Detection of *Opisthorchis viverrini* in experimentally infected bithynid snails and cyprinoid fishes by a PCR-based method

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SUMMARY

A PCR procedure for the detection of *Opisthorchis viverrini* in experimentally infected bithynid snails and cyprinoid fishes was developed. This procedure was based on primers designed from a pOV-A6 specific probe sequence giving a 330 base-pair product. The detection was accomplished in host tissue homogenates to which a single cercaria or metacercaria was introduced. PCR can detect as little as a single cercaria artificially inoculated in a snail or a single metacercaria artificially inoculated in a fish sample. The method gave a 100% positivity rate for all infected snails or fishes. The method did not yield a 330 base-pair amplified product with other digenean fluke DNAs such as *Haplorchis taichui*, *Centrocestus* spp., *Echinostoma malayanum*, *Fasciola gigantica*, animal schistosomes, *Paragonimus heterotremus* or *Haplorchoides* spp. The assay has great potential for application in epidemiological surveys of both snail and fish intermediate hosts as well as for investigation of foodborne parasites in freshwater fishes.

Key words: *Opisthorchis viverrini*, PCR, detection, bithynid snail, cyprinoid fish.

INTRODUCTION

Opisthorchiasis, an infection caused by a liver fluke, *Opisthorchis viverrini*, is endemic along the Mekong River Basin e.g. Laos, Cambodia and Thailand. It is estimated that the number of current human infections is in the order of 9×10^6 cases (WHO, 1995). The disease has become more known in developed countries with the influx of Asian immigrants (Woolf *et al*. 1984; Schwartz, 1986). In Thailand, a recent survey shows that 21·5% of the population are infected (Jongsuksuntigul & Imsomboon, 1998). Infection occurs when raw or inadequately cooked infected freshwater fish are ingested. The parasites can produce morbidity with symptoms like abdominal pain, dyspepsia and fatigue. In heavily infected cases, pyogenic cholangitis, biliary calculi, obstructive jaundice and even cholangiocarcinoma can develop (Schwartz, 1980; Harinasuta, Riganti & Bunnag, 1984; Pungpak *et al*. 1994).

The life-cycle of *O. viverrini* is complex involving 2 intermediate hosts (bithynid snails and cyprinoid fishes) and 1 definitive host (humans and other fish-eating mammals) (Wykoff *et al*. 1965). From parasitological surveys in any defined freshwater bodies in Thailand, only 0·11% of snails were estimated to be infected, while up to 25–100% of fishes may contain metacercariae (Vichasri, Viyanant & Upatham, 1982; Brockelman *et al*. 1986; Waikagul, 1998). However, the parasitological technique is only reliable when the intensity of infection is high and when the procedure is performed by experienced laboratory personnel. In addition, the microscopical examinations for the presence of cercariae in snails or metacercariae in fishes are laborious and time consuming. Even experienced personnel can make a false positive diagnosis if the intermediate hosts are concomitantly infected with other trematodes. Typically, an underestimation of the true prevalence of infection in snails results because some snails harbour pre-patent infections that remain undetected by the cercarial shedding method. We have sought to develop a reliable technique for determining the presence of *O. viverrini* in infected snails and fishes, one that is based exclusively on PCR methodology. The method was derived from the recent development of a sensitive and specific PCR method using a pair of primers named OV-6F/OV-6R for the detection of *O. viverrini* in

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infected hamsters (Wongratanacheewin *et al*. 2001). The primers were designed based on the pOV-A6 specific DNA probe sequence (Sermswan *et al*. 1991) which gave a 330 base-pair product. The probe sequence was obtained from a highly repeated sequence of *O. viverrini* DNA which showed no significant homology to other related parasites such as *Clonorchis sinensis*, *Paragonimus siamensis*, minute intestinal flukes or any other sequences of parasite DNAs in the GenBank (NCBI-GenBank Flat File Release 129.0; April 15, 2002). The PCR method can detect a single *O. viverrini* egg in artificially inoculated feces or as little as 2×10^{-17} ng *O. viverrini* genomic DNA. Based on this information, we developed a PCR technique not only for the detection of *O. viverrini* in experimentally infected bithynid snails, but also in experimentally infected cyprinoid fishes. The technique could prove to be of practical diagnostic value for those interested in checking for *O. viverrini* contamination in fish intended for human consumption and is well suited for epidemiological surveys.

MATERIALS AND METHODS

Breeding and rearing of bithynid snails

Pure and young laboratory bred of *Bithynia siamensis goniomphalus*, used for maintenance of the life-cycle of *O. viverrini* were cultured and reared in the laboratory aquarium of the Applied Malacology Centre, Faculty of Tropical Medicine, Mahidol University. The culture and snail rearing methods of Liang & Kitikoon (1980) and Kruatrachue *et al*. (1982) were followed throughout.

Experimental animals

Golden Syrian hamsters (*Mesocricetus auratus*) of both sexes aged 1–5 months old were used as the experimental definitive hosts of *O. viverrini*. These hamsters were maintained under standard laboratory conditions in an Animal Section at the Faculty of Medicine, Khon Kaen University. Hamsters were housed in groups of 4 or 5 in plastic box cages and provided with rodent chow and water *ad libitum*. The maintenance and care of all animal experiments complied with the ethic regulations set by the Animal Experimentation Committee, Khon Kaen University.

Cyprinoid fish, *Puntius gonionotus*, 2–3 cm in length, with an approximate age of 2 months, were purchased from the Department of Fisheries, Ministry of Agriculture and Co-operatives, Thailand. They were kept at least 1 week in the laboratory for acclimatization before starting the experiment. Random samples of each newly bought lot of fishes were examined for parasites and all of them were found free from any parasites.

Experimental infection of snails and fishes and maintenance of the O. viverrini *life-cycle*

The pure bithynid snails, *B. s. goniomphalus*, were allowed to eat the *Opisthorchis* ova from infected hamsters. After about 2 months, the snails were checked for the presence of cercariae by shedding. The cercariae were used to infect the fishes (50–200 cercariae per 1 fish). Infected cyprinoid fish, *P. gonionotus*, with an infection period of at least 21 days were required for full maturation of metacercariae. The fishes were then checked for *O. viverrini* infection by examination for the presence of metacercariae at the caudal fin under a stereomicroscope. The infective metacercariae were harvested from the fishes and fed to each hamster by gastric intubation. Fecal specimens of the individual hamsters were checked for parasite eggs after about 2 months. Hamsters that were positive for *O. viverrini* ova in their feces were kept as stock for the maintenance of the *O. viverrini* life-cycle. The free cercariae or metacercariae were kept and artificially inoculated in tissue of individual snails or individual caudal fins of fishes (approximately 0·1 g) for determination of the capability of detection by PCR test. The whole bodies of infected snails as well as caudal fins (approximately 0·1 g) cut from infected fishes were kept at $-20\,^{\circ}$C for PCR detection.

Source of parasites and DNA preparation

Fresh or frozen adult worms of *O. viverrini*, *Haplorchis taichui*, *Centrocestus* spp., *Fasciola gigantica*, *Echinostoma malayanum*, and *Paragonimus heterotremus* as well as metacercariae of *Haplorchoides* spp. and cercariae of animal schistosomes were separately extracted and purified using the Nucleospin Tissue kit (Machenery-Nagel GmbH & Co., Duren, Germany) under the recommended conditions. The DNA was resuspended in 5 mM Tris–HCl, pH 8·5 and used as positive control DNA as well as for specificity evaluation of the PCR amplification.

Standardization of detection

To determine the capability of detection by PCR, non-infected ground snail tissue equivalent to 1 snail, or the chopped caudal fin of non-infected cyprinoid fish (approximately 0·1 g) were separately prepared. Then aliquots of each snail were separately inoculated with increasing amounts of 1, 5, 10 and 30 *O. viverrini* cercariae while individual caudal fin samples were separately inoculated with increasing amounts of 1, 2, 4 and 8 *O. viverrini* metacercariae.

For specificity determination of the detection method, 1 ng of genomic DNA from each of *Centrocestus* spp., *H. taichui*, *F. gigantica*, *E. malayanum*, *P. heterotremus*, *Haplorchoides* spp. and animal schistosomes were used in the PCR.

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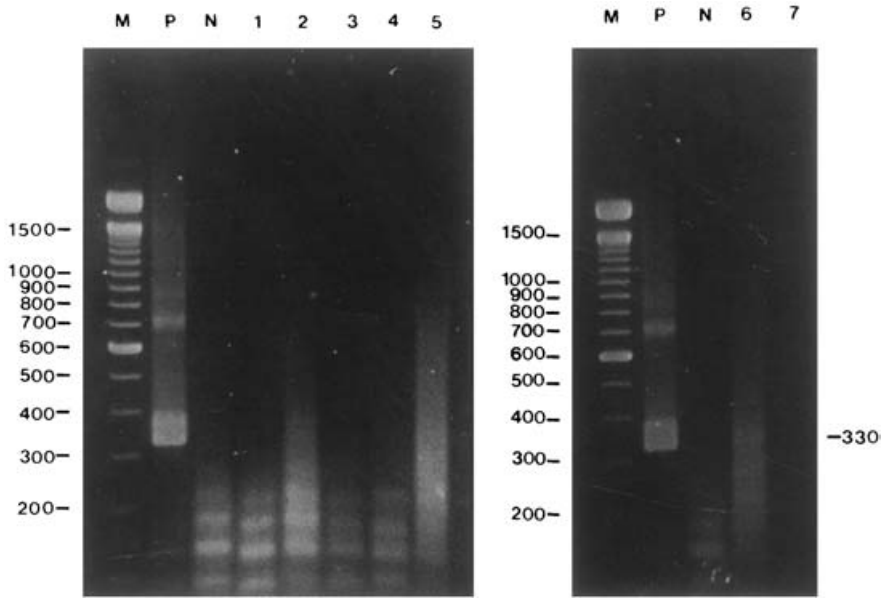

Fig. 1. Specificity of the PCR for the detection of *Opisthorchis viverrini*. Ethidium bromide staining patterns of the PCR products on a 2% agarose gel. Lane M: DNA size markers (100 bp ladder from GibcoBRL, Life Technologies); lanes N and P: negative and positive controls containing no DNA and 1 ng of *O. viverrini* DNA, respectively. PCR products from *Haplorchis taichui* (lane 1), *Centrocestus* spp. (lane 2), *Echinostoma malayanum* (lane 3), *Fasciola gigantica* (lane 4), animal schistosomes (lane 5), *Paragonimus heterotremus* (lane 6) and *Haplorchoides* spp. (lane 7). The 330 bp product is marked on the right.

Preparation of specimens for PCR

DNA was extracted from each of the non-infected or infected snails including shell, artificially inoculated snails, non-infected or infected caudal fins of fishes and artificially inoculated caudal fins of fishes. Each specimen was placed in a 1·5 ml microcentrifuge tube and homogenized with disposable polypropylene pestles (Bellco Glass Inc., Vineland, NJ), followed by extraction using the Nucleospin Tissue kit (Machenery-Nagel GmbH & Co.). The eluted DNA was resuspended in 5 mM Tris–HCl, pH 8·5 and 1 μl was used in the PCR reaction.

PCR amplification

The PCR was based on the DNA sequence, pOV-A6, a specific DNA probe. The OV-6F (5′-CTGAATCTCTCGTTTGTTCA-3′) and OV-6R (5′-GTTCCAGGTGAGTCTCTCTA-3′) primers were used (Wongratanacheewin *et al*. 2001) with a Tm in the range of 56 to 60 °C, which was used to amplify 330 bp of inserted DNA in pOV-A6. The PCR reaction was performed using a DNA thermal cycler (2400; Perkin-Elmer, Norwalk, CT). The reaction was carried out in a 25 μl volume containing PCR buffer (20 mM Tris–HCl, pH 8·4, 50 mM KCl, 1·5 mM $MgCl_2$), 200 μM of each dNTPs, 1 μM of each of the primers, 1 μl of the sample and 1·5 units of *Taq* DNA polymerase. The DNA template was initially denatured at 94 °C for 5 min. The amplification procedure comprised 30 cycles at 94 °C for 30 sec (denaturation), 52 °C for 30 sec (annealing) and 72 °C for 45 sec (extension). Amplified products were analysed by electrophoresis in a 2% agarose gel using 1 × TBE buffer, pH 8·0.

RESULTS

Standardization of O. viverrini *detection*

Each of the bithynid snails, artificially inoculated with 1, 5, 10 and 30 *O. viverrini* cercariae, respectively and caudal fins of cyprinoid fishes, artificially inoculated with 1, 2, 4 and 8 *O. viverrini* metacercariae respectively, were amplified. As little as 1 single cercaria could be clearly detected in 1 bithynid snail as a 330 bp band in the ethidium bromide-stained gel (figure not shown). The primers could also detect as little as 1 metacercaria in 1 caudal fin of the fish (figure not shown). The primers did not show the 330 bp amplified product with DNA from *H. taichui*, *Centrocestus* spp., *E. malayanum*, *F. gigantica*, animal schistosomes, *P. heterotremus* and *Haplorchoides* spp. genomic DNA (Fig. 1, lanes 1–7).

PCR detection in experimentally infected bithynid snails and cyprinoid fishes

Thirty infected and 20 non-infected snails as well as 30 infected and 20 non-infected fins of fishes were separately tested. All of the infected snails and infected fishes were positive by the PCR method, while all non-infected snails and non-infected fishes were negative. Both the sensitivity and the specificity of the PCR method are 100%.

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DISCUSSION

The PCR-based method has been successfully used for the detection of digenean flukes in intermediate hosts e.g. detection of *Schistosoma mansoni* in *Biomphalaria* (Hanelt *et al*. 1997) and detection of *F. hepatica* in intermediate hosts (Rognlie, Dimke & Knapp, 1994). This report describes the first use of a PCR-based technique for the detection of *O. viverrini* infection in the intermediate hosts i.e. snails and fishes. The high sensitivity of the PCR, which can detect as little as 1 cercaria or 1 metacercaria, might be attributed to the high amount of copies of the target sequence (Sermswan *et al*. 1991). It could therefore be useful for determining the prevalence of *O. viverrini* even in areas with a low burden of infection.

Pertaining to the specificity of the procedure, we have shown that the primers did not amplify the 330 bp band when DNAs from other digenean flukes belonging to different families than *O. viverrini* were used. Our specific PCR could be used for distinguishing *O. viverrini* from those other flukes that are common causes of human infection in Asian countries i.e. *Centrocestus* spp., *H. taichui*, *F. gigantica*, *E. malayanum*, *P. heterotremus*, and animal schistosomes (WHO, 1995; Anantaphruti, 2001).

The PCR protocol described here provides an alternative to available classic and more modern molecular or immunological methods for the detection of *O. viverrini* in snails and fishes. By virtue of using PCR methodology, the entire protocol starting from extraction of DNA from specimens to photographic visualization of the results can be completed within 1 day. The method also obliviates the need for the laborious and time-consuming microscopical examinations by experienced personnel. The method is independent of the subjective bias sometimes encountered in microscopical examinations and avoids confusion with other digenean flukes.

In conclusion, a specific and sensitive PCR method for the detection of *O. viverrini* cercariae and metacercariae in the 2 intermediate hosts (snails and fishes) has been demonstrated. The test is suitable for epidemiological studies and eradication programmes for the whole intermediate host range of the parasite. More importantly, this method might be used for the detection of *O. viverrini* contaminated freshwater fishes, the major protein diet of people in Asian countries (WHO, 1995). This is a crucial issue for an effective food safety strategy program. Further tests should be carried out in epidemiological surveys, not only for the detection in intermediate hosts, but also for cercarial surveys of natural water bodies.

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REFERENCES

ANANTAPHRUTI, M. T. (2001). Parasitic contaminants in food. *Southeast Asian Journal of Tropical Medicine and Public Health* **32** (Suppl.), 218–228.

BROCKELMAN, W. Y., UPATHAM, E. S., VIYANANT, V., ARDSUNGNOEN, S. & CHANTANAWAT, R. (1986). Field studies on the transmission of the human liver fluke, *Opisthorchis viverrini* in Northeast Thailand: population changes of the snail intermediate host. *International Journal for Parasitology* **16**, 545–552.

HANELT, B., ADEMA, C. M., MANSOUR, M. H. & LOKER, E. S. (1997). Detection of *Schistosoma mansoni* in *Biomphalaria* using nested PCR. *Journal of Parasitology* **83**, 387–394.

HARINASUTA, T., RIGANTI, M. & BUNNAG, D. (1984). *Opisthorchis viverrini* infection: pathogenesis and clinical features. *Arzneimittelforschung* **34**, 1167–1169.

JONGSUKSUNTIGUL, P. & IMSOMBOON, T. (1998). Epidemiology of opisthorchiasis and national control program in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **29**, 327–332.

KRUATRACHUE, M., JANTATAEME, S., RATANATHAM, S., VICHASRI, S. & UPATHAM, E. S. (1982). A culture method for *Bithynia* (Prosobranchia: Bithyniidae), snail hosts for the trematode *Opisthorchis viverrini*. *Malacological Review* **15**, 63–67.

LIANG, Y. S. & KITIKOON, V. (1980). Cultivation of *Lithoglyphopsis aperta*, snail vector of *Schistosoma mekongi*. *Malacological Review* **13** (Suppl.), 35–45.

PUNGPAK, S., CHALERMRUT, K., HARINASUTA, T., VIRAVAN, C., SCHELP, P. F., HEMPFLING, A., SCHLATTMANN, P. & BUNNAG, D. (1994). *Opisthorchis viverrini* infection in Thailand: symptoms and signs of infection – a population-based study. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**, 561–564.

ROGNLIE, M. C., DIMKE, K. L. & KNAPP, S. E. (1994). Detection of *Fasciola hepatica* in infected intermediate hosts using RT-PCR. *Journal of Parasitology* **80**, 748–755.

SCHWARTZ, D. A. (1980). Helminths in the induction of cancer: *Opisthorchis viverrini*, *Clonorchis sinensis* and cholangiocarcinoma. *Tropical and Geographical Medicine* **32**, 95–100.

SCHWARTZ, D. A. (1986). Cholangiocarcinoma associated with liver fluke infection: a preventable source of morbidity in Asian immigrants. *American Journal of Gastroenterology* **81**, 76–79.

SERMSWAN, R., MONGKOLSUK, S., PANYIM, S. & SIRISINHA, S. (1991). Isolation and characterization of *Opisthorchis viverrini* specific DNA probe. *Molecular and Cellular Probes* **5**, 399–407.

VICHASRI, S., VIYANANT, V. & UPATHAM, E. S. (1982). *Opisthorchis viverrini*; intensity and rates of infection in cyprinoid fish from an endemic focus in Northeast Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **13**, 138–141.

WAIKAGUL, J. (1998). *Opisthorchis viverrini* metacercaria in Thai freshwater fish. *Southeast Asian Journal of Tropical Medicine and Public Health* **29**, 324–326.

WONGRATANACHEEWIN, S., PUMIDONMING, W., SERMSWAN, R. W. & MALEEWONG, W. (2001). Development of a PCR-based method for the detection of *Opisthorchis viverrini*

in experimentally infected hamsters. *Parasitology* **122**, 175–180.

WOOLF, A., GREEN, J., LEVINE, J. A., ESTEVEZ, E. G., WEATHERLY, N., ROSENBERG, E. & FROTHINGHAM, T. (1984). A clinical study of Laotian refugees infected with *Clonorchis sinensis* or *Opisthorchis viverrini*. *American Journal of Tropical Medicine and Hygiene* **33**, 1279–1280.

WORLD HEALTH ORGANIZATION (1995). *Control of Foodborne Trematode Infections*. WHO Technical Report Series No. 849. WHO, Geneva.

WYKOFF, D. E., HARINASUTA, C., JUTTIJUDATA, P. & WINN, M. M. (1965). *Opisthorchis viverrini* in Thailand. The life cycle and comparison with *O. felineus*. *Journal of Parasitology* **51**, 207–214.

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