogonimus ovatus* were tested as the DNA from intermediate hosts snails *B. fuchsiana* and *Melanoides tuberculata* and the cyprinid fishes *P. parva* and *Carassius auratus auratus*, respectively.

The PCR assays were carried out with a final volume of 50 µl with 1× PCR buffer containing Tris–HCl, KCl, $(NH_4)_2SO_4$ and 1.5 mM $MgCl_2$; pH 8.7 (Qiagen) and 1.25 U Taq DNA polymerase (Qiagen), 200 µM of each dNTP and 0.4 µM of each CS primer. PCR assays were performed in a PTC-0150 MiniCycler (MJ Research, Watertown, MA, USA) with the following cycling conditions: initial denaturation step at 94°C for 3 min, three-step cycling by denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min. The number of cycles conducted was 40. A final extension step was carried out at 72°C for 10 min before cooling the reaction mix to 4°C.

The sensitivity of the primers was determined by diluting genomic *C. sinensis* DNA at which a dilution up to an amount of 10^{-13} g could be detected (Fig. 1). Reports on molecular methods to detect opisthorchiid liver flukes are scarce. To our knowledge for *C. sinensis*, only one study with PCR has been published (Le et al. 2006). There, a mitochondrial-based multiplex PCR for the identification of *C. sinensis* and *O. viverrini* was described, and 7.8×10^{-10} g of genomic parasite DNA has been found to be the detection limit. The primer pair developed for *C. sinensis* in the present study is, however, much more sensitive and required only 1×10^{-13} g DNA.

In order to test the specificity of the PCR system, the DNA was isolated from 10 specimens of *C. sinensis* originating from Korea. The PCR always yielded a single DNA fragment of a typical product size of 315 base pairs (bp). The examination of DNA from closely related opisthorchiid liver fluke species, other digenea and various intermediate hosts mentioned above showed no cross-reactions with the primers specific for *C. sinensis.*

PCR may contribute to an unambiguous identification of *C. sinensis*, while this is difficult by light microscopy. The present techniques may enable a better tracking of the relevant pathways of transmissions by identifying host species and infection reservoirs. With respect to the expanding worldwide trade of fresh water fish from aquacultures, it is desirable to have a means for control inspections for fish in order to avoid importation of fish being infected with *C. sinensis* metacercariae (WHO 1995).

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