AN ENZYME-LINKED IMMUNOSORBENT ASSAY AS SCREENING TOOL FOR HUMAN INTESTINAL CAPILLARIASIS

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Abstract. Human intestinal capillariasis caused by *Capillaria philippinensis* is characterized by chronic diarrhea which may lead to death if left untreated. The mortality is highest among patients who are negative by conventional stool examination. Therefore this study explored the application of an enzyme-linked immunosorbent assay (ELISA) as a screening test for human intestinal capillariasis. The ELISA was developed using *Trichinella spiralis* soluble antigen for the detection of antibodies against *C. philippinensis*. A cut-off level at the upper 99% limit of the absorbance values of the healthy controls was established for positivity. All intestinal capillariasis sera showed positive ELISA, demonstrating 100% sensitivity, while all healthy control sera gave absorbance values below the cut-off level, resulting in 100% specificity. The ELISA was also positive with 75% of trichinellosis, 13.9% of strongyloidiasis, 9.1% of trichuriasis, and 4.2% of opisthorchiasis sera. The ELISA and immunoblot were in agreement in 91.1% of the sera tested. It was suggested that the here-presented ELISA is capable to detect intestinal capillariasis cases in endemic areas whose coproscopy is negative for worm eggs, larvae or adults.

Key words: ELISA, *Capillaria philippinensis*, screening test

INTRODUCTION

Infection of small intestines by the nematode *Capillaria philippinensis* has been well recognized since the first description of the parasite isolated from a human autopsy during an epidemic in the northern and central Philippines from 1967 to 1990 (Chitwood *et al*, 1968; Cross, 1992). Subsequently, cases have been reported from other Asian countries, such as Thailand (Pradatsundarasar *et al*, 1973; Saichua *et al*, 2008), Taiwan (Lu *et al*, 2006), Indonesia (Chichino *et al*, 1992), and Korea (Lee *et al*, 1993; Hong *et al*, 1994). In addition, a few cases were reported from other countries such as Egypt (Youssef *et al*, 1989; Mansour *et al*, 1990), Iran (Hoghooghi-Rad *et al*, 1987), India (Kang *et al*, 1994), and Colombia (Dronda *et al*, 1993). In Thailand, the endemic area covers 19 provinces in the northeast. Patients usually present with chronic diarrhea and the diagnosis is made by routine stool examination for *Capillaria* eggs, larva or adults (Cross, 1992). The

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accuracy of the conventional stool examination is low as patients can be misdiagnosed due to inexperienced microscopists or due to a scarcity of worm eggs in the stool specimens.

Recently, an immunoblot assay using *Trichinella* antigen has reported to be highly useful in supporting the diagnosis of intestinal capillariasis (Intapan *et al*, 2006). Thus the present study explored the application of a conventional enzyme-linked immunosorbent assay (ELISA) as a screening test for human intestinal capillariasis.

MATERIALS AND METHODS

Sera

Forty-three intestinal capillariasis, 23 opisthorchiasis, 22 trichuriasis, and 36 strongyloidiasis sera were collected from patients attending Srinagarind Hospital, Khon Kaen, Thailand whose stool specimens revealed worm eggs, larvae or adults by the formalin-ethyl acetate concentration technique (FECT) (Erdman, 1981; Intapan *et al*, 2005). Twenty trichinellosis sera were obtained from patients in Chiang Rai, Thailand 23 days after being infected in a trichinellosis outbreak (Morakote *et al*, 1991) and from patients in northeastern Thailand admitted to a hospital with clinical signs and symptoms compatible with trichinellosis 14-16 days after raw meat consumption. Negative control sera were obtained from 23 healthy adults from northeastern Thailand with a mean age of 31.6 ± 8.3 years (range 22-48 years) and from 34 children from southern Thailand with a mean age of 4.5 ± 1.1 years (range 3-6 years), all of whom revealed no parasites in their stool specimens by FECT. All sera were kept in a repository of the Department of Parasitology, Faculty of Medicine, Khon Kaen University. The use of these sera was approved by the Khon Kaen University Ethics Committee of Human Research (HE501115).

Antigen

Crude *Trichinella spiralis* antigen was prepared as described previously (Intapan *et al*, 2006). In brief, larvae were isolated from mouse carcasses by acid pepsin digestion method, then homogenized in a saline solution containing 0.1 mM phenyl-methyl sulfonyl fluoride, 0.1 mM tosylamide-2-phenylethyl-chloromethylketone, and 1 μM N-(N-[L-3-transcarboxyoxiran-2-carbonyl]-L leucyl)- agmatine, followed by sonication. The preparation was centrifuged at 10,000*g* for 30 minutes at 4ºC and the supernatant fluid was aliquoted and stored at -40ºC for use as antigen in the study. The protein content of the antigen preparation was determined by the method of Lowry *et al* (1951).

Immunoblot analysis

Immunoblot was performed as described previously (Intapan *et al*, 2006). The presence of 36.5, 40.5 and 54 kDa bands was diagnostic for intestinal capillariasis.

Enzyme-linked immunosorbent assay

The protocol of the indirect enzyme-linked immunosorbent assay used in this study followed those described previously (Voller *et al*, 1986; Morakote *et al*, 1991). The optimal antigen, serum and conjugate dilution, determined by checker board titration, was 5 μg protein/ml, 1:1,600, and 1:20,000, respectively. Each well of a flat-bottom 96-well microtiter plate (Costar, Corning, NY) was filled with 100 μl of optimal *Trichinella* antigen concentration in 0.1 M carbonate buffer, pH 9.6, and left overnight at 4ºC. After washing with phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T), 250 μl of 3% bovine serum albumin (BSA) in PBS-T were added to each well and left at room

temperature for one hour. The plate was washed again and 100 µl of optimally diluted sera at 1:1,600 in 1% BSA in PBS-T were added and incubated at 37°C for one hour followed by washing five times with PBS-T. One hundred µl of optimally diluted peroxidase-conjugated goat anti-human IgG antibody (Zymed Laboratories, San Francisco, CA) in 1% BSA-PBS-T were added and the solution was incubated for one hour at 37°C. Then, the plate was washed five times with PBS-T and all wells were filled with 100 µl of substrate-chromogen solution (0.03% hydrogen peroxide, 0.2 mg/ml *o*-phenylene diamine hydrochloride in citrate-phosphate buffer, pH 5.0) and the plate was left in the dark for 30 minutes. The reaction was stopped by adding 50 µl of 8N sulfuric acid and the absorbance of each well was determined with an ELISA microplate reader (Tecan, Salzburg, Austria) at a wavelength of 492 nm. Pooled capillariasis sera and healthy control sera were included in all plates to control day-to-day interplate and intraplate variation.

Statistical analysis

Mean absorbances between two groups were compared using *t*-test. Sensitivity, specificity and predictive values were determined as described by Zou *et al* (2007). Kappa statistics was used to demonstrate the agreement between two tests.

RESULTS

Distribution of absorbance values

ELISA results for intestinal capillariasis, healthy controls, trichinellosis, trichuriasis, strongyloidiasis, opisthorchiasis and mixed infections are presented in Fig 1. The absorbance values of the intestinal capillariasis sera were higher than those of the healthy control sera. The distribution of these values of the two groups did not overlap. The distribution of the helminthiases sera was wide in range with a few sera producing high values. Table 1 summarizes the means and standard deviations of the absorbance values of each

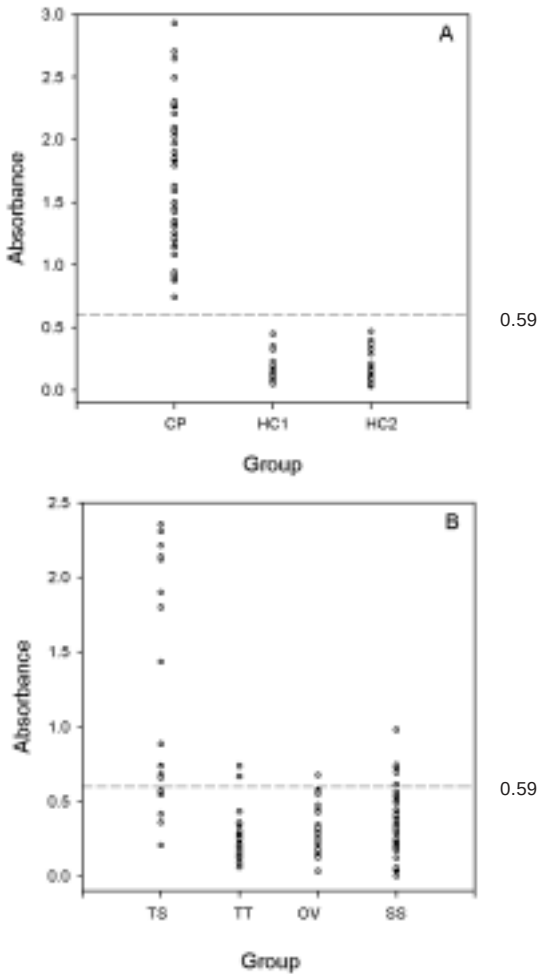

Fig 1–Distribution of ELISA absorbance values in (A) intestinal capillariasis patients and healthy controls, (B) helminthiases sera. The broken line represents the cut-off level of the ELISA. CP, Intestinal capillariasis sera; HC1, healthy control sera from northeastern provinces; HC2, healthy control sera from southern provinces; TS, trichinellosis; TT, trichuriasis; OV, opisthorchiasis; SS, strongyloidiasis sera.

Table 1
ELISA absorbance values among helminthiases and healthy control sera.

Group of sera	*n*	Mean ± SD	Range	No. positive (%)
Healthy controls	57	0.15 ± 0.11	0.03 - 0.47	0 (0%)
Northeastern	23	0.15 ± 0.10	0.05 - 0.47	0 (0%)
Southern	34	0.15 ± 0.11	0.03 - 0.47	0 (0 %)
Intestinal capillariasis	43	1.67 ± 0.5	0.74 - 2.93	43 (100%)
Trichuriasis	22	0.27 ± 0.17	0.06 - 0.74	2 (9.%)
Trichinellosis	20	1.16 ± 0.76	0.21 - 2.35	15 (75%)
Opisthorchiasis	24	0.32 ± 0.6	0.03 - 0.68	1 (4%)
Strongyloidiasis	36	0.35 ± 0.22	0.00 - 0.98	5 (14%)

SD, standard deviation

serum group. The mean absorbance of the intestinal capillariasis group was the highest (1.67 ± 0.55), followed by the trichinellosis group (1.16 ± 0.76). As expected, the mean absorbance of the healthy control group was the lowest (0.15 ± 0.11). The ELISA values of the healthy control from the northeastern provinces were not significantly different from those from the southern provinces (0.15 ± 0.10 *vs* 0.15 ± 0.11, $p > 0.05$).

Sensitivity, specificity and predictive value of ELISA

Using the mean absorbance value plus 4 SD of the healthy control sera (0.59) as a cut-off value, all intestinal capillariasis sera were positive, resulting in 100% sensitivity, while all healthy control sera were negative, resulting in 100% specificity (Table 1). The test gave 100% positive and negative predictive values. Above the cut-off value, 75% of trichinellosis sera were positive. Other helminthiasis groups gave lower rates of cross-positivity, *ie*, 4.2% for opisthorchiasis, 9.1% for trichuriasis, and 13.9% for strongyloidiasis.

Agreement with immunoblot analysis

Sera were tested also using immunoblot as a comparison for the ELISA and the results are summarized in Table 2. An agreement was found in 184 cases (91.1%), *ie*, 64 from 202 sera (31.7%) were positive with both tests while 120 sera (59.4%) were negative with both tests. A kappa value of 0.81 was obtained demonstrating an excellent agreement between immunoblot and ELISA. Of special interest was that 14 ELISA-negative trichuriasis sera gave positive immunoblot results suggesting that the latter detected antibody below the cut-off level of the ELISA.

Application of ELISA for differential diagnosis of chronic diarrhea cases

Out of 48 patients who came to the hospital with the chief complaint of chronic diarrhea but whose fecal examination by FECT could not reveal *C. philippinensis* eggs and /or larvae and /or adults, 4 patients with clinical features compatible with chronic intestinal capillariasis, were positive by ELISA and immunoblot.

DISCUSSION

An earlier attempt to develop a serologic test for the diagnosis of human intestinal capillariasis employed *C. opsignata* antigen, a parasite of poultry (Banzon *et al*, 1975). The indirect hemagglutination

Table 2
Agreement between immunoblot and ELISA.

Group of sera	*n*	ELISA/Immunoblot +/+	+/-	-/+	-/-
Healthy controls	57	0	0	0	57
Intestinal capillariasis	43	43	0	0	0
Trichuriasis	22	2	0	14	6
Trichinellosis	20	14	1	1	4
Opisthorchiasis	24	1	0	1	22
Strongyloidiasis	36	4	1	0	31
Total	202	64	2	16	120

assay (IHA) gave 85.9% positivity among 151 confirmed cases of intestinal capillariasis. The test also gave positive titers with trichinellosis and schistosomiasis sera. Subsequently, an ELISA using *C. philippinensis* adult and larval worm antigen obtained from experimentally infected gerbil was applied to 82 patients and 26 controls in the Philippines. The ELISA value distribution of the two groups apparently overlapped and the use of a more purified antigen was suggested (Cross and Chi, 1977).

For the sustainability of a diagnostic test, however, the availability of antigen is crucial. *Trichinella* antigen is a good substitute for *Capillaria* antigen as the worm can be maintained in a laboratory and both worms have common antigens. The use of *Trichinella* antigen was supported by its positive reactivity with capillariasis sera in IHA and precipitin tests (Banzon *et al*, 1975), and another ELISA (Morakote *et al*, 1991). Such substitution is only useful if the test is applied in areas where intestinal capillariasis is prevalent but trichinellosis is not endemic, such as in northeastern Thailand. Stichocytes, which are common among trichuroideas, have been proven to be an important source of diagnostic antigens of *Trichinella* (Despommier and Müller 1976; Takahashi *et al*, 1992; Bioreau *et al*, 1997) and can be shared among worms in the superfamily Trichuroidea (Roach *et al*, 1988). This is supported by evidence that rabbit anti-*Trichinella* serum reacted with *Capillaria* and *Trichuris* extracts (Cross and Chi, 1977). With such knowledge, an immunoblot using *Trichinella* antigen was successfully developed for the serodiagnosis of human intestinal capillariasis with a sensitivity of 100% (Intapan *et al*, 2006). Yet the test was only available in a research laboratory and the interpretation required expertise. The development of a more conventional assay such as an ELISA was needed.

In the present study, a microplate ELISA using *Trichinella* antigen gave high absorbance values for intestinal capillariasis patients. Even the lowest value of 0.74 was well above the cut-off point of 0.59. On the other hand, all healthy control sera from the southern province, a place where intestinal capillariasis was not endemic, were negative. The same applied to healthy control sera from the northeastern provinces. Analyzing the data from the intestinal capillariasis and healthy control groups, the ELISA had 100% sensitivity, 100% specificity, and 100% predictive

values. Thus this microplate ELISA using *Trichinella* larval extract as an antigen could be highly useful in *C. philippinensis* endemic areas for screening patients with chronic diarrhea whose coproscopy was negative for eggs, larvae or adults. The successful commercial development of this ELISA, in comparison to that of Cross and Chi (1977), may involve optimizing the conditions of the test, such as antigen, serum and conjugate dilutions. Our method could detect 4 patients with clinical features compatible with chronic intestinal capillariasis but who were copro-negative for *Capillaria* infection. All patients responded to mebendazole treatment (400 mg twice daily for 20 days). A medical history of these patients revealed that the copro-negative results in these cases were probably due to partial treatment with anthelminthic drugs before visiting the hospital.

The positive cross-reactions of the ELISA with other helminthiases may raise concern, but the clinical presentations of trichinellosis and trichuriasis, such as facial edema and myalgia, should greatly reduce the possibility of misinterpreting the ELISA results (Limsuwan and Siriprasert, 1994). Moreover, epidemiologically the majority of trichinellosis cases (77.9%) are in the northern part of Thailand (Kaewpitoon *et al*, 2008). As other helminthiases, 1 from 24 opisthorchiasis sera (4.2%) was ELISA-positive with an absorbance value of 0.68. In addition, 5 of 36 strongyloidiasis sera gave positive ELISA results (Table 1). All 5 of these sera showed specific bands by immunoblot indicating that these patients might in fact have intestinal capillariasis. Patients could not be followed up to confirm the infections since the sera came from the repository. Thus the positivity of the ELISA with other helminthiases might have been due to asymptomatic intestinal capillariasis or a failure of diagnosis by coproscopy or because of previous anthelminthic treatment. Evidence supporting this notion was a report of 8 cases of intestinal capillariasis in Taiwan that were undetected for more than a year (Lu *et al*, 2006) and a case in Thailand that required repeated stool examinations and endoscopy before worm eggs could be detected (Wongsawasdi *et al*, 2002). These findings emphasize the need for serologic screening tests. Most importantly, intestinal capillariasis is characterized by chronic diarrhea ranging from weeks to years, and if cases are left untreated it may lead to death due to electrolyte imbalance or septicemia from secondary bacterial infection (Cross, 1992). Thus ELISA using *Trichinella* antigen is practical for hospitals in endemic areas to supplement coprological diagnosis of intestinal capillariasis.

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