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Immunodiagnosis of Egyptian Human Schistosomiasis Using Leucine Aminopeptidase

[1]Noha Mahana, [1]Mennat Allah Samy, [2]Tarek Diab, [2]Ibrahim Rabia and [1]Somaya El Deeb

[1]Department of Zoology, Faculty of Science, Cairo University, Egypt
[2]Theodore Bilharz Research Institute, Imbaba, Giza, Egypt

Abstract: The schistosomiasis caused by *Schistosomiasis* (*S.*) *mansoni* is one of the major public health problems in the world including Egypt. Immunodiagnostic methods are more applicable for their better sensitivity and specificity than other methods. The detection of leucine aminopeptidase (LAP) in serum could be more valuable in diagnosis; hence early treatment could be applied before irreversible damage takes place. *S. mansoni* LAP antigen was purified from excretory/secretory (ES) products by DEAE-ion exchange chromatography and was injected into rabbits to raise specific polyclonal antibodies (pAb) which was used as a primary capture to coat ELISA plates. The secondary capture pAb was prepared by conjugation of primary pAb with horse-raddish peroxidase (HRP). Sandwich-ELISA was done for *S. mansoni* infected mice and Egyptian patients' serum samples and showed sensitivity of 89.29% and 85%, respectively and specificity of 88.89% and 80%, respectively, by comparing with those infected with other parasites and control. From all data in this study, the sandwich ELISA techniques appear to be sufficiently sensitive assays for the detection of schistosomiasis. LAP detection in the patients' serum could be used for the early diagnosis of schistosomiasis.

Key words: Schistosomiasis *mansoni* · Leucine aminopeptidase (LAP) · Immunodiagnosis · Polyclonal antibody

INTRODUCTION

The most wide spread tropical diseases that cause worldwide morbidity and mortality are firstly malaria and is followed by schistosomiasis [1- 3]. Schistosomes infect about 200 million of the world's population [4] specially, Egyptians at different ages [5, 6].

Exposure to contaminated water is the main cause of infection as the cercariae penetrate the skin causing the infection [7].

Detection of parasite ova (parasitological methods), proved a direct method for diagnosis of schistosomiasis. Whereas, detection of specific antibodies or circulating schistosome antigens (CSA) in patients' serum or urine (immunological methods) are considered indirect means of diagnosis that are of high sensitivity and specificity [8].

The most commonly used immunodiagnostic assays for detection of light infection are enzyme linked immunosorbent assay (ELISA) [9, 10], Western blotting (WB) and immunofluorescence [11].

Leucine aminopeptidases (LAPs) are exopeptidases that are of critical biological importance due to their proteins ability to degrade which is done by hydrolysis of amino acid residues at the amino terminus of polypeptide chains [12].

Since, the LAPs activity, detected in the various stages of *Schistosoma* (eggs, schistosomula and adult worm), were responsible for miracidial emergence, scientists were directed to block this activity. It was found out that be statin, which was widely distributed, ubiquitous in nature and a general inhibitor of aminopeptidases, blocked LAP activity [13-15].

LAP was detected amongst a number of proteins and glycoproteins within the schistosome eggs [16].

Immunolocalization studies showed that the *S. mansoni* LAP was synthesized in the gastrodermal cells surrounding the gut lumen, so peptides generated in the lumen of the schistosome gut were absorbed into the gastrodermal cells and were cleaved by LAP to free amino acids before being distributed to the internal tissues of

Corresponding Author: Ibrahim Aly, Department of Parasitology, Theodor Bilharz Research Institute (TBRI), Imbaba,Giza, Egypt. E-mail: ibrahimshalash@yahoo.com.

the parasite. Since LAP was localized to the surface tegument, it might play an additional role in surface membrane re-modeling [15]. So this study aims to detect *S. mansoni* LAP antigen in sera of infected patients to evaluate its efficacy in early diagnosis of *S. mansoni*.

MATERIALS AND METHODS

Animals: Fifty six, eight-week (wk) old, male Swiss albino mice of the CD-1 strain, weighing 20±2 grams (g) were obtained from the *Schistosoma* Biologic Supply Center (SBSP), Theodore Bilharz Research Institute (TBRI, Imbaba, Giza, Egypt). Mice were infected by tail immersion with 100 cercariae of an Egyptian strain of *S.mansoni* supplied from SBSP/TBRI. Uninfected, untreated animals (25) were used as normal control, in addition to 20 mice were infected with other parasites such as *Entamoeba histolotica* (n= 10) and *Giardia lamblia* (n= 10). All animals were sacrificed12 wk post infection, blood was collected, serum was separated by centrifugation at 3000 xg for 10 minutes (min) and stored at -20°C until use.

New Zealand white male rabbit, weighing approximately 2 kg and about 3 months age, was examined before the experiments (free from *Schistosoma* and other parasitic infections) and maintained at SBSP/TBRI. It was kept under standard laboratory care. Handling and treatment of animals were conducted according to internationally valid guidelines and ethical conditions [17].

Study Population: This study was conducted on 90 patients from Outpatients Clinic and Hospital at TBRI. By parasitological examination they were divided into 60 infected with *S. mansoni*, 30 infected with other parasites (*Fasciola, Echinococcus* and *Ancylostoma)*, in addition, 20 individuals of the medical staff at TBRI served as parasite free-healthy negative control. Blood samples were collected from all cases and sera were separated, aliquoted and kept at -70°C until use.

Preparation and Purification of *S. mansoni* LAP Antigen: Mature *S. mansoni* worms were incubated for 16 hrs at 37°C in RPMI 1640 (PH 7.3) (Sigma, St. Louis, MO) containing 2% glucose, 30 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) and 25 mg of gentamycin (Sigma). The suspension was centrifuged at 14,900 x g for 30 min and the supernatant (ES) was collected and stored at -20°C as aliquots until use [18].

Antigen was purified by two steps DEAE sephadex G-50 ion exchange chromatography and gel filtration chromatography on sephadex-G-100 HR column [19]. Absorbance of each fraction was measured at 280 nm and the purity of the produced protein was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli [20]. Protein content of excretory secretory (ES) LAPantigen was measured by the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA) [21].

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Purified ES antigens were electrophoresed on 12% SDS-PAGE, under reducing condition according to Laemmli [20].The antigen bands and molecular weight markers were visualized in polyacrylamide gel by staining with Comassie brilliant blue R-250 (Sigma).

Production and Purification of Polyclonalantibody (pAb): One mg of *S. mansoni* LAP product was mixed with an equal vol. of complete Freund's adjuvant (CFA) and injected intramuscularly (i.m.) into each of 2 rabbits according to Guobadia and Fegbami [22]. Booster doses [0.5 mg mixed with an equal vol. of incomplete FA (IFA)] were i.m. administered at weeks 2, 3 and 4 after the initial dose according to Fagbemi and Guobadia [23]. Blood samples were examined from the rabbit's ear before injection and before each boosting injection to detect the titer of antibodies produced. When the titer became high (~4 days post last injection), the animals were sacrificed and blood samples were collected. Antisera were pooled and heat-inactivated then stored as aliquots at -20°C till use [24].

Pab in sera was purified by ammonium sulfate precipitation methods according to [25]. The gamma protein was further purified from serum proteins (IgG) by caprylic acid treatment [26, 27]. Protein content was estimated after each purification according to Bradford [21]. The purity of the produced IgG was identified by 12% SDS-PAGE (1mm) under reducing conditions [20].

Reactivity of pAb to *S.mansoni* LAP Antigen by Indirect ELISA: Microtitre plate was coated overnight at 4°C with 30 ug/ml LAP in carbonate coating buffer, blocked with 0.1% BSA in PBS then 100 ul/well of serially diluted pAb (1:50 to 1-3200) in washing buffer was added. Hundred µl/well of anti-rabbit IgG peroxidase conjugate (Sigma) diluted in washing buffer (1/1000) was dispensed. Fifty µ1/well of 8N H_2SO_4 was added to stop the enzyme

substrate reaction. The absorbance was measured at 492 nm using ELISA reader (Bio-Rad microplate reader, Richomond, Ca). After each step, there were washing 5 times and the incubation was 1hr at 37°C.

Reactivity of *S. mansoni* pAb LAP in Patients' Sera by Sandwich ELISA: Labeling of pAb with HRP was performed by periodate method according to Tijssen and Kurstak [28]. Sandwich ELISA, originally described by Engvall and Perlmann [29], was performed. Wells of microtitre plates were coated with 100 µl/well of purified 10 µg/ml pAb IgG in carbonate buffer, pH 9.6, then blocked after washing with 200 µl/well 2.5% fetal calf serum (FCS) (Sigma)/0.1 M PBS/T for 2 hr and incubated at 37°C. Hundred µl of pooled positive and negative sera, was added individually to each well and incubated for 2 hr at 37°C. The plates were washed trice with washing buffer. Hundred µl/well of peroxidase-conjugated IgG antibodies of dilution 1/50, 100, 250, 500 and 1000 was dispensed and plates were incubated for 1 hr at 37°C and then were washed 5 times with washing buffer. Color appearance was done by addition of 100 µl/well substrate buffer and the plates were kept in dark at room temperature for 30 min., then the enzyme reaction was stopped by 50 µl/well of 8 NH_2SO_4. The absorbance was measured at 492nm using ELISA reader.

Statistical Analysis: The data are presented as mean±standard deviation (M±SD). Correlation coefficient (r) was used to find the relation between the ELISA optical density (OD) and parasitological data according to Snedecor and Cochran [30]. The data were considered significant if *P* values were equal to or less than 0.05. Statistical analysis was performed with the aid of the SPSS computer program (version windows 6.0).

RESULTS

Antigen Purification by DEAE-Sephadex A50-ion Exchange Chromatography: The OD_{280} profile of the antigen fractions obtained following purification by DEAE Sephadex A-50 ion exchange chromatography. The eluted antigen is represented by a single peak with maximum OD value approximately equal to 2.8 at fraction number (no) 9 (Fig. 1).

Purification by Sephacryl S-200 Column Chromatography (Gel Filtration Column): The antigen fraction no 9 were further purified by DEAE sephacryl S-200 gel filtration column chromatography and one peak was obtained represented the column elution volume fractions which contain LAP with OD value 1.5 at fraction no 7 (Fig. 2).

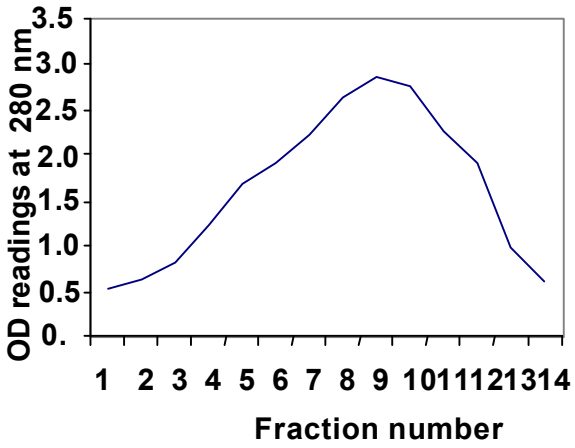

Fig. 1: OD_{280} profile of the antigen fractions obtained following purification by DEAE Sephadex A-50 ion exchange chromatography.

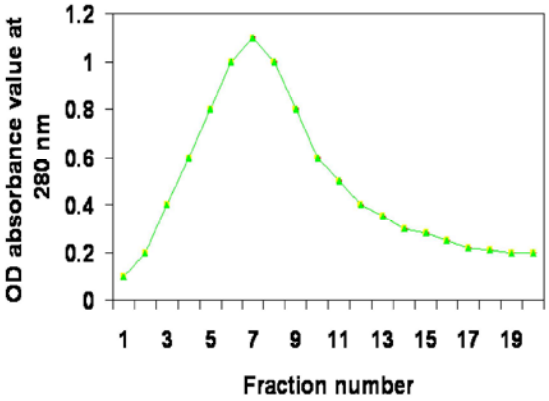

Fig. 2: OD_{280} profile of the antigen fractions obtained following Purification by sephacryl S-200 column chromatography

Characterization and Identification of LAP

Antigen Profile: The eluted protein fractions resulted from the different purification methods was analyzed by 12% SDS-PAGE under reducing condition and showed only one band at ~97.5 kDa which representing LAP antigen (Fig. 3). The protein content of the purified LAP antigen was 2.8 mg/ml.

Reactivity of Target Antigen by Indirect ELISA: Serum samples from human-infected with *S. mansoni* gave significant ($P<0.001$) strong reactivity against *Schistosoma* LAP with mean OD reading equal to 2.29 and no cross reactions were recorded with sera of

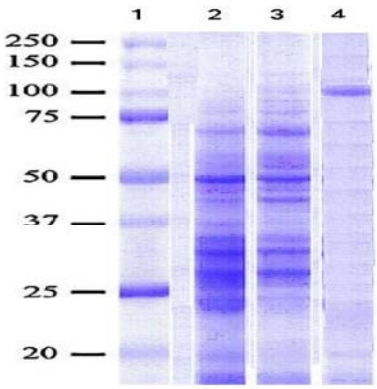

Fig. 3: SDS-PAGE (12%) of LAP eluted from affinity chromatography columns. Lane 1: Low molecular weight standard; Lane 2: E/S product; Lane 3: Purified LAP by DEAE-ion exchange chromatography; Lane 4: purified LAP.

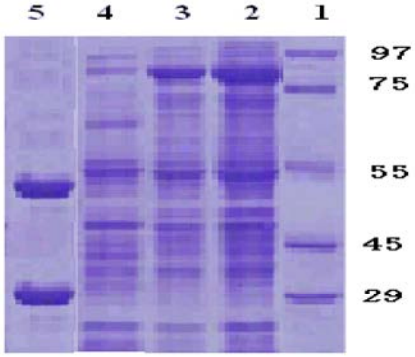

Fig. 4: 12% gel of anti-LAP IgG antibody before and after pAb purification stained with commassieblue.Lane 1: Molecular weight of standard protein; Lane 2: Crude anti-*S. mansoni* LAP IgG pAb; Lane 3: Precipitated proteins after 50% ammonium sulfate treatment; Lane 4: Purified IgG antibodies after 7% caprylic acid treatment; Lane 5: Purified LAP after DEAE.

patients infected with other parasites e.g., *Fasciola*, *Echinococcus* and *Ancylostoma,* by indirect ELISA (Table 1).

Purification of IgG pAb: The purity of the crud IgG pAb (with a protein content 12.5 mg/ml) after each purification step was assayed by 12% SDS-PAGE under reducing conditions. Analysis of 50% ammonium sulfate-precipitated proteins showed several bands (5.9 mg/ml), then further purified by 7% caprylic acid (3.1 mg/ml). Finally, the highly purified anti-*Schistosoma* IgG pAb subjected to ion exchange chromatography method (DEAE sephadex A-50 ion exchange column chromatography) was represented by only 2 bands, L and H-chain bands at 31 and 53 kDa, respectively. The protein content of highly purified pAb was 2.9 mg/ml and appeared free from other proteins (Fig. 4).

Table 1: Reactivity of purified target antigen by indirect ELISA

Serum samples	M of OD readings at 492 nm ☐ SD
Schistosomiasis	2.29±0.342***
Fasciolosis	0.264±0.201
Echinococcosis	0.106±0.094
Ancylostomiasis	0.182±0.082

OD= optical density; SD= standard deviation; M= mean

*** $P< 0.001$ high significant increase between shistosomiasis and all other parasites groups using purified target LAP antigen.

Table 2: Specificity of polyclonal antibody against LAP

Serum samples	OD readings at 492 nm±SD
Schistosomiasis	2.525±0.22***
Fasciolosis	0.462±0.151
Echinococciasis	0.406±0.112
Ancylostomiasis	0.310±0.021

OD= optical density; SD= standard deviation; M= mean

*** $P< 0.001$ high significant increase between shistosomiasis and all other parasites groups using purified target LAP antigen.

Reactivity of pAb Against *Schistosoma* LAP: Indirect ELISA recorded an increasing antibody level started one wk after the first booster dose. Three days after the 3rd booster dose immune sera gave a high titer against *Schistosoma* LAP with OD of 2.97.

Specificity of Polyclonal Antibody Against LAP: The produced anti-*Schistosoma* antibodies gave significant ($P< 0.001$) increase strong reactivity to *Schistosoma* LAP. The OD readings at 492 nm for *Schistosoma* were 2.525 compared to 0.462, 0.406 and 0.310 for *Fasciola*, *Echinococus* and *Ancylostoma,* respectively, where they gave no considerable cross reaction to *Schistosoma* LAP (Table 2).

Sandwich ELISA: In order to measure the incidence of positivity for *S. mansoni* LAP in the studied sera, the cut off point for positivity was measured asmean OD reading of negative controls +2SD. Tested samples showing OD values more than cut off value were considered positive for *S. mansoni* LAP in sera of schistosomiasis group.

Detection of *Schistosoma* Antigen in Serum of Infected Mice: The OD values of *S. mansoni* infected animals were significantly ($P<0.001$) higher than both negative controls and other parasites infected animals. Fifty out of 56 mice were positive. Regarding mice infected with other

Table 3: Detection of circulating *S.mansoni* antigen in different studied groups using purified anti-LAP *Schistosoma* IgG antibody in animals'sera using sandwich ELISA

	Positive cases		Negative cases	
Group	No.	M±SD	No.	M±SD
Healthy control (n= 25)	-	-	25	0.209±0.121
S. mansoni (n= 56)	50	2.23±0.358***	6	0.279±0.119
Enamoeba histolotica. (n= 10)	3	0.519±0.193	7	0.231±0.108
Giardia lamblia (n= 10)	2	0.622±0.233	8	0.257±0.180

M= mean, SD= Standard deviation, No= number.
Cut off= 0.45.
*** $P<0.001$ high significant increase between circulating antigen in serum samples of *S. mansoni* infectd mice gruop and all other parasite mice groups using purified polyclonal antibody against LAP.

Table 4: Detection of circulating *S.mansoni* antigen in different studied groups using purified anti-LAP *Schistosoma* IgG antibody in human's sera using sandwich ELISA

	Positive cases		Negative cases	
Group	No.	M±SD		No.
Healthy control (n= 20)			20	0.290±0.05
S. mansoni (n= 60)	51	1.12±0.410***	9	0.245±0.113
Fasciola (n= 10)	5	0.521±0.135	5	0.134±0.107
Echinococcus (n= 10)	4	0.39±0.109	6	0.281±0.145
Ancylostoma (n= 10)	3	0.469±0.139	7	0.311±0.132

M= mean, SD= Standard deviation, No= number.
Cutoff= 0.39.
*** $P<0.001$ high significant increase between circulating antigen in serum samples of *S. mansoni* patient's gruop and all other parasites patient's groups using purified polyclonal antibody against LAP.

parasites, there was a degree of cross reactivity as mice infected with *Giardia lamblia* showedhigher value than those infected with *Enamoeba histolotica.* The sensitivity and specificity of the assay were 89.29% and 88.89%, respectively, the positive and negative predictive values (PPV and NPV) were 90.91% and 86.96%, respectively (Table 3).

Infected Human: The OD values of *S. mansoni* infected patients were significantly ($P<0.001$) higher than both negative controls and other parasitic patients. Fifty one out of 60 cases were positive samples, the remaining 9 were negative. Regarding patients with other parasites, there was a degree of cross reactivity as fasciolosis showed the highest one, followed by ancylostomiasis and echinococcosis, respectively.

The detection of serum LAP antigens by sandwich ELISA gave sensitivity of 85%and specificity of 80%. PPV andNPV were calculated coprologicallyas a reference test for diagnosis of human schistosomiasis. LAP IgG-ELISA gave PPV= 80.95% and NPV= 84.21% (Table 4).

DISCUSSION

The immunological method proved to be more sensitive and accurate than parasitological methods for diagnosis of schistosomal infection, especially in early or low-intensity infected patients [31, 32]. So this study focused on the antigen detection in sera of infected patients to evaluate its efficacy in early diagnosis of *S. mansoni*.

Affinity chromatography has been shown to be a very effective tool for isolation of candidate diagnostic and vaccine molecule [22, 23, 33]. In this study the LAP was purified from the *S. mansoni* by affinity chromatography.

The purified LAP was reasonable in comparison with that of purified antigen from any biological fluid following similar purification procedures [34]. In agreement with [35], the eluted protein fractions showed only one band at 97.5 kDa which represented LAP.

In the present study, a relatively large series of controls and other parasite infected patients were included to avoid conjunction with other infectious diseases [36, 37], cross-reactive antibodies [38, 39] and autoimmune antibodies [40].

ELISA test, for its high sensitivity and specificity [41], was considered to be a most useful immunodiagnostic test for epidemiological studies in low endemic areas [42-44].

Specificity is a very important parameter for a diagnostic test to outline subjects without the targeted disease, thus the higher specificity the more reliable is the test [45]. The present study showed specificity in both

mice and human (88.89% and 80%, respectively) compared to the study of [46], which recorded a higher specificity percentage (98.2%). This difference could be attributed to the samples with other infectious diseases in endemic area where they may be undergo cryptic schistosomiasis given the high prevalence of infection with *Schistosoma* in areas of endemicity, but in the previous study it sure come from non endemic area, also both high and low specificity with ELISA have previously been reported [47, 48, 49].

The specificities of the evaluated tests were mainly influenced by the cross-reactivity with specimens from fascioliasis, echinococcosis or ancylostomiasis patients. These observations suggested that when a positive result is obtained for a patient from an area co-endemic with schistosomiasis and one of the previous diseases, the history of infection with, or exposure to, the other two diseases should be considered also.

In making a choice between producing pAbs or mAbs, the desired application of the antibody and the time and money available for production should be considered. The fact that a polyclonal antiserum can be obtained within a short time (4-8 wk) with little financial investment favors its use, whereas it takes about 3 to 6 months to produce mAbs. Many research questions can be answered by using a polyclonal antiserum. mAbs are specific for an epitope, which can be essential in specific cases[50].

The prevalence of the disease could influence the predictive values. The higher the prevalence, the more likely a positive test would be predictive of the disease [51, 52]. Thus, the PPV of the detection of LAP antigen by ELISA, in serum of both mice and humans, could be high in areas where the prevalence of *S. mansoni* is high as it had high results in the present study (90.91% and 80.95%, respectively), whereas it is low in low prevalence areas[53]stated that, as the transmission of disease drops to very low levels, the PPV for any test is decreased. On the other hand, the NPV is expected to be low as the prevalence of the disease increases as observed in this study, where NPV recorded slightly low for serum of both mice and humans (86.96% and 84.21%, respectively).

Our results, with agreement with recent study by Oniya *et al*. [54], showed that there was a significant difference in the individuals with *S. mansoni* and those without, thus showing that this ELISA test actually does differentiate between individuals who have been infected and those who have not. The importance of our study in choice of the circulating *Schistosoma* LAP antigen, revealed that, the sensitivity and specificity of ELISA in schistosomiasis diagnosis has been documented.

In Conclusion, the sandwich ELISA and indirect ELISA techniques appear to be sufficiently sensitive assays for the detection of schistosomiasis in both mice and human. Moreover, they proved to be the most accurate technique for estimation of antigen and antibody concentrations in their serum samples using pAb raised against purified *S. mansoni* LAP antigens and determination of disease activity.

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