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Improved performance and quantitative detection of copro-antigens by a monoclonal antibody based ELISA to diagnose human opisthorchiasis

Nattaya Watwiengkam[a,b], Jiraporn Sithithaworn[c], Kunyarat Duenngai[d], Banchob Sripa[b,e], Thewarach Laha[a], Maria Vang Johansen[f], Paiboon Sithithaworn[a,b,*]

[a] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[b] Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[c] Department of Clinical Microscopy, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand
[d] Department of Public Health, Faculty of Science and Technology, Phetchabun Rajabhat University, Phetchabun, Thailand
[e] Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[f] Section for Parasitology and Aquatic Diseases, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

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ABSTRACT

Copro-antigen detection has been advocated as a promising method for diagnosis of opisthorchiasis, particularly in people that harbored light infection or have had recent drug treatment. This study aimed to improve performance of a monoclonal antibody-based enzyme-linked immunosorbent assay (Mab-ELISA) for detection of *Opisthorchis viverrini* copro-antigen and assess the correlation between copro-antigen and intensity of infection. Four different treatment methods of 71 samples from the Lawa endemic area, Khon Kaen province were assessed for copro-antigen detection, namely (1) phosphate buffer saline (PBS), (2) heating (70 °C 30 min), (3) alkaline (pH 9.6 in carbonate buffer), and (4) trichloroacetic acid (TCA) treatment. Comparison of these protocols showed that the TCA method gave the best performance in discriminating *O. viverrini* positive and negative samples with high sensitivity (97.9%) and moderate specificity (54.2%) compared with other methods. Application of TCA-based Mab-ELISA method for antigen detection in fecal samples obtained from an endemic area of opisthorchiasis revealed that 86 of 141 samples (61.0%) were positive compared with 68 (48.2%) by PBS-based protocol, while the formalin ethyl-acetate concentration technique (FECT) yielded a positive proportion of 71.6%. Among 40 egg-negative samples confirmed by a gold standard parasitological method (FECT) from the same endemic area, 19 (47.5%) were positive by the TCA-based while only 6 (15%) were positive by PBS-based Mab-ELISA protocol. In addition, levels of antigen detection significantly correlated with intensity of infection (egg per gram feces). The results show that the improved Mab-ELISA method has high sensitivity and also quantifiable diagnosis of opisthorchiasis.

1. Introduction

The liver fluke, *Opisthorchis viverrini*, is the causative agent of opisthorchiasis, a neglected tropical disease affecting at least 10 million people in Thailand and Lao PDR (Sithithaworn et al., 2012). The infection has also been reported from Cambodia and Vietnam (WHO, 1995). Chronic infection with *O. viverrini* associates with hepatobiliary disease, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis and has been implicated in cholangiocarcinoma (Mairiang and Mairiang, 2003; Shin et al., 2010; Sithithaworn and Haswell-Elkins, 2003). Control of opisthorchiasis is achieved mainly with chemotherapy by praziquantel (PZQ) treatment (Jongsuksuntigul and Imsomboon, 2003), hence, sensitive and accurate diagnosis is required for long term and sustainable control programs (Johansen et al., 2010).

To date, conventional parasitological methods are the gold standards for diagnosis of opisthorchiasis such as the formalin ethyl-acetate concentration technique (FECT) or the Kato-Katz technique (KK) (Elkins et al., 1990; Viyanant et al., 1983). However, fecal examination requires experienced microscopists as it is difficult to distinguish eggs of *O. viverrini* from eggs of other zoonotic fish borne trematodes, for example, minute intestinal flukes. These

* Corresponding author at: Department of Parasitology and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Tel.: +66 43348387; fax: +66 43202475.
E-mail addresses: paibsit@gmail.com, paib_sit@kku.ac.th (P. Sithithaworn).

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conventional parasitological methods are not sensitive enough in cases of light infection and also for individuals who have recently received chemotherapy. To circumvent these problems, molecular and immunological diagnostic methods have been developed. Both polymerase chain reaction (PCR) (Duenngai et al., 2008; Umesha et al., 2008; Wongratanacheewin et al., 2001, 2002) and by loop-mediated isothermal amplification (LAMP) (Arimatsu et al., 2012) have revealed higher sensitivity in detecting *O. viverrini* infected persons than conventional parasitological tests. In addition to DNA-based diagnosis, copro-antigen detection by monoclonal antibody based ELISA (Mab-ELISA) has been introduced for diagnosis of opisthorchiasis with promising results in endemic communities (Sirisinha et al., 1995).

The aim of the present study was to improve the performance of a copro-antigen detection method by Mab-ELISA for diagnosis of opisthorchiasis by using different antigen extraction protocols to select, establish and optimize the most appropriate Mab-ELISA. It was applied to assess the community-based prevalence of opisthorchiasis and examine the relationship between *O. viverrini* copro-antigen and intensity of infection.

2. Materials and methods

2.1. Sample population and fecal specimen collection

Sample population recruited for this study was from participants enrolled in an ongoing project on morbidity of opisthorchiasis in Khon Kaen Province, northeast Thailand. The average age of the participants ($n = 141$) was 47.7 years and ranged from 29 to 59, which included 62 males and 79 females. The fecal specimens were collected in plastic containers and were kept in a chilled ice box and transported to the laboratory within one day. At the laboratory, the specimens were aliquot and 2 g aliquots were processed for examination by quantitative formalin ethyl-acetate concentration technique (FECT) (Elkins et al., 1990). The remaining aliquots allocated for antigen detection were kept at −20 °C for subsequent analysis.

2.2. Sample preparation experiment

A sub-sample of 71 samples with adequate fecal volumes was used for the sample preparation experiment. Examination of these samples by FECT revealed that 47 (66.2%) were positive for *O. viverrini* and 24 were parasite-negative. Sample preparation was done by adding phosphate buffer saline (PBS) pH 7.2 to each sample (1:6 by volume), mixed and centrifuged at 2000 rpm at 4 °C for 30 min. The resulting supernatants were then transferred into new vials and centrifuged at 10,000 rpm at 4 °C for 5 min and further processed to remove interfering components by one of the 4 treatment methods as follows: (1) PBS treatment, the samples that received no treatment which served as controls. (2) Heat treatment the samples were heated for 30 min at 70 °C without any additive (Kahama et al., 1998). (3) Alkaline treatment, which was done by adding an equal volume of 0.244 M carbonate buffer (pH 9.6) to each sample and thoroughly mixed (Kahama et al., 1998). (4) Trichloroacetic acid (TCA) treatment (De Jonge et al., 1987), which was done by adding equal volumes of 4%TCA solution to samples and incubated for 20 min at room temperature and subsequently neutralized with equal volume of 0.244 M carbonate buffer (pH 9.6). The samples prepared by these protocols were then further processed for antigen detection by Mab-ELISA as detailed below. Calculations of diagnostic efficacies based on PBS, heat, alkaline and TCA-based protocol for antigen detection were performed using FECT as a gold standard method.

2.3. Copro-antigen detection by Mab-ELISA

Monoclonal antibody, clone KKU505, which specifically recognizes molecules present in the excretory–secretory (ES) antigens of *O. viverrini* at molecular weights of 70 and 118 kDa was used for Mab-ELISA (Sithithaworn, unpublished). The assay was performed in polystyrene microtitration plates (Maxisorp®; Nunc) which were coated with 5 μg/ml Mab in carbonate buffer, pH 9.6 (100 μl/well) overnight at 4 °C. Plates were washed three times with PBS pH 7.4 containing 0.05% Tween 20 (PBST) and uncoated site were blocked with a blocking solution of 5% dried skimmed milk in PBST. The plates were incubated for 1 h; each incubation step was carried out at 37 °C. Washing was repeated thrice with PBST. Then, 100 μl of undiluted sample was added and incubated for 2 h at 37 °C and the plates were washed five times with PBST. IgG rabbit anti crude *O. viverrini* antigen (10 μg/ml) was added and incubated for 2 h at 37 °C. After washing, goat anti-rabbit IgG-peroxidase conjugate (Zymed, USA) (1:4000 dilutions) was added and incubated for 1 h at 37 °C. Thereafter, the plates were washed three times and 100 μl of substrate (*O*-phenylenediamine hydrochloride) was added and incubated for 20 min in the dark at room temperature. The reaction was terminated by the addition of 100 μl of 2 M sulfuric acid and the optical densities (OD) were read spectrophotometrically at 492 nm, with an ELISA reader (Tecan Sunrise Absorbance Reader, Austria). Since ES-antigen of *O. viverrini* in PBS gave higher signal than heating, alkaline, and TCA, ES-antigen of *O. viverrini* diluted in PBS was used as a positive control and PBS was used as a negative control. The average OD of positive control was then used to calculate adjusted OD value of tested samples. Furthermore, if fecal suspension was used to dilute ES-antigen, a lower signal was observed, hence PBS was a more suitable diluting solution for controls. Fecal samples were considered positive if the optical densities (OD) were higher than cutoff point derived from receiver operation characteristic curve (ROC) analysis.

This Mab-ELISA protocol was used for antigen detection in the samples prepared following different protocols as well as in field collected samples.

2.4. Receiver operation characteristic (ROC) curve

Known negative and positive fecal samples (20 each) of *O. viverrini* determined by repeated examination by FECT were used to construct the ROC curves in order to obtain the cutoff point for Mab-ELISA. For each protocol of sample treatment a cutoff value was calculated and used for diagnosis, namely, 0.225 for PBS, 0.230 for heating, 0.226 for alkaline and 0.210 for TCA based-Mab-ELISA as determined by ROC analysis using Med Calc version 9.6.3. The sensitivity, specificity, positive and negative predictive values were calculated and compared between diagnostic protocols.

2.5. Detection limit and standard curve construction

The excretory–secretory antigen of *O. viverrini* (ES antigen) was prepared from short terms *in vitro* culture of adult worms obtained from experimentally infected hamsters as previously described (Amornpunt et al., 1991). The antigen was used for spiking fecal specimens and used as positive control in Mab-ELISA. Parasite free-fecal samples obtained from laboratory staff were prepared and processed for detection limit experiments and standard curve construction.

The detection limit of Mab-ELISA was assessed by measurement of antigen in spiked samples with ES antigen of *O. viverrini* serially diluted with PBS or TCA treated fecal extract. The lowest concentration in comparison with negative controls was determined and considered as the detection limit of the Mab-ELISA in PBS