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Research Article

Detection of Specific Antibodies for Amoebapore in Serum of Patients with Amoebic Liver Abscess

González-Canto A[1,2]*, Escalona-Montaño AR[2], Ruiz-Remigio A[2], Ximénez-García C[2], Del Callejo-Canal D[3], Gutiérrez-Ruíz C[4], López-Vancell R[2] and Pérez Tamayo R[2]

[1]Posgrado de Biología Experimental, Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana Iztapalapa, México

[2]Unidad de Investigación en Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México,

[3]Instituto de Investigación de Estudios Superiores Económicos y Sociales de la, Universidad Veracruzana, Mexico

[4]Departamento de Ciencias de la Salud, División de Ciencias Biológicas de la Salud, Universidad Autónoma Metropolitana Iztapalapa, México

*Corresponding author

González-Canto A, Unidad de Investigación en Medicina Experimental, Facultad de Medicina, UNAM, Dr. Balmis 148, Colonia Doctores, C.P. 06726, Ciudad de México, México, Tel: 52-56-23-2652; Fax: 52-57610249.
Email: metaugus@yahoo.com

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- Clinical diagnosis

Abstract

The presence of anti-amoebapore antibodies was detected through an enzymatic immunoassay linked to an enzyme (ELISA) and immune electro transference (Western Blot) in patients with a clinical diagnosis of amoebic liver abscess (ALA).

Twenty-five samples of serum of patients from General Hospital of Mexico with a previous clinical diagnosis of ALA were assessed. In addition to this, the samples obtained from other 22 subjects were evaluated to form the 4 control groups that are described as follows: 9 patients with pyogenic liver abscess (PLA), 3 with leishmaniasis, 3 with trypanosomiasis, and, 7 healthy ones. Out of the patients previously diagnosed with ALA, 60% tested positive in the actual ELISA analysis; the same outcome was obtained in the Western Blot (WB) assay. The statistical analysis with the Receiver Operating Characteristic Curve (ROC) showed values of 100% of sensitivity and 100% of specificity in the ELISA technique using pure amoebapore. Therefore, the ELISA with the amoebapore protein seems to be a good choice for the serum diagnosis of ALA.

INTRODUCTION

Amoebiasis is a disease caused by the *Entamoeba histolytica,* which is a protozoan parasite with worldwide distribution. It is believed that this parasite infects around 50 million people every year, and that 110,000 of them die from complications. One percent of the infected people may develop pathologies such as acute amoebic colitis or amoebic liver abscess (ALA) [1-5]; having this latter a 10-times higher frequency in men than in women [6,7]. ALA shows up when the parasite invades the liver from the intestine, probably through via porta; most of the cases exist with only one abscess generally located in the right hepatic lobe, which receives most part of the portal circulation [8,9]. The origins of the liver abscess in a patient may be amoebic or not [10], thus to make a specific diagnosis requires lab tests such as the ultrasound and microbiological and immunological analyses to corroborate the etiology [11-14].

Amoebiasis diagnosis based on the parasite´s morphology through a microscopic analysis is highly useful; however, it requires a series of fresh samples and the results tend to vary a lot, thus several analyses are needed in order to give a morphological diagnosis [15].

The *E. histolytica* parasite produces an immune response in humans: cellular as well as humorally; antibodies are produced in patients with symptomatic amoebiasis but also in asymptomatic patients, probably as a result of the invasion of such pathogen [16,17].

The presence of antibodies against *E. histolytica* in patients with extraintestinal amebiasis [13,18-20] has been identified by different techniques such as complement fixation (CF), counter immunoelectrophoresis, indirect immunofluorescence, ELISA and Western Blot (WB).

It has been shown the increase of circulating antibodies in patients with ALA, mainly of the IgG type, which may be detectable one week after symptoms start, in humans as well as in experimental animals [21]. These antibodies endure for years after invasive amoebiasis is resolute [22,23] probably due to the

persistence of amoebic antigens in the monocyte macrophage system cells [17].

The increased titles of antibodies are not related to the clinical seriousness or to the prognosis, but they do coincide with early stages of the disease; however, such variables decrease with treatment [24]. Statistical analysis shows a relationship between active amoebiasis and an increase in the serum IgG concentration; ALA patients show higher levels of IgG than those with amoebic colitis or of healthy patients ($p < 0,001$) [25,26].

There are seroepidemiological studies in which 81-100% of ALA patients and a lower percentage of patients with amoebic colitis (50%) develop specific IgG antibodies to *E. histolytica* [26-28]. A low percentage of false negatives may be due to the late production of antibodies and false positive results are more frequently found in endemic areas, due to past infections [16]. Overall, from all immunoglobulins, the increase in IgG has been the most consistent in patients with symptomatic amoebiasis [25]. Several research groups have detected, through ELISA, certain amoebic antigens in feces and pus from liver abscesses as well as antibodies in serum and saliva [29-32].

In some assays, a complete *E. histolytica* lysate was used to identify an immunological reaction [33,34]. In regards to the use of pure molecules for this type of assays, Gal/GalNAc lectin is the most common [34,35], even though there are other molecules derived from the amoeba used for this purpose, such as the serine-rich antigen [36] or the lipophosphoglycan [37]. On the other hand, WB allows the detection of *E. histolytica* specific antigens, which are recognized by sera from symptomatic and asymptomatic patients [38].

ELISA and WB techniques were used in this research work for the detection of specific antibodies for amoebapore in serum from patients with a clinical diagnose of ALA based on the results of ELISA using as antigen a membrane rich extract.

MATERIAL AND METHODS

Purified amoebapore

As described by Diamond *et al*., we got the lysate and amoebic supernatant from the axenic culture of *Entamoeba histolytica* HM1: IMSS trophozoites [39-41]. The purification of the amoebapore was carried out in a similar way to the method used by Leippe [42], with two adaptations: 1) the fraction was eluted with 0.1M NaCl in the ionic exchange chromatography; and, 2) in the last stage of purification, native electroelution was performed. Fractions were analyzed in silver stained 16% polyacrylamide gels electrophoresis (PAGE), in each purification phase; amoebapore activity was monitored fluorometrically by the dissipation of a valinomycin-induced diffusion potential in liposomes [42,43].

In order to corroborate de molecule´s purity, the porin sample was sequenced by a mass spectrometry (MS) in the Sequencing Unity at the Medicine School of the National Autonomous University of Mexico.

Anti-amoebapore rabbit IgG antibodies

To prepare rabbit antibodies, pure amoebapore was used as antigen following the protocol described elsewhere [44]. At the end of the immunization scheme, rabbits were bled and anti-amoebapore IgGs were purified.

The specificity of IgGs anti-amoebapore was corroborated by Western Blot analysis [45] against total lysates of *E. histolytica* HM1: IMSS trophozoites.

Specificity for anti-amoebapore IgGs assay over *Leishmania*, *Trypanosomes* and *Entamoeba histolytica* extracts

A WB assay was carried out from a complete lysate of *L. mexicana* and *T. cruzi* button cells; each sample was treated with 10 mM EDTA, 100 µM Iodoacetamide and 0.2 µM E-64. An amoeba lysate, treated in the same way, and a pure sample of amoebapore (3.2 µg) were included as a positive control. All samples were adjusted to 10 µg of protein and treated with 10 % β-mercaptoethanol. The transference was carried out in nitrocellulose and 1.25 µg/mL anti-amoebapore rabbit IgGs were used. And lastly, it was treated with a 1:120,000 dilution of the peroxidase-conjugated secondary antibody (goat anti-rabbit IgG's). The strips were revealed by chemiluminescence [46].

Sera

The examined 47 human sera were classified into four groups. Group A included 25 patients´sera with ALA clinical diagnosed; in group B, 7 healthy subjects´sera were obtained; group C constituted of 9 pyogenic liver abscess`s (PLA) sera; and, lastly, group D consisted of 6 sera: 3 from patients with leishmaniasis and other three with trypanosomiasis. Sera were obtained from 5 mL of blood from each patient.

ELISA

The antigen (0.6 µg of pure amoebapore) was fixed by microwell (each serum to be analyzed in triplicate) on a 96 plaque. A 100 µL dilution of 1:100 was added by microwell of each one of the sera belonging to each one of the four groups previously described. Additional 100 µL of dilution 1:12,000 was added by microwell of the secondary peroxidated antibody (rabbit anti-human IgA, IgG and IgM), which were revealed by TMB peroxidase substrate (Tetramethylbenzidine) and peroxidase solution B. The reaction was stopped with 100 µL of phosphoric acid 1M. The reading of the samples was carried out at a wavelength of 450 nm and the cut point of each analyzed sample was calculated taking into consideration the standard deviation (SD) and applying the following formula: (3 x SD + the average of negative control). Next, the cut point was deducted from the average value of each sample and when the result was higher to the cut point, it was considered positive. Otherwise, if the result was lesser or equal to the cut point, it was considered negative.

Western Blot

A denaturing electrophoresis was carried out at 16 % polyacrylamide with 40 µg of a pure amoebapore sample available at the only lane formed by a one-single tooth comb. The run was stopped when the advancing front reached the end of the gel. The gel was then transferred to a nitrocellulose membrane

and blocked with 5% nonfat milk in a TBST buffer for one hour at room temperature. After rinsing, the membrane was cut in strips in order to incubate each of the sera. Each strip was incubated during all night with a 1:200 dilution of the corresponding serum; in all, 47 sera were analyzed. A 1: 5,000 dilution of a secondary antibody (peroxidated rabbit anti-human IgA, IgG and IgM antibody) was used. Strips were revealed by chemiluminescence.

STATISTICAL ANALYSIS

The statistical analysis was made with a ROC curve (Receiver Operating Characteristics Curves). We wanted to evaluate the quality performance of two diagnostic tests: ELISA using the membrane rich extract of amoebic trophozoites as antigen (Table 1) and ELISA using pure amoebapore (Table 2). For this reason, the sensitivity and specificity values of these two assays were calculated with the WB result (corroborative method) as a true condition status.

Table 1: ELISA assay using the membrane rich extract of amoebic trophozoites as antigen.

Sera	Average 490 nm	Cut point	Results	Reading
A81	0.90	0.52	>cp	+
A82	0.83	0.52	>cp	+
A83	0.54	0.52	>cp	+
A84	1.20	0.52	>cp	+
A89	0.73	0.52	>cp	+
A101	1.40	0.52	>cp	+
A119	1.20	0.52	>cp	+
A121	0.65	0.52	>cp	+
A123	1.30	0.52	>cp	+
A126	1.14	0.52	>cp	+
A129	1.10	0.52	>cp	+
A131	0.71	0.52	>cp	+
A133	0.65	0.52	>cp	+
A138	0.63	0.52	>cp	+
A139	0.90	0.52	>cp	+
A140	0.80	0.52	>cp	+
A141	0.59	0.52	>cp	+
A148	1.30	0.52	>cp	+
A149	1.00	0.52	>cp	+
A150	0.73	0.52	>cp	+
A154	0.90	0.52	>cp	+
A155	0.90	0.52	>cp	+
A158	0.90	0.52	>cp	+
A160	0.73	0.52	>cp	+
A162	0.60	0.52	>cp	+
N129	0.40	0.52	<cp	-
N131	0.30	0.52	<cp	-
N132	0.18	0.52	<cp	-
N134	0.45	0.52	<cp	-
N136	0.50	0.52	<cp	-
N137	0.20	0.52	<cp	-
N138	0.10	0.52	<cp	-
N139	0.10	0.52	<cp	-
N152	0.34	0.52	<cp	-

Sera of ALA clinical diagnosis (**A**). Clinical suggestion of PLA sera (**N**); >cp higher than the cut point →positive; <cp lower than the cut point →negative.

Table 2: Anti-amoebapore antibody identification in 47 sera analyzed.

Groups	Sera	Average 450 nm	Cut point	Results	Reading
A ALA (CS)	A81	0.213	0.264	0	-
	A82	0.990	0.329	0.669	+
	A83	0.164	0.251	0	-
	A85	1.164	0.386	0.777	+
	A89	0.273	0.395	0	-
	A101	0.674	0.268	0.405	+
	A119	1.634	0.366	1.267	+
	A121	1.944	0.348	1.595	+
	A123	1.173	0.306	0.866	+
	A126	0.618	0.405	0.212	-
	A129	1.585	0.271	1.313	+
	A131	1.439	0.256	1.182	+
	A133	2.450	0.280	2.169	+
	A138	0.434	0.253	0.180	-
	A139	1.421	0.272	1.148	+
	A140	1.124	0.364	0.759	+
	A141	0.961	0.265	0.695	+
	A148	0.681	0.279	0.401	+
	A149	1.322	0.304	1.017	+
	A150	1.145	0.240	0.904	+
	A154	0.280	0.263	0.016	-
	A155	0.249	0.172	0.076	-
	A158	0.524	0.320	0.204	-
	A160	0.238	0.286	0	-
	A162	0.254	0.322	0	-
B (H)	H1	0.163	0.190	0	-
	H2	0.376	0.199	0.176	-
	H3	0.192	0.230	0	-
	H4	0.227	0.198	0.028	-
	H5	0.219	0.212	0.006	-
	H6	0.352	0.181	0.17	-
	H7	0.264	0.184	0.079	-
C (PLA)	N129	0.509	0.666	0.057	-
	N131	0.338	0.194	0.143	-
	N132	0.188	0.426	0	-
	N134	0.649	0.742	0	-
	N136	1.295	0.355	0.939	+
	N137	0.264	0.179	0.084	-
	N138	0.747	0.407	0.339	-
	N139	0.558	0.339	0.218	-
	N152	0.373	0.426	0	-
D (T)	T1	0.098	0.273	0	-
	T2	0.299	0.336	0	-
	T3	0.269	0.261	0.007	-
(L)	L1	0.130	0.270	0	-
	L2	0.141	0.188	0.3	-
	L3	0.298	0.263	0.37	-

ELISA assay using amoebapore antigen (0.6 µg) with different sera groups: Group A, patients with clinical suggestion of Amoebic Liver Abscess (ALA); Group B, healthy subjects´ sera (S); Group C, Patients with Pyogenic Liver Abscess (PLA); Group D, patients with *Leishmaniasis* (L) and *Trypanosomiasis* (T). Results: negative (-) and positive (+).

RESULTS

Amoebapore purification

Amoebapore was purified from of *Entamoeba histolytica* HM1: IMSS trophozoites, as already described [47]. The pure sample was analyzed electrophoretically in gels at 16% of polyacrylamide and silver stained (Figure 1). A band with a

molecular weight of 10 kDa was obtained. Sequencing of 8 amino acid residues by mass spectrophotometry, the result: KLIQIEKV, matched the Blastp in 100 % with the "peptide precursor of amoebaopore A" of *Entamoeba histolytica* strain HM1: IMSS, with the number of access: XP_653265.1. In regards to its activity verification, the depolarizing assay of liposomal membranes showed porin activity (Figure 2).

Specificity of the antiamoebapore antibodies for total lysates from *E. histolytica*, *Leishmania* and *Trypanosome*

Figure (3) shows as expected, our antibodies recognized pure amoebapore (lane 4), and also in the *E. histolytica* lysate (lane 1), but there was no cross reactivity with *Leishmania* (lane 2), nor *Trypanosome* lysatee (lane 3)

Quantitative assays of human sera

Consider the 34 patients group (originally classified with liver abscess) to compare the two ELISA assays with the WB result (as a corroborative method). The results obtained in ELISA with the membrane rich extract indicated that 25 sera were positive for ALA and 9 were negative, but positive for PLA (Table 4). The ELISA performed to the same sera, but using pure amoebapore detected 16 positive for ALA and 18 negative (Table 5). The first assay (ELISA using membrane rich extract) shows 10 cases (55.55%) as false positives, and 1 case (6.25%) as false negative, while the second assay (ELISA performed with pure amoebapore) shows no false negatives nor false positives.

The sensitivity and the specificity of two assays are shown in the Figure (1), one can see the ELISA using pure amoebapore detecting anti-amoebapore antibodies in the sera is better as the same assay using the membrane rich extract constituted by multiple peptides.

Qualitative assays using human sera

The results obtained in the WB assays, with the porin of the amoeba and the different sera groups (A-D) of the participants, were identical to those obtained through the ELISA assays. Figure (5) shows some representative strips of WB of each of the analyzed groups.

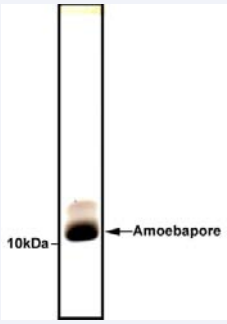

Figure 1 Purity of the amoebapore of *E. histolytic*, strain HM1:IMSS. Electrophoretic profile gel at 16 % of purified Amoebapore was stained with silver a histolytica.

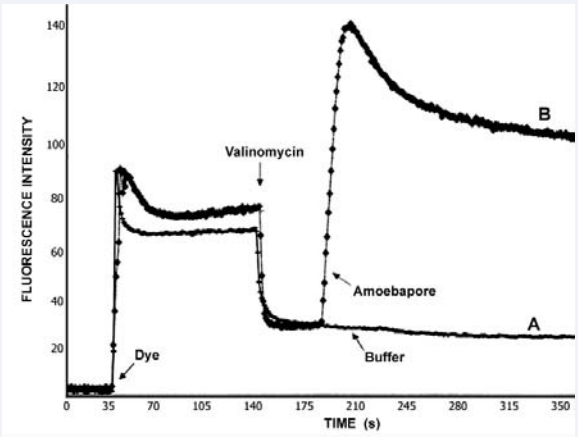

Figure 2 Temporary course of the liposome depolarization by Amoebapore A. A) Measured fluorescence at adding a TRIS suspension (20 mM, pH 7.8) to liposomes. B) In this case, Amoebapore molecule was added to the cuvette with liposomes. A radical change is observed in the fluorescence measure caused by the diffusion of the cyanine dye from the liposomes into the medium, which implies that the molecule shows activity.

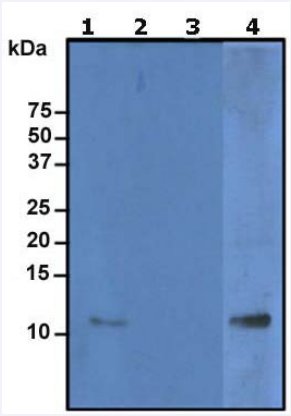

Figure 3 Immunoelectrotransference profile of total lysate of parasite samples. Anti-amoebapore antibody specificity in extracts from different protozoan parasites by WB. 1) *E. histolytica,* 2) *L. mexicana,* 3) *T. cruzi,* 4) Amoebapore.

DISCUSSION

Liver abscess is by far the most common manifestation of extraintestinal amoebiasis. As afore mentioned, the increase of circulant antibodies in ALA patients may be detected soon after symptoms appear. As it is a complication that puts patients´life in danger, its fast and precise diagnosis is crucial, thus serodiagnosis in patients with probable ALA may be of great help in the clinical decision and the spare of costs at avoiding other treatments and the unnecessary prolonged hospitalization. Although the search for antigens of *Entamoeba histolytica* recognized by immune sera from patients with amoebic liver abscess was first referred by Joyce et al in 1988 [48] there are recent works [49,50] setting different conditions for serological assays in the diagnosis of ALA patients. The aforementioned reflects that there is still a need of a reliable diagnosis method for ALA. ELISA, using an undefined

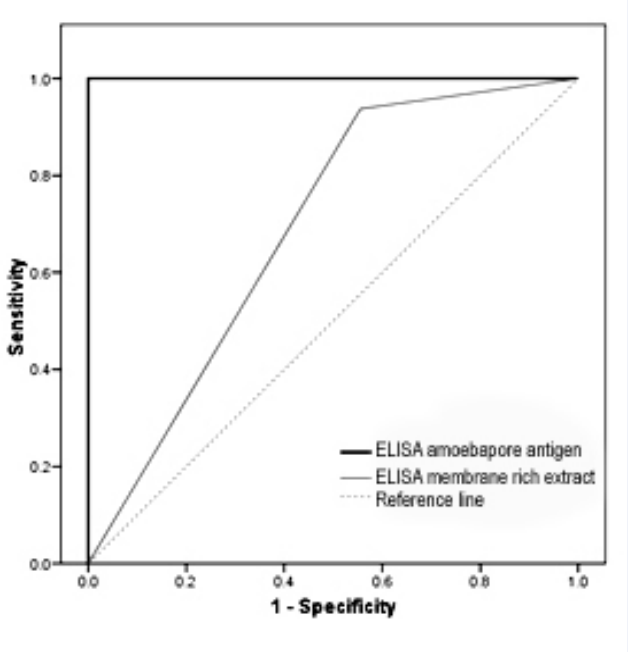

Figure 4 ROC curve for the two ELISA methods. Using the SPSS 23.0.

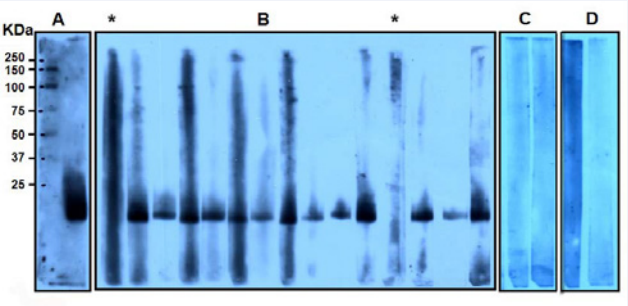

Figure 5 WB showing identification of amoebapore by specific antibodies present in CS-ALA patients'sera A) anti-amoebapore IgG's (rabbit); B) CS-ALA patients´ sera that came out positive; C) healthy subjects´sera; D) patients´sera with Leishmaniasis and an asterisk. (*) CS-ALA patients´ sera that came out negative.

mixture of *Entamoeba histolytica* as antigen is one of the most popular serological methods used by Diagnostic Labs, as it is highly sensitive and specific aside from being quantitative

At the General Hospital of Mexico, patients are diagnosed at first, in the Medical Service, based on clinical symptoms and further tests such as ultrasound imaging thereafter, at the Experimental Medicine Service (EMS) in the same Hospital, an indirect ELISA is performed on the patients serum samples to achieve a differential diagnosis with pyogenic liver abscess (PLA). The indirect ELISA is performed with a membrane rich extract [51] as antigen, from trophozoites of E. histolytica HM1: IMSS axenically cultured. The HM1: IMSS strain is considered as a reference virulent strain, and is commonly used in amebiasis research.

A group of 34 serum samples from patients of the General Hospital, was diagnosed (by ultrasound imaging) as having liver abscess; from them, 25 were further confirmed at the EMS with ALA by their positive result with the indirect ELISA using membrane rich extract as antigen, and the remaining 9 with PLA [52]. We have previously reported amebopore purification in its native conformation and with pore forming activity from *E. histolytica* HM1:IMSS trophozoites [47]; in this study, we have performed to the same 34 serum samples from patients mentioned above, an indirect ELISA and Western Blot using as antigen the purified amebopore. The results obtained by the indirect ELISA and the Western Blot using purified amebopore were identical: those sera positive or negative by ELISA gave the same result by WB. We evaluated the sensitivity and specificity of the indirect ELISA using the membrane rich extract as antigen or the purified amebopore. To do this, we considered the WB result as a true condition status.

The sensitivity and specificity of the indirect ELISA using amebopore was 100%, and indicated that 10 serum samples that were considered positive with the indirect ELISA using the membrane rich extract were "false positives", so those patients would have been treated unnecessarily. On the other hand, one of the serum samples originally detected as PLA, turned out as positive for ALA (false negative), although we don´t have a reasonable explanation for this result.

There are in the literature serologic studies for diagnosis of ALA in which recombinant proteins are used; for example: it has been reported [53] the recombinant pyruvate phosphate dikinase (rPPDK) from *E. histolytica* as a potential diagnostic marker for ALA, however, although the specificity of WB using the rPPDK was 100% when compared to the recombinant Gal/GalNAc lectin, the sensitivity of the assay was 93%, and the specificity of 100% is only achieved when they use as the secondary antibody, one to the IgG4 subtype. Although the predominant IgG subtypes in amebiasis have been reported to be: IgG1 and IgG4, the sera from patients with ALA show ELISA values for all antibody subtypes higher than those of healthy controls [54] so a specificity of 100% should be obtained with a mixture of all the antibodies present in the patients sera. A clear advantage of using a purified antigen in

Table 3: Decision matrix: ELISA, Using membrane rich extract of trophozoites from *E. histolytica.*

		True condition status (WB)		
		Positive	**Negative**	**Total**
Test Result	Positive	15	10	25
	Negative	1	8	9
	Total	16	18	34
	Sensitivity	93.75%		
	Specificity	44.44%		

Table 4: Decision matrix: ELISA assay using pure amoebapore detected anti-amoebapore antibodies.

		True condition status (WB)		
		Positive	**Negative**	**Total**
Test Result	Positive	16	0	16
	Negative	0	18	18
	Total	16	18	34
	Sensitivity	100%		
	Specificity	100%		

its native conformation over a recombinant one in a serological assay is that you assure that the antibodies you are going to detect are exactly those that were generated in the humoral response. On the other hand, the native structure of the amebopore is also corroborated by its confirmed hability to form pores.

The results here by obtained allow us to propose amebopore purified from *E. histolytica* as a potential diagnostic marker for ALA.

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