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Short communication

Discrimination of *O. viverrini*, *C. sinensis*, *H. pumilio* and *H. taichui* using nuclear DNA-based PCR targeting ribosomal DNA ITS regions

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ABSTRACT

Small liver and minute intestinal flukes are highly prevalent in Southeast Asia, and in mixed infections, their eggs are difficult to differentiate morphologically in fecal samples. PCR assays targeting the ITS regions in ribosomal DNA were designed to identify and differentiate species. The PCR amplicons of *Opisthorchis viverrini*, *Clonorchis sinensis*, *Haplorchis pumilio*, and *Haplorchis taichui* were 800, 820, 1250, and 930 bp for the ITS1 region, and 380, 390, 380, and 530 bp for ITS2, respectively. The ITS1-region amplicon sizes successfully differentiated 4 species, while only *H. taichui* were significantly different from the other 3 species in the ITS2 region. PCR assays were employed for preliminary analysis using fecal samples diagnosed as having "small trematode eggs" by modified thick smear, showing 76.2% sensitivity for ITS1 and 95.2% for ITS2.

1. Introduction

Opisthorchiid trematodes and heterophyid intestinal flukes are highly prevalent in Southeast Asian countries (Wongratanacheewin et al., 2001; Chai et al., 2005). Since the source of infection may be the same, mixed infections often occur in one patient (Chai et al., 2005; Waikagul and Radomyos, 2005). These infections have a major public health impact. The morphological characters of the eggs are difficult to differentiate using normal stool examination methods, even for experienced technicians (Kaewkes et al., 1991).

PCR assays have been developed for these fish-borne trematodes (Wongratanacheewin et al., 2001, 2002; Le et al., 2006; Parvathi et al., 2007; Thaenkham et al., 2007) to overcome the deficiencies of microscopic examination, and to assure an accurate diagnosis. However, the diagnostic sensitivity of PCR has not proved it to be a suitable diagnostic tool compared with simple fecal examination (Stensvold et al., 2006).

In this study, PCR assays, based on ITS regions, were developed to broadly discriminate between species of parasites in the Superfamily Opisthorchoidea, and to generate a new diagnostic tool capable of determining the prevalence of each fluke.

2. Materials and methods

2.1. Sampling

The study protocol was approved by Mahidol University Ethical Review Committee (Approval numbers: MUTM 2006-040 and 2006-065) and written and oral informed consent were obtained from the participants. The adult worms were collected from human hosts; *Clonorchis sinensis* from Nam Dinh Province, Vietnam, *Opisthorchis viverrini* from Sakeo Province, Thailand, and *Haplorchis taichui* from Nan Province, Thailand. Metacercariae of *Haplorchis pumilio* were collected from *Trichogaster trichopterus* caught in Nakhon Prathom Province, Central Thailand. Twenty-one human fecal specimens, diagnosed as having small trematode eggs by modified thick smear method, were collected in Sakaeo Province. Adult worms and stool samples were preserved in 70% and metacercariae in 20% ethanol, respectively, then stored at −20 °C until used.

2.2. DNA preparation

Adult *O. viverrini* and *C. sinensis* worms were digested with lysis buffer containing 25 mM Tris buffer (pH 7.5), 0.5% sodium dodecyl sulfate, 100 mM sodium chloride, 10 mM EDTA and 0.1% Proteinase K at 56 °C in a water bath overnight. The lysate was extracted by phenol/isoamyl alcohol/chloroform method, and precipitated with ethanol. Finally, the DNA was resuspended with 200 μl of distilled water (DW). The *H. pumilio* metacercariae and adult *H. taichui*

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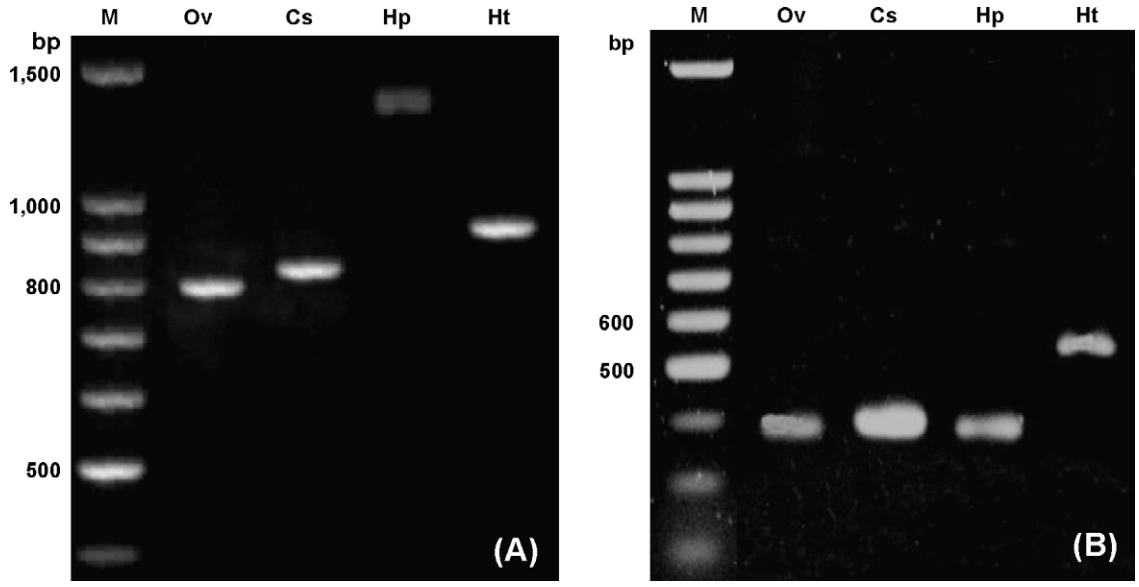

Fig. 1. Species-specific amplicons. Lane 1: 100 bp DNA marker (Promega). Lane 2: *O. viverrini* (Ov), Lane 3: *C. sinensis* (Cs), Lane 4: *H. pumilio* (Hp) and Lane 5: *H. taichui* (Ht). (A) Using primer set ITS1-F and ITS1-R targeting rDNA ITS1 region in 2.0% agarose gel electrophoresed for 2.5 h. (B) Using primer set ITS2-F and ITS2-R targeting rDNA ITS2 region in 1.0% agarose gel electrophoresed for 1.5 h.

worms were ground and put into a PCR tube with 10 μl sterile DW, then used directly in PCR. Ten samples were examined per species.

2.3. PCR

The primers were designed manually from the sequences of intestinal flukes, *H. taichui* ribosomal RNA (GenBank accession no. AY245705) and *H. pumilio* ribosomal RNA (GenBank accession no. AY245706), targeting the complete ITS regions, ITS1 region: 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′, ITS2 region: 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′. The reaction was carried out in 50 μl volume, with a final PCR mixture concentration of 5 mM dNTP, 1.5 mM $MgCl_2$, 1.5 U *Taq* polymerase and 1 mM primers. The DNA samples were initially denatured at 94 °C for 4 min, followed by 40 amplification cycles, consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and elongation at 72 °C for 2 min. Amplicons were electrophoresed in 1.0% agarose gel or/and 2.0% agarose gel.

2.4. Analysis of fecal samples

Fecal samples of 0.5 ml per experiment were washed twice by DW to remove fixative, and frozen at −80 °C. The frozen feces were crushed three times with a glass bar to break any eggs. DNA was extracted from the broken eggs by Qiagen Stool kit (Qiagen) with half a tablet of inhibitEX (Stensvold et al., 2006). The DNA was resuspended with 50 μl DW and used as a template.

2.5. Statistical analysis

The results of modified thick smear and PCR methods using fecal samples were analyzed by kappa test, to measure the agreement between the two methods.

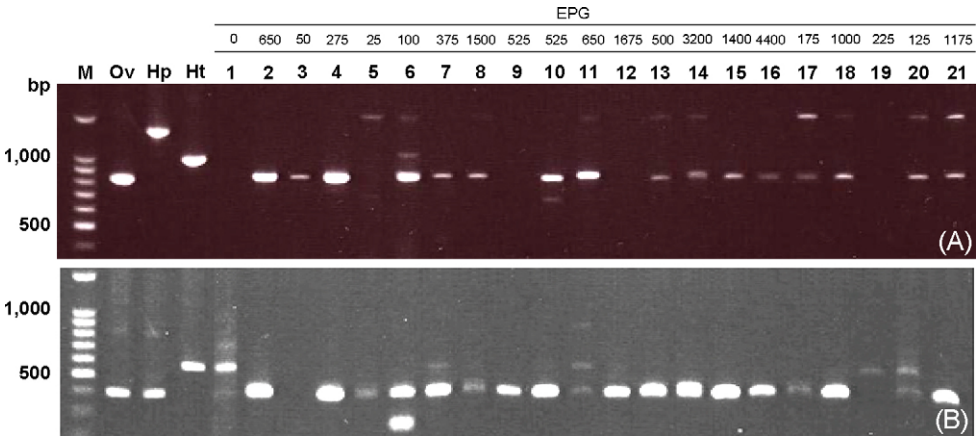

Fig. 2. PCR results of fecal samples targeting ITS region. The PCR products were electrophoresed in 1.0% agarose gel electrophoresed for 1.5 h. Lane M: 100 bp DNA marker (Promega), Lane Ov: *O. viverrini*, Lane Hp: *H. pumilio*, Lane Ht: *H. taichui* and Lanes 1–21: fecal samples. (A) ITS1 region and (B) ITS2 region.

3. Results

3.1. Species discrimination

The positive PCR bands were confirmed in 1.0% agarose. No intraspecies variation was observed in 10 samples per species and region; then, amplicons of 4 species were electrophoresed together. For the ITS1 region, the size of the PCR amplicons from *O. viverrini* and *C. sinensis* was approximately 800 and 820 bp, respectively. The PCR amplicon of *H. pumilio* was about 1250 bp, and that of *H. taichui* 930 bp (Fig. 1A). However, *O. viverrini* and *C. sinensis* showed a small difference when separated using 2.0% agarose gel. The ITS2-region amplicon sizes of *O. viverrini*, *C. sinensis*, *H. pumilio,* and *H. taichui,* were approximately 380, 390, 380, and 530 bp, respectively (Fig. 1B). The amplicon size of *H. taichui* was clearly different from the other 3 species.

3.2. Stool examination

With the ITS1 PCR, an 800 bp band, the same size as *O. viverrini*, was found in 16 of 21 samples (76.2%), and the 930 bp band of *H. taichui* appeared in 1 sample (Fig. 2A). For the ITS2 PCR, 16 of 21 showed the 380 bp band, the same size as *O. viverrini* and/or *H. pumilio*, and 4 samples had bands of 530 bp, the same as *H. taichui*; both band sizes appeared in 3 samples (Fig. 2B). In all, the samples showed positive results in 20 of 21 samples (95.2%). *C. sinensis* was excluded, since the parasite is not prevalent in the study area. Eggs per gram (EPG) ranged between 0 and 4400 (Fig. 2).

The kappa test was used to study agreement between the two methods. The kappa value was 0.75 for ITS1 PCR, and 0.95 for ITS2, indicating a medium and a high association with the modified thick smear method. The PCR targeting ITS1 was less sensitive than the standard fecal-smear method, while ITS2 exhibited a high capacity to detect flukes in the Superfamily Opisthorchoidea.

4. Discussion

In this study, PCR products were amplified for the target region ITS1 and discriminated *O. viverrini*, *C. sinensis*, *H. pumilio*, and *H. taichui*, even though the difference in size between *O. viverrini* and *C. sinensis* was only about 20 bp. Using the ITS2 PCR, the DNA from 4 species of flukes could be amplified; however, it was difficult to utilize the results to discriminate species, since only the amplicon of *H. taichui* was significantly different in size from the other 3 species.

In the laboratory setting, the sensitivity of the PCR was measured as 100% for detecting *O. viverrini* in human feces (Wongratanacheewin et al., 2002); however, in the field survey, its sensitivity was lower than the formalin–ethanol concentration and modified thick smear methods (Stensvold et al., 2006). Fecal DNA extraction using cetyl trimethyl ammonium bromide (CTAB) was used to remove the PCR inhibitor and when compared with the commercial stool kit method, showing a sensitivity of 79.3% (Duenngai et al., 2008). Without using CTAB, our examination of fecal samples by PCR yielded 76.2% positive for ITS1 and 95.2% for ITS2, it is not necessary to use CTAB to improve the sensitivity of our ITS PCR methods. ITS1 is specific for the discrimination of the parasite species, but less sensitive for diagnosis, whereas ITS2 is less specific but highly sensitive. Several unspecific bands were noted, which may have been due to DNA from other parasites or fecal material. One of 5 samples showed negative results; 1 sample even showed a high level of EPG (1675). By contrast, some samples with low EPG levels showed clear positive bands. These controversial results might be caused by inhibitors remaining in the fecal samples. The kappa test showed a medium association ($\kappa = 0.75$) between the PCR ITS1-region assay and the modified thick smear method, indicating that the PCR with ITS1 was less sensitive than the thick smear method. A high association ($\kappa = 0.95$) was obtained for the ITS2 region PCR and the modified thick smear method, so that PCR targeting the ITS2 region might be used as a diagnostic tool instead of the modified thick smear method. A combination of ITS1 and ITS2 PCR is recommended, the ITS2 for screening and ITS1 for discriminating small liver flukes and intestinal flukes.

However, many other intestinal flukes have been reported in Southeast Asian countries (Waikagul et al., 1997). These parasite species must be collected and analysed for the diagnostic sensitivity and specificity of ITS1 and ITS2 PCR. Further studies are required before they can be used practically as a copro-diagnostic tool in the epidemiological study of Opisthorchiid trematodes in definitive and intermediate hosts.

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