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Evaluation of PCR based coprodiagnosis of human opisthorchiasis

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Abstract

In this study, a recently developed PCR test for the detection of *Opisthorchis viverrini* in human faecal samples was evaluated using two parasitological methods as references. During a survey of foodborne trematodes (FBT) in the Vientiane Province, Lao PDR, 85 samples were collected and evaluated for FBT eggs by the Kato Katz (KK) technique, the formalin ethyl acetate concentration technique (FECT) and a PCR analysis for the distinction between *O. viverrini* and other FBT. The two parasitological methods did not differ in the ability of detecting FBT eggs, and a single KK reading was characterized by a sensitivity of 85% when compared to two FECT readings. The PCR tested positive only in cases where eggs had been demonstrated by parasitological examination. However, the PCR tested negative in some samples with very high egg counts. Demonstrating a PCR sensitivity of approximately 50% in samples with faecal egg counts > 1000, the previously reported PCR sensitivity based on in vitro studies was not supported. It is believed that technical problems rather than diagnostic reference related issues were responsible for the relatively low PCR performance. Further studies should aim at optimizing DNA extraction and amplification, and future PCR evaluation should include specificity control such as the scanning electron microscopy of eggs in test samples or the expulsion of adult trematodes from PCR tested individuals.

Keywords: *Opisthorchis viverrini*; Parasitological methods; Molecular techniques; Coproantigen

1. Introduction

Fishborne liver fluke infections due to *Opisthorchis viverrini* are endemic in Thailand, the Lao PDR and Cambodia (Kaewkes, 2003). It has been estimated that the number of infections caused by *O. viverrini* amounts to 9 million cases (WHO, 1995). Mixed infections with *O. viverrini* and minute intestinal flukes (MIF) of heterophyid and lecithodendriid families are common (Giboda et al., 1991; Radomyos et al., 1998; Sirisinha et al., 1995; Sukontason et al., 2001) but infections with only the heterophyid fluke *Haplorchis taichui* have also been reported (Sukontason et al., 2001). Distinguishing opisthorchiid eggs from MIFs by parasitological methods is very difficult, and eggs can only be characterized as '*Opisthorchis*-like' (Giboda et al., 1991; Radomyos et

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al., 1998; Sukontason et al., 2001). A definitive morphological diagnosis requires scanning electron microscope (Tesana et al., 1991; Ditrich et al., 1992) or identification of adult worms expelled after treatment and purgation of infected individuals (Radomyos et al., 1998). Prevalence estimates of fishborne liver fluke infections are nevertheless based on parasitological methods, such as the formalin ethyl acetate concentration technique (FECT) or the Kato Katz (KK) technique, which raises a fundamental question of diagnostic accuracy. Moreover, the diagnostic sensitivity of available parasitological techniques is relatively low, impeding the detection of light infections. The KK method is considered less sensitive than the FECT by some authors (Sithithaworn et al., 1994) and equally or more sensitive by others (Viyanant et al., 1983; Hong et al., 2003) for the detection of small trematode eggs although the KK technique is not considered reliable for quantitative examination of, e.g. clonorchiasis (Hong et al., 2003). The FECT is more laborious and time consuming than the KK technique typically used in large-scale surveys.

Recently, a PCR method for the detection of *O. viverrini* in human stool specimens was developed as a suitable tool for the investigation of a large number of human faecal samples at one time (Wongratanacheewin et al., 2002). The method was developed for the detection of *O. viverrini* in hamsters (Wongratanacheewin et al., 2001; Sermswan et al., 1991) and was characterized by a specificity of 97.8%, a sensitivity of 100% in moderate-severe infections (more than 1000 eggs per gram (EPG) faeces) and a maximum sensitivity of 68.2% in the detection of light infections (EPG < 1000) with a detection limit of 200 eggs (Wongratanacheewin et al., 2002). The aim of the present study was to evaluate the PCR technique as a diagnostic tool for the detection of human opisthorchiasis.

2. Study subjects, materials and methods

2.1. Stool sampling, preparation and parasitological examination

In a foodborne trematodes (FBT) survey instituted by WHO and national health authorities, 85 stool samples were obtained from previously praziquantel-treated residents of four villages in two districts (Thoulakhom and Keo Oudom) in the Vientiane Province, Lao PDR. All faecal samples were evaluated for the presence of *O. viverrini*-like eggs by the quantitative method of KK. The KK method was performed in accordance with procedures described by WHO (1994). KK slides were examined 2–4 h after preparation and estimated infection intensity expressed as *O. viverrini*-like eggs per gram faeces (EPG = faecal egg count/slide × 24). For the FECT, 0.5–2 g of each of the 85 samples was fixed in 7 ml of formalin (10%) and processed according to Elkins et al. (1991). Two egg counts were made for each sample. Both FECT readings were used for evaluation of the KK method, i.e. if a sample was egg positive by only one of the FECT readings, it was designated as positive, and average egg counts were used. FBT infections were classified into three sub groups: light infections (EPG < 1000), moderate infections (EPG from 1000–10,000) and heavy infections (EPG > 10,000) according to Upatham et al. (1984). For PCR analysis, 0.5 g of faeces was obtained after thorough mixing. The samples were kept in 2 ml cryotubes (CM-Lab, Vordingborg, Denmark) and stored at −20 °C for PCR analysis.

2.2. Extraction of DNA for PCR

Faecal samples (100–500 mg) were mixed with 1 ml normal sterile saline (NSS) and 200 μl of ethyl acetate, and the mixture was centrifuged at 8000 rpm for 5 min. The pellet was washed with 1.5 ml of NSS, mixed (vortex) and incubated in 500 μl of 0.5N NaOH for 60 min at room temperature, autoclaved for 60 min (121 °C), mixed and centrifuged for 5 min at 8000 rpm. Samples were evaluated for the presence of broken trematode eggs after autoclavation. The supernatant was transferred to a 1.5 ml micro centrifuge tube. Sodium acetate (250 μl of 3 M) was added to the supernatant and pH was adjusted to 7–8. Half of one InhibitEX tablet (QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany)) was added to reduce potential inhibition and the mixture was centrifuged for 5 min at 14,000 rpm. The supernatant was transferred into a new tube, and the pellet was discarded. Ten microlitres of Proteinase K was added to the supernatant. After adding 200 μl Buffer AL and mixing, the suspension was incubated at 70 °C for 10 min. Finally, 200 μl of absolute ethanol was added, and the DNA solution cleaned and eluded by using a QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturers. Five microlitres was used in the PCR.

2.3. Polymerase chain reaction (PCR)

PCR was performed in a DNA thermal cycler (MJ PTC-2000). The reaction was carried out in a 25 μl volume containing 15 μl distilled water, 5 μl of extracted DNA and 2.5 μl of each OV-6 primer (5′-CTGAATCTCTCGTTTGTTCA-3′ and 5′-GTTCCAGGTGAGTCTCTCTA-3′) (Wongratanacheewin