

COPRO-DNA DIAGNOSIS OF *OPISTHORCHIS VIVERRINI* AND *HAPLORCHIS TAICHUI* INFECTION IN AN ENDEMIC AREA OF LAO PDR

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Abstract. The utility of differential copro-DNA diagnosis using modified sample preparation steps of small liver and minute intestinal fluke infections was tested. Fecal samples containing parasite eggs were washed extensively with diluted detergent solution. Parasite eggs were concentrated by sedimentation and broken by microwaving before DNA extraction. PCR targeting ITS1 and ITS2 regions were performed using primer specific for *Opisthorchis viverrini*, *Haplorchis taichui* and other related species. Of 125 fecal samples, 94 were positive for small trematode eggs by a modified cellophane thick smear method. By ITS1-PCR, 52 samples were positive for *O. viverrini*, 12 *H. taichui* and 7 mixed infection. By ITS2-PCR, 63 were positive for *O. viverrini*, 17 *H. taichui*, and 19 mixed infection. The ITS-PCR assay identified a higher number of opisthorchiasis cases than those with *O. viverrini* expelled after treatment, but for *H. taichui*, ITS-PCR identified less than half of the worm expelled cases. These results showed that copro-DNA diagnosis was useful for the differential diagnosis of *O. viverrini* and *H. taichui* infection, which could not be discriminated by microscopy.

Key words: copro-DNA diagnosis, *Opisthorchis viverrini*, *Haplorchis taichui*, Lao PDR

INTRODUCTION

The small liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, are prevalent in many Asian countries. *O. viverrini* infection, in particular, is a serious public-health problem in the Indochinese penin-

sula, because heavy, chronic infection often results in cholangiocarcinoma (Bunnag *et al*, 2000; Sripa, 2003). Early diagnosis and correct treatment are important to prevent the development of cancer. Minute intestinal flukes, such as *Haplorchis* species, which belong to the family Heterophyidae, are distributed in many Asian countries (Waikagul, 1991). Thus, areas endemic for small liver and minute intestinal flukes often overlap, and the incidence of mixed infection is high (Radomyos *et al*, 1998; Belizario *et al*, 2004; Chai *et al*, 2005).

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The gold standard for the diagnosis of small liver and minute intestinal flukes is the detection of eggs in fecal samples. However, it is difficult to differentiate and identify species because of the morphological similarities of their eggs (Kaewkes *et al*, 1991). In *O. viverrini* endemic areas, small fluke eggs are often identified as *O. viverrini* eggs based on examinations of feces. Thus, the prevalence of *O. viverrini* infection might be overestimated in areas where intestinal flukes are co-endemic. Conversely, minute intestinal fluke infection might be overlooked in such areas. Therefore, molecular approaches to the differential diagnosis of liver and intestinal flukes have been tested in laboratory settings (Wongratanacheewin *et al*, 2001, 2002; Le *et al*, 2006; Thaenkham *et al*, 2007; Sato *et al*, 2009a). However, the practical sensitivity of molecular diagnosis has not been entirely satisfactory because of problems of DNA extraction and subsequent PCR; as the fecal egg count is not high because of the low egg-production rate of Opisthorchioidea parasites (WHO, 1995), their eggshell is thick in relation to their small size, and fecal samples contain substances that interfere with the PCR process (Stensvold *et al*, 2006). Despite several attempts to improve the sensitivity and specificity of copro-DNA diagnosis for these fluke infections (Wongratanacheewin *et al*, 2002; Stensvold *et al*, 2006; Möller *et al*, 2007; Duengngai *et al*, 2008), the reagents and/or equipment are costly, and the procedure is time-consuming. Moreover, the sensitivity of the improved copro-DNA diagnoses remains lower than fecal egg examination.

In this study, we report a cross-sectional investigation of small fluke infection in an endemic area of Lao PDR. PCR was performed on humans fecal samples randomly collected using primer sets

targeting the ITS previously designed for the differential diagnosis of small liver flukes and minute intestinal flukes (Sato *et al*, 2009a). The results of PCR were compared with stool examination and adult worm expelled post-treatment.

MATERIALS AND METHODS

Fecal sample collection and examination

Human fecal specimens were randomly collected in Lahanam Village, Sonkon District, Savannakhet Province, Lao PDR, after obtaining oral and written informed consent. This study was approved by the Lao Medical Ethics Committee (172/NECHR), and Mahidol University Ethics Committee (MUTM 2008-022-01). Fecal examinations were conducted at the sampling site by modified cellophane thick smear method (Kato-Katz, KK) (Katz *et al*, 1972). The entire slide was examined under a microscope and all helminth eggs counted and the number of eggs per gram of feces (EPG) was calculated.

Anthelmintic treatment and collection of expelled worms

All 125 participants were treated with a single 40 mg/kg dose of oral Praziquantel (PZQ) (Opticide-FC® The Medicpharma, Thailand). Purgation was done with 60 ml of saturated magnesium sulphate solution. All fecal samples were collected and sediments were washed extensively with tap water by repeated sedimentation/decanting. Parasites recognizable by the naked eye were separated, identified, and preserved in 70% ethanol, and all residual sediments were preserved in 70% ethanol. Samples were transferred to the laboratory in Bangkok, where parasites were collected and identified under a light microscope.

DNA preparation

About 2 g of feces were passed through a wire sieve and placed in a 15 ml Falcon™ tube. Tap water containing 2% liquid dish-washing detergent was added to a level of 12 ml and the sample was washed repeatedly until the supernatant was clear. Then the supernatant was removed and 8 ml aliquot of 70% ethanol was added to the sediment for transport back to the laboratory. The sediment then was washed with distilled water (DW) to remove ethanol and re-suspended in 3 ml of DW. A 0.5 ml aliquot of the suspension was transferred to a 1.5 ml microfuge tube and used for DNA extraction. To disrupt parasite eggs, the suspension was microwaved at 700W (MS-1812C, LG, Korea) for 60 seconds. DNA was extracted using Genomic DNA Mini Kit (Geneaid, Taiwan) following the manufacturer's instructions. For adult helminths, *Echinostoma* and *Opisthorchis*, DNA was prepared by phenol/ chloroform technique, followed by ethanol precipitation. Minute intestinal flukes, *Haplorchis*, *Phaneropsolus* and *Prosthodendrium*, were ground and boiled, then used directly as DNA template.

PCR

Primers used were ITS1-F (5'-GTA TGC TTC GGC AGC TCG ACC GG-3') and ITS1-R (5'-GGC TGC GCT CTT CAT CGA CAC ACG-3') to amplify ITS1 region, and ITS2-F (5'-CTT GAA CGC ACA TTG CGG CCA TGG G-3') and ITS2-R (5'-GCG GGT AAT CAC GTC TGA GCC GAG G-3') for ITS2 region (Sato *et al*, 2009a). PCR was conducted in thermalcycler (Eppendorf Mastercycler Personal, Germany) in a total volume of 25 µl, using commercial PCR reagents (Roche, Germany). The final concentration of dNTP, MgCl₂, *Taq* polymerase and primers was 5 mM, 1.5 mM, 1.5 U and 1 mM, respectively. One µl of DNA preparation was used as template. DNA samples were initially heated at 94°C for 4 minutes,

and then subjected to 30 amplification cycles consisting of 94°C for 1 minute, 60°C for 30 seconds, and 72°C for 2 minutes. Amplicons were electrophoresed in 2.0% agarose gel. ITS1 amplicon of *O. viverrini*, *Phaneropsolus bonnei* and *Prosthodendrium molenkampii*, *Echinostoma* sp and *H. taichui* adult worm was 790, 720, 610, 580, and 930 bp, respectively, and that of ITS2 380, 390, 330, 510, and 530 bp, respectively.

Statistical analysis

Agreement between diagnostic methods was measured by McNemar test.

RESULTS

Stool egg examination and worm collection

In Lahanan village, the most prevalent (94 of 125 samples, 75%) parasite eggs were those of small trematodes (Table 1). Hookworm eggs were found in 36 (29%) samples and *Taenia* spp eggs in 5 (4%). After PZQ and purgative treatments, 122/125 (98%) participants were found to be infected with *H. taichui* (Table 1). The second-most prevalent parasite was *O. viverrini* (48/125, 38%), followed by *Taenia saginata* (23/125, 18%). Trematodes, *Echinostoma* sp, *P. bonnei*, and *P. molenkampii*, were also expelled. Although the participants were treated with PZQ alone, *Enterobius vermicularis* (16/125, 13%) and *Ancylostoma duodenale* (1/125, 1%) were found in the stool. Nematode egg-positive cases were treated further with albendazole.

Application of PCR to human fecal samples

Of 125 fecal samples 71 (57%) were ITS1-PCR-positive and 99 were ITS2-PCR-positive (Table 2). There is no difference in the prevalence of small fluke infection detected by ITS2-PCR and fecal thick smear method ($\chi^2=0.5$, $p>0.05$). On the other hand, the prevalence measured by ITS1-PCR was significantly lower than microscopic examination ($\chi^2= 13.82$, $p<0.05$).

Table 1
Prevalence of helminth infections in Lahanam Village, Savannakhet Province, Lao PDR by modified cellophane thick smear method and actual worm recovery.

Parasites	Fecal egg positive (%)	Worm positive (%)
Trematodes (small eggs)	94 (75)	
<i>Opisthorchis viverrini</i>		48 (38)
<i>Haplorchis taichui</i>		122 (98)
<i>Phaneropsolus bonnie</i>		5 (4)
<i>Prosthodendrium molenkampi</i>		5 (4)
(big eggs)		
<i>Echinostoma</i> sp	0 (0)	11 (9)
Tapeworm eggs	5 (4)	
<i>Taenia saginata</i>		23 (18)
Hookworm eggs	36 (29)	
<i>Ancylostoma duodenale</i>		1 (1)
<i>Enterobius vermicularis</i>	0 (0)	16 (13)

A total of 125 villagers participated in the study. Fecal egg examination was performed by modified cellophane thick smear method. Worms were counted and identified for total stool collected post-praziquantel treatment (40 mg/kg body weight).

Table 2
Comparison of modified cellophane thick smear method and PCR for diagnosis of small trematode eggs (125 samples)

PCR	Fecal egg detection		Total no. (%)	
	Positive	Negative		
ITS1	Positive ^a	65	6	71 (57)
	<i>O. viverrini</i> alone	47	5	
	<i>H. taichui</i> alone	12	0	
	mixed	6	1	
	Negative	29	25	54 (43)
	Total (%)	94 (75)	31 (25)	125 (100)
ITS2	Positive ^a	81	18	99 (79)
	<i>O. viverrini</i> alone	49	14	
	<i>H. taichui</i> alone	14	3	
	mixed	18	1	
	Negative	13	13	26 (21)
	Total (%)	94 (75)	31 (25)	125 (100)

^aSpecies identification was based on the molecular size of PCR products on agarose gel electrophoresis.

To examine the applicability of PCR diagnosis for cases of mixed infection, *O. viverrini* and *H. taichui* eggs were artificially

mixed at various ratios (*O. viverrini*: *H. taichui* = 1:1, 1:10, 1:100, 1:1,000, 10:1, 100:1, and 1,000:1), into parasite egg-free stool and

these samples processed for DNA extraction and PCR examination. After electrophoresis in agarose gel, the amplicons displayed two distinctive bands for all ratios of mixed infection (data not shown).

For species discrimination among 71 ITS1-PCR positive cases, 52 (73%) were *O. viverrini*-positive, 12 (17%) *H. taichui*-positive, and 7 (10%) were positive for both species. Among 99 ITS2-PCR positives, 63 (64%) were positive for *O. viverrini*, 17 (17%) *H. taichui*, and 19 (19%) mixed infection. However, both ITS1- and ITS2-PCR did not detect other fluke species.

The sensitivity of copro-DNA detection by PCR depended on the numbers of parasite eggs in feces. Detection ability of PCR diagnosis increased with increasing EPG of fecal samples (Fig 1). ITS2 was a better marker for small fluke detection than ITS1, and maximum yield was reached at intensity >1,000 EPG.

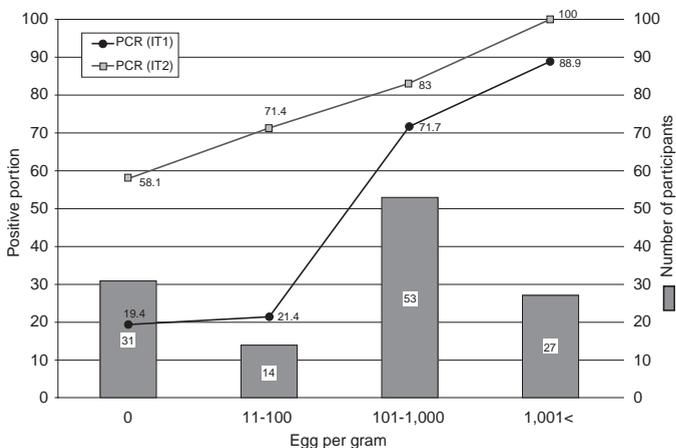


Fig 1–Detection of *Opisthorchis viverrini* and *Haplorchis taichui* in 125 samples (Lahanum Village, Savannakhet Province, Lao PDR) by worms recovered after treatment with Praziquantel, ITS1-PCR and ITS2-PCR. PCR was carried out as described in Materials and Methods.

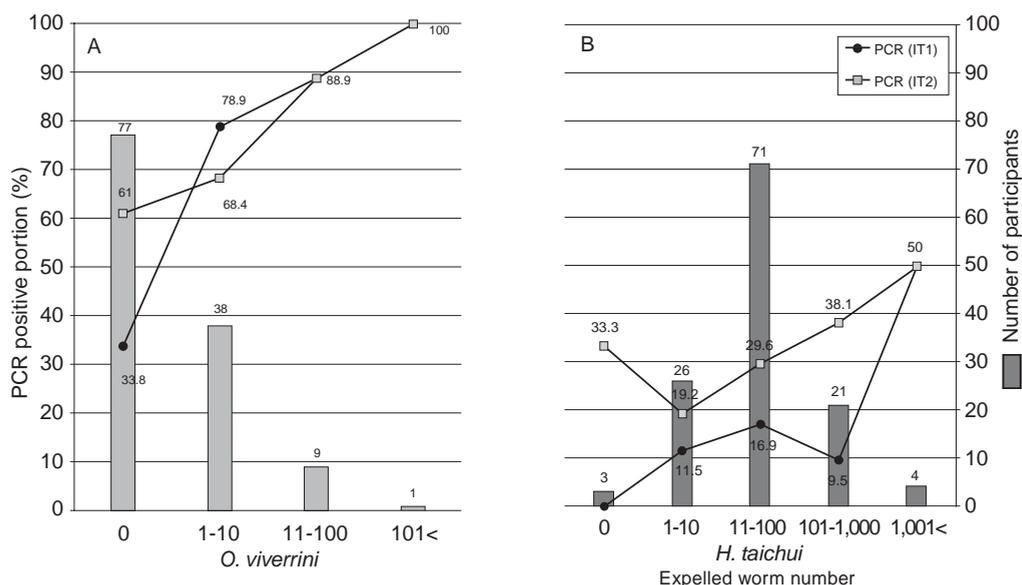


Fig 2–Positive portion of *Opisthorchis viverrini* and *Haplorchis taichui* based on expelled worm number and on ITS1-PCR and ITS2-PCR assays.

All participants were treated with PZQ and expelled worms collected and identified. The PCR results were compared with the actual numbers of worms recovered after PZQ treatment (Fig 2). For detection of *O. viverrini*, both ITS1- and ITS2-PCR showed better results than the worm-expelled cases, but for *H. taichui* both markers were less able than worm recovery.

DISCUSSION

In this study, the procedure for preparing fecal sample for DNA extraction was a new technique applied to the diagnosis of small fluke infection. Hitherto, the preparation of DNA from small trematode eggs has commonly been treated by NaOH treatment and autoclaving, followed by DNA extraction using commercial fecal DNA kits (Wongratanacheewin *et al*, 2002; Stensvold *et al*, 2006). However, with this method, the sensitivity of the copro-DNA method was not sufficiently high to surpass that of microscopic fecal egg examination. Recently, a protocol using cetyl trimethyl ammonium bromide (CTAB) to remove PCR inhibitors, followed by *O. viverrini*-specific PCR, improved the sensitivity of up to 79.3% (Duengngai *et al*, 2008). High sensitivity (minimum detection limit of 5 *O. viverrini* eggs/g feces) was also obtained by another *O. viverrini*-specific PCR protocol using the same CTAB DNA extraction procedure (Umesha *et al*, 2008). The CTAB method appears effective but is time-consuming; also breaking the eggs by autoclaving was unsatisfactory because about 50% remain intact (Möller *et al*, 2007). Compared with these methods, the DNA preparation protocol described here was simple, easy, and inexpensive. The method required only dish-washing detergent, which is supposed to remove PCR inhibitors in feces, and an ordinary micro-

wave oven to break the eggs within only 1 minute. Although a commercial DNA extraction kit was used, it may be possible to extract DNA with phenol/chloroform to minimize cost.

Lovis *et al* (2009), using COI marker to detect *O. viverrini* and *H. taichui* infections, showed that PCR gives better results than single fecal examination (1KK), but poorer than three thick smear examinations (3KK). Although the results of 3KK give more accurate infections data than 1KK in general practise it is unlikely applicable in any large scale examination.

In the present study, a number of participants were predicted to have mixed *O. viverrini* and *H. taichui* infection, based on the presence of two amplicons of different molecular sizes specific to each species. Seven cases of mixed *O. viverrini* and *H. taichui* infection were found by ITS1-PCR, and 19 by ITS2-PCR. Such mixed infections could not be detected by a general microscopic examination alone, because the eggs of these two species, and of other minute intestinal flukes, are morphologically similar and practically indistinguishable. Therefore, even if the sensitivity of fecal egg examination and copro-PCR detection is comparable, PCR methods is valuable for identifying parasite species.

After PZQ treatment, almost all (122/125) participants were *H. taichui*-infected. Thus all *O. viverrini*-worm positive cases were concurrently infected with *H. taichui* to varying degrees, in which all cases were not detected by PCR. In the present study, copro-PCR diagnosis detected more (3-5 times higher) *O. viverrini* than *H. taichui*. The reasons for such conflicting results among actual worm burden, fecal egg count and PCR egg detection remain unclear. One possibility is that the daily reproduction rate (egg production) of

H. taichui adults is extremely low at 82 eggs/worm/day (Sato *et al*, 2009b), so that most low-density infections were not detected by fecal egg examination or PCR. Another possibility is that even when there were many worms, most of them were still immature, and had not yet laid eggs. However, the latter is unlikely, because over 70% of worms recovered post-PZQ treatment were mature, and the uteri contained eggs. To clarify the discrepancy between *H. taichui* worm burden and egg production, more biological data are required, eg, life span and reproductive capacity, for this parasite.

Several flukes were found post-PZQ treatment, namely, *P. bonnie*, *P. molenkampi*, and *Echinostoma* sp. However, the DNA of these parasites was not amplified in the copro-PCR assay. The number of expelled worms was less than five. The reproduction of Lecithodendriidae in humans has not been described. *Echinostoma hortense*, produces about 1,500 eggs daily (Lee *et al*, 1988). Thus, it is possible that the numbers of these parasites were below the detection limit of the ITS-PCR assay.

In summery, we successfully improved the sensitivity of copro-DNA detection by PCR for *O. viverrini* cases using simple and inexpensive methods for the pre-treatment of fecal specimens before DNA extraction. Copro-PCR assay revealed several fecal samples having mixed infection of *Opisthorchis viverrini* and *Haplorchis taichui*. However, for *H. taichui* cases, further improvements are necessary, based on clarification of the parasite's biological characteristics.

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