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Original Research Article

DIAGNOSIS OF HUMAN PARAGONIMIASIS IN COSTA RICA USING INTRADERMAL TEST AND IMMUNOBLOT

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Abstract: Excretory-secretory antigen (ESA) and adult somatic antigen (ASA) were obtained from adult worms of *Paragonimus mexicanus.* These antigens were characterized, tested and compared for its use in serodiagnosis of human paragonimiasis. ESA proteins with 30-32.5 kDa and ASA proteins with 30-35 kDa molecular weight reacted consistently with positive control sera, these proteins were determined as specific for detection of antibodies against *P. mexicanus* in immunoblotting. ASA was also used to prepare an intradermal test. In an indigenous community, 100 children were administered the intradermal test, 23 reacted positive. ESA immunoblot carried out with blood samples of the children detected 11 sera positive, while immunoblot with ASA detected 14 sera positive. Results of ESA immunoblot showed good correlation (0.86) with ASA immunoblot results, but both immunoblots showed moderate correlation (0.50 and 0.58) to the intradermal test. Although both antigens, seemed to be adequate for its use in serodiagnosis, we recommend the use of ESA for serological diagnosis of paragonimiasis in Costa Rica, since cross-reactions with other parasitic infection, especially with other trematode infections, did not occur. Furthermore, intradermal tests should be used for monitoring population at risk, and ESA immunoblot to confirm active infections of *P. mexicanus*.

Key words: *Paragonimus mexicanus***,** secretory-excretory antigen, adult somatic antigen, immunoblot, intradermal test

Introduction: Human paragonimiasis is an important zoonotic foodborne disease caused by trematodes of the genus *Paragonimus*[1]. Definitive hosts are wild mammals and domestic carnivores. Animals and humans acquire the parasite by eating raw freshwater crabs or crayfish containing metacercariae of these trematodes; adult parasites are commonly found in the lungs of the definitive hosts[2]. Two species of *Paragonimus* have been reported in wild animals and humans of Costa Rica, *Paragonimus mexicanus* and *Paragonimus caliensis*[3,4]. However, pathogenesis and

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prevalence of these parasites in humans from Costa Rica is unknown, due to the difficulty of diagnosing clinical cases and lack of laboratory diagnostic techniques in the country.

The first two cases of paragonimiasis were reported in two adult males with pulmonary complications, one of which died[5]. Summarizing the data published since then, a total of 28 clinical cases have been recorded in Costa Rica, occurring mainly in rural areas and in 90% of the cases affecting children[6]. Most cases reported in Costa Rica presented the pulmonary form (50%), whereas 26% were extrapulmonary (mainly cerebral and pericardial form) cases, and 24% presented mixed forms (pulmonary and extra-pulmonary cases)[7].

Diagnosis of pulmonary paragonimiasis is through detection of eggs in feces or sputum of patients[8], however, absence of eggs is possible in early infections, also in ectopic paragonimiasis[9]. The intradermal test is useful as a screening method and in differentiating paragonimiasis from other diseases, however an active infection cannot be distinguished from a past infection, and positive reactions can be recorded 10 to 20 years after complete recovery[10]. In addition, cross-reactions with other trematodes were reported[11]. Immunological techniques are useful in detecting antibodies against *Paragonimus*, immunoblot assay has been used in the diagnosis of paragonimiasis in humans from Asia[8,12,13,14].

In the present study, excretory-secretory antigen (ESA) and adult somatic antigen (ASA) of *P. mexicanus* were used to standardize an immunoblot, and perform serodiagnosis of human paragonimiasis in children of an indigenous community.

Materials and Methods: Production of excretory-secretory antigen (ESA)

Metacercariae of *P. mexicanus* were obtained from naturally infected freshwater crabs (Ptychophallus tristani), recollected in Balsa de Atenas, Alajuela, and resuspended in sterile saline solution. Subsequently six cats (*Felis domesticus*), three months old, were infected with 30-40 metacercariae by oral administration. The animals were kept in individual stainless steel cages, food and water was provided *ad libitum*. Two months post-infection, all six cats tested positive for *Paragonimus* eggs in feces, the animals were euthanized with pentobarbital. Lungs were washed several times with sterile saline solution and adult worms were recovered, washed three times with sterile saline solution, and ten worms placed in culture bottle (25 cm^2) containing 2.5 ml Eagle's basal medium (BME), and incubated at 37°C, 5% CO_2 as described previously[15]. Motility of adult flukes was recorded every 2 hours during 24 hours, when BME was recollected and changed. A fluke was considered dead, when its appearance was opaque, and motility was not recorded.

The harvested BME fractions were centrifuged for 15 minutes at 10.000 x g, and pellets resuspended with 500 µl of phosphate-buffered saline (PBS, 0.01M, pH 7.4), analyzed for egg presence, eggs counted microscopically, and expressed as number of eggs released per worm per two hours (EPWP2H). The supernatants of all BME fractions were dialyzed against PBS during 48 hours at 4°C, and stored at –20°C. Protein content was determined by optical density at 280nm and by the Folin-phenol method described by Lowry *et al.*[16].

To determine ESA in the supernatants of the culture fluids, immunodiffusion test was carried out (1% agarose, PBS, 0.02% sodium azide) as described by Ouchterlony[17], using control sera from human patients infected with *P. heterotremus*, kindly donated by Dr. Dekumyoy, Faculty of Tropical Medicine, Mahidol University, Thailand. Forty-three µl of the reactants were placed in each well and left to diffuse for 48 hour at room temperature in a moist chamber. The agar was then washed with distilled water for 72 h, stained with 0.25% Coomassie blue R-250, 45% methanol, 10% acetic acid, and destained with 20% methanol, 7.5% acetic acid, 10% ethanol.

Production of adult somatic antigen (ASA)

Flukes were washed three times with sterile saline solution and lyophilized. The dried

worms were macerated, suspended in distilled water, centrifuged at 10.000 x g for 15 minutes at 4°C, and the supernatant stored at -20°C until used[13]. Protein content was determined by the Folin-phenol method[16].

Analyzed samples: Control sera: Fifteen sera from patients with confirmed *P. heterotremus* infection were used as positive controls, whereas sera from 15 healthy humans were used as negative controls. These samples were kindly donated by Paron Dekumyoy, Faculty of Tropical Medicine, Mahidol University, Thailand, and used to implement the immunoblot. Also sera from 29 humans from Costa Rica, who were infected with cysticercosis [8], ancylostomiasis (larva migrans) [6] ascariasis [2], uncinariasis [4], fascioliasis [1], and mixed infections (ascariasis, tricocephaliasis, amibiasis, giardiasis [8]), kindly donated by Andrea Urbina, School of Veterinary Medicine, Universidad Nacional, Costa Rica, were used as negative controls in the immunoblot.

Tested population: A total of 100 indigenous children, 7 to 12 years old, from the primary school of Alto Katsi, in Talamanca, Limón province (9°30'N Latitude and 82°55'W Longitude), were tested by an intradermal test. Sera was collected at the same time, in addition fecal samples were obtained from 20 children. The region of Alto Katsi is known as an endemic region of paragonimiasis in Costa Rica, and ingestion of raw crabs is common part of the diet of indigenous people living in this region[18,19,20].

SDS-PAGE and immunoblotting technique

Excretory-secretory and adult somatic antigen were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 12% gels (1.5M Tris-HCl, pH 8.8) prepared as described by Laemmli[21]. Samples containing 50µg protein were boiled for 5 min in sample buffer (0.01M Tris-HCl, 2% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.02% bromophenol blue, pH 6.8) before loading onto the gel. After electrophoresis, the characterized polypeptide bands were either revealed with silver stain or transferred to nitrocellulose membranes for use in immunoblotting. Molecular weights were determined with standard markers ranging 6.5 to 205 kDa (Sigma, #M4038, St. Louis, USA). Immunoblot was carried out as described by Towbin et al. (1979)[22]. After protein transfer onto the nitrocellulose membrane, the membrane was immersed in blocking solution (4% hydrolyzed casein) for 6 hours and cut vertically into strips. Each strip was incubated with each serum sample, diluted 1:50, in PBS, 0.05% Tween-20 overnight, with gentle agitation. The strips were then washed 3 times with PBS, 0.05% Tween-20, and subsequently incubated with anti-human IgG-peroxidase (SIGMA, #A-0170) diluted 1:4000 in PBS, 0.05% Tween-20 for two hours, and washed as mentioned above. The immune complexes were visualized with 3'3 diaminobenzidine substrate as orange-brown bands, the reaction was stopped by washing with distilled water.

Intradermal test: The antigen for the intradermal test was prepared according to Sawada et al.[23]. Three grams of lyophilized *P. mexicanus* adult worms were powdered, resuspended in 100ml of 0.01M sterile PBS (pH 7.4), and centrifuged at 10.000 x g for 30 minutes at 4°C two times. To both supernatants (containing Fraction F) 10% Trichloroacetic acid (pH 3.5-4.0) was added, and kept in an ice box for 30 minutes, centrifuged at 10.000 x g for 30 min at 4°C, and supernatants dialyzed against distilled water for 24 hours. Finally, supernatants were centrifuged at 10.000 x g for 30 min at 4°C and lyophilized. The lyophilized antigen was suspended in sterile saline solution in a concentration of 50µg/ml, and tested for sterility with Thioglycolate and Sabouraud. Using individual tuberculin syringes, 0.01 to 0.02 ml of antigen was applied into the skin of the flexor surface of the forearm of the children. Furthermore, 0.01 to 0.02 of sterile saline solution was injected as negative control in the other forearm. The reaction was considered positive when the circumference of the wheal increased more than 5 mm within 20 minutes after injection (weak positive reactions [+]: 5 mm-7 mm; positive reactions [++]: 7 mm-10 mm, strong positive reactions [+++]:

>10 mm). The reaction was evaluated again 72 hours after application.

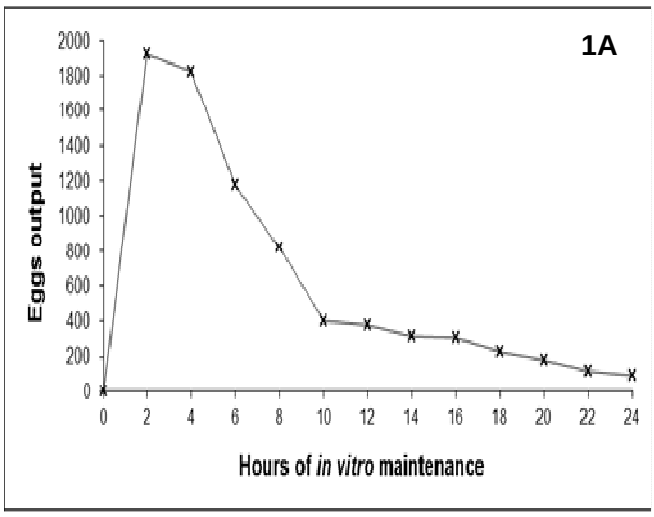
Statistical analysis: Statistical analysis of the results between immunoblot of ESA and intradermal test was performed with Cohen's kappa coefficient using Winepiscope 2.0 software. The same statistical test was used to correlate results of the immunoblot of ASA and immunoblot of ESA. *In vitro* maintenance of adult *Paragonimus* worms was analyzed by descriptive statistics using SPSS 8.0 software, also results of intradermal test, and parasitological analysis of fecal samples of children from Alto Katsi.

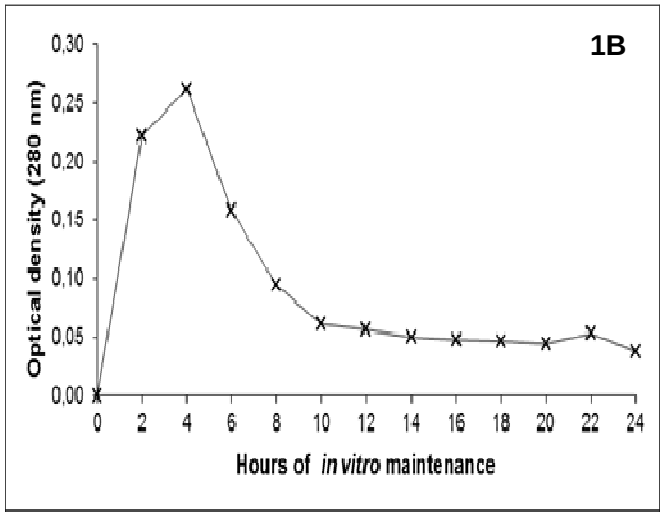
Results: Production of excretory-secretory antigen (ESA)

The worms seemed healthy and with normal appearance during the first 6 hours of cultivation. The worms became smaller and paler, and died within the first 26 hours of incubation.

The mean quantity of eggs released by adult worms in culture medium reached a maximum peak after 2 hours of incubation, and declined considerably after 6 hours of incubation (Figure 1A). The microscopic appearance of eggs released by worms during *in vitro* incubation was identical as the eggs found in feces of experimental infected cats. Likewise, the protein content of excretory-secretory antigen produced by ten adult worms during *in vitro* maintenance declined after 6 hours (Figure 1B), *P. mexicanus* antigen was determined with immunodiffusion in the first three fractions (2h, 4h, 6h of *in vitro* maintenance) (Figure 1C), in the fractions of the subsequent hours, precipitation bands were not observed. The protein content of ESA in the different fractions determined by Lowry assay is shown in Figure 1C.

Figure **1A.** Number of eggs released by *P. mexicanus* worms maintained *in vitro.* **1B.** Protein content determined by optical density at 280nm per 10 adult worms of *P. mexicanus* maintained *in vitro* 1C. Results of the different cultivation fractions of *P. mexicanus* in immunodifusion (ID) against positive control sera and protein content determined by Lowry assay.





1C

Hours *in vitro* maintenance	0	2	4	6	8	10	12	14	16	18	20	22	24
ID	-	+	+	+	-	-	-	-	-	-	-	-	-
Lowry assay	0	0.47	0.56	0.34	0.07	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.02

SDS-PAGE and immunoblot analysis of the ESA and ASA

SDS-PAGE analysis of *P. mexicanus* ESA revealed at least ten major proteins with molecular weights ranging from 21kDa to 84 kDa (21, 23, 25, 28.5, 30, 32.5, 35, 66, 74, 84 kDa). Immunoblot analysis with positive human control sera showed reaction of antibodies against nine major proteins with molecular weights of 21, 23, 28.5, 30, 32.5, 35, 66, 74, 84 kDa. The reactivity of each protein of the excretory-secretory antigen of *P. mexicanus* with sera from humans infected with different parasitosis is summarized in Table 1A. Proteins with 30 and 32.5 kDa molecular weight reacted with all positive control sera of *P. heterotremus*, and not with negative control sera and sera from patients with other parasitic infections (Table 1A).

SDS-PAGE analysis of *P. mexicanus* ASA revealed at least nine major proteins with molecular weights ranging from 21kDa to 120 kDa (21, 25, 30, 35, 36, 54, 55, 65,120 kDa). Immunoblot analysis with positive control sera showed reaction of antibodies against nine major proteins with molecular weights of 21, 25, 30, 35, 36, 54, 55, 65,120 kDa. Frequencies of reactivity of each antigen with sera from humans infected with different parasitosis is summarized in Table 1B. The proteins with 30 and 35 kDa reacted consistently with all positive control sera of *P. heterotremus*, and not with negative control sera and sera from patients with other parasitic infections (Table 1B).

Intradermal skin test, stool examination, and immunoblot analysis of native children

Twenty three (23%) out of 100 children reacted positive with the intradermal test. Eight of the positive children showed weak positive reactions, nine children positive and six strong positive reactions (Table 2). Reactions against the negative intradermal control were not recorded. Positive reactions were observed during 24 hours, when they began to decline. All positive cases were detected within 20 minutes, no new positive cases were found 72 hours after inoculation.

Eleven (11%) sera reacted positive in the ESA immunoblot, these sera reacted with proteins of 30 and 32.5 kDa molecular weight, and belonged to children that reacted positive in the intradermal test (Table 2). Of the total of sera examined by the immunoblot using adult somatic antigen, 14 (14%) yielded positive results, these sera reacted with proteins of 30 and 35kDa molecular weight. However, in this group, three sera yielded negative results in the intradermal test and immunoblot using excretory-secretory antigen (Table 2). The concordance of results of immunoblot with ESA and immunoblot with ASA, measured by the Kappa statistic, was of 0.86, whereas Kappa between intradermal test and ESA immunoblot and ASA immunoblot was 0.58 and 0.50, respectively.

From the total of stool samples collected, eight belonged to children that reacted positive to the intradermal test and in ESA and ASA immunoblotting, whereas 12 samples corresponded to negative reacting children in the intradermal test and ESA immunoblot, however in this group three children reacted positive in ASA immunoblot. *P. mexicanus* eggs were not detected, however, *Ascaris lumbricoides* (15/20, 75%), Uncinariariasis (12/20, 60%), and *Giardia lamblia* (10/20, 50%), were found in the stool samples.

Table 1. Recognition of antigenic components of *P. mexicanus* excretory-secretory antigen (**A**) and adult somatic antigen (**B**) by sera from humans infected with different parasitosis using immunoblotting.

Control sera	n	**A.** Number of control sera reacting with ESA proteins (in kDa) of *P. mexicanus*									**B.** Number of control sera reacting with ASA proteins (in kDa) of *P. mexicanus*								
		p21	**p25**	**p30**	**p35**	**p36**	**p54**	**p55**	**p65**	**p120**	**p21**	**p25**	**p30**	**p35**	**p36**	**p54**	**p55**	**p65**	**p120**
Paragonimiasis	16	16	16	16	16	16	16	14	16	15	16	16	16	16	16	16	16	14	16
Cysticercosis	8	8	8	0	0	0	8	0	5	0	8	8	8	0	0	0	8	0	5
Ancylostomiasis (larva migrans)	6	0	6	0	0	0	6	5	3	1	6	0	6	0	0	0	6	5	3
Ascariasis	2	1	0	0	0	0	2	0	2	2	2	1	0	0	0	0	2	0	2
Uncinariasis	4	4	4	0	0	0	3	4	0	0	4	4	4	0	0	0	3	4	0
Fasciolasis	1	1	1	0	0	1	0	0	0	0	1	1	1	0	0	1	0	0	0
Mixed infections	8	8	8	0	0	0	8	6	0	3	8	8	8	0	0	0	8	6	0
Negative control sera	65	65	65	0	0	0	0	0	0	0	65	65	65	0	0	0	0	0	0

Table 2. Comparison of results of intradermal test, ESA immunoblot, and ASA immunoblot of *P. mexicanus* of children from Katsi School, Talamanca (only positive results are shown).

Child	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Intradermal test	++	+++	+++	+	+++	+	+	++	+	++	+++	+++	+	+	++	++	++	++	+++	+	++	+	-	-	-	-
ESA immunoblot	-	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-
ASA immunoblot	-	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	+

Discussion

To implement a serological diagnosis of paragonimiasis in Costa Rica, excretory-secretory and adult somatic antigens were obtained from adult *P. mexicanus* flukes. To our knowledge, this is the first time, that *P. mexicanus* was maintained *in vitro* for production of ESA. Although the parasites showed a high metabolic activity during the first 6 hours, which also correlated with a high egg release and excretory-secretory protein production, it decreased afterwards, and lead to the death of the flukes between 8 and 24 hours of incubation. These results are similar to that obtained by Ikeda (1998)[24], and are probably due lack of supplementation of the medium. Although ESA protein was produced, it was a relatively small amount. It is recommended to improve the incubation period, supplementing with sera, essential amino acids and vitamins that besides adding a nutritional value to the medium, may be important to prevent changes in pH and osmolarity[15]. Finally, more frequent changes of the medium may have contributed to a longer survival of the flukes, since accumulated excretion and secretion products may have been removed. It is recommended to produce excretory-secretory proteins, and subject them to cloning.

The electrophoretic pattern determined for ESA of *P. mexicanus* was similar to that reported for *P. heterotremus*[14], whereas the immunoblot using ESA of *P. mexicanus*, determined the proteins with molecular weights of 30 and 32.5 kDa as specific, since they were recognized by all positive control sera and none of the negative control sera. These results agree with Maleewong et al. (1992)[14], who reported an ESA protein of *P. heterotremus* with molecular weight of 31.5 kDa reacting consistently with most of the sera of patients with paragonimiasis, and recommended its use in cloning experiments in future studies.

The electrophoretic pattern determined for ASA of *P. mexicanus* was similar to that reported for *P. heterotremus*[14], whereas the immunoblot with ASA determined proteins of 30 and 35 kDa as specific, since they were detected by all positive control sera and not by negative control sera[8,13].

The results obtained with the intradermal test applied to 100 indigenous children showed that 23% had been probably exposed to *P. mexicanus* infection. Although the intradermal test represents an easy, economic and fast assay[10,25], it has the disadvantage, that not only active infections were diagnosed, since an individual may react positively for a large period of time, even when recovered from the infection[10]. Furthermore, weak positive and false positive reactions have been reported[11]. For this reason, only children that reacted positive in the intradermal test and in the immunoblot should be considered with an active infection[25]. Excretory-secretory and adult somatic antigen immunoblots showed good correlation between them (0.86), but a moderate correlation compared to the intradermal test (0.50 to 0.58). However, ESA immunoblot seemed to be more adequate to detect active *P. mexicanus* infections in Costa Rica, since the ASA immunoblot detected three sera as positive that belonged to children that reacted negative in the intradermal test. These false positive reactions are probably due to cross-reactions with other parasitic infections, such as schistosomes or *Fasciola*[9,13,26].

Examination of fecal samples from the children yielded negative results, no *P. mexicanus* eggs were found, and that would have been the definitive diagnosis. Finding eggs was not expected in children of Alto Katsi, since eggs are shed intermittently in feces[27], and they are not detected in ectopic cases (50% of paragonimiasis cases in Costa Rica are extrapulmonary)[20]. On the other hand, presence of other parasitic infections were determined in the fecal examination, leading to the conclusion, that these may have caused some positive reactions in the intradermal test and the ASA immunoblot, what has been widely reported in the literature. In contrast, nonspecific reactions with ESA has never been reported[9,11,13,26].

The results of this study led to the conclusion, that the intradermal test is a useful tool for monitoring a population at risk with *Paragonimus* infection, although serological diagnosis is needed for the clinical diagnosis of the parasitic infection. We recommend the use of ESA for serological diagnosis of paragonimiasis in Costa Rica, since cross-reactions with other parasitic infections, especially with trematode infections, did not occur. This test appears to be sensitive and specific. Future investigations should explore the possibility of producing cloned ESA for use in serological assays.

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Conflict of interest:

No conflict of interests is declared.

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