Standardization and Evaluation of Elisa for the Serodiagnosis of Amoebic Liver Abscess

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An ELISA test for the serological diagnosis of amoebic liver abscess (ALA) was standardized and evaluated in sera from three groups of patients: (1) three patients with diagnosis confirmed by isolation of the parasite, (2) thirty seven patients with diagnosis established by clinical findings and ultrasound studies and (3) seven patients whose diagnosis were established by clinical findings and a positive double immunodifusion test. Ninety one serum samples from healthy subjects and 22 from patients with other liver or parasitic diseases were also included in the study.

The optimum concentration of Entamoeba histolytica *antigen was 1.25 µg/ml and optimum dilutions of serum and anti-human IgG -alkaline phosphatase conjugate were 1:400 and 1:4000 respectively. The cut-off point of the ELISA test in this study was an absorbance value of 0.34. The test parameters were: sensitivity = 95.7%, specificity = 100%, positive predictive value = 100% and negative predictive value = 98.2%.*

The ELISA test was found to be of great use as a diagnostic tool for the establishment of amoebic etiology in patients with clinical supposition of ALA. The test could also be used for seroepidemiological surveys of the prevalence of invasive amoebiasis in a given population, since it allows the processing of a greater number of samples at a lower cost than other serological tests.

Key words: amoebic liver abscess – ELISA – serodiagnosis amoebiasis

Entamoeba histolytica, which is widely distributed throughout the world, causes high morbidity and mortality rates due to amoebic colitis and liver abscess. An estimated 10% of the world's population are asymptomatic carriers but only a small number of patients are afflicted by amoebic disease (Walsh 1986).

Amoebic liver abscess (ALA) is the invasion of the liver by *E. histolytica* from an intestinal focus. In most cases there is only a single abscess, generally localized in the right superior hepatic lobe which receives the greater part of the portal circulation. Its frequency is three times greater in men than in women, generally affecting young adults who live in endemic tropical areas (Thompson et al. 1985).

The prevalence rates of invasive amoebiasis in the general population, as measured by the presence of amoebic antibodies, are 5.95% according to a Mexican serological survey (Gutiérrez et al. 1976) and 7% in Colombia (Corredor 1980). Amoebic liver abscess is thought to be responsible for 30 to 37% of all cases of invasive intestinal or extraintestinal amoebiasis (Ochsner & Debakey 1943, Belding 1952, Duque 1965, Rowland 1967).

The diagnosis of ALA is suggested clinically, but requires further paraclinical tests such as ultrasound, radioisotope scanning, computarized tomography scanning and magnetic resonance imaging for the identification of abscesses in the liver. The most precise and valid method for the diagnosis of amoebiasis is the demonstration of the etiological agent.

Parasitological confirmation, however, is a risk for the patient who has to undergo invasive procedures such as liver biopsy aspiration or surgery to obtain samples for the identification of *E. histolytica* trophozoites. These methods are the most specific and allow a differential diagnosis with pyogenic liver abscess (Greenstein & Sachar 1988, Botero & Restrepo 1992) but are seldom employed because of the inherent risk and also because parasite identification is not always possible. Currently, they are used as therapeutical measures with precise indications when drug therapy has failed.

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Due to the above mentioned difficulties, serological techniques that help in the etiological diagnosis of ALA were developed to detect specific antibodies such as: indirect haemagglutination (Kessel et al. 1961), immunodiffusion (Maddison 1965), complement fixation (Kessel et al. 1965), counterimmunoelectrophoresis (Samrejrongroj & Tharavanij 1985), indirect immunofluorescence (Bos et al. 1975), immunoenzymatic assay (Gandhi et al. 1987, Samrejrongroj & Tharavanij 1985, Nagano et al. 1990) and Dot ELISA (Sharma et al. 1988) or circulating immune complexes (Gandhi et al. 1988). They all show high sensitivity and specificity for the diagnosis of ALA (Healy 1986), but immunoenzymatic assays offer better sensitivity, specificity, ease of proccesing of a greater number of samples (Voller et al. 1976) and lower costs than the other immunodiagnostic methods.

This study describes the standardization and evaluation of ELISA for the serodiagnosis of ALA.

MATERIALS AND METHODS

Patients – A total of 47 patients with ALA diagnosis were studied; three patients were diagnosed by direct observation of the parasite in liver biopsies or aspirates, 37 by clinical findings and ultrasound and seven by clinical features and positive immunodiffusion test.

Furthermore, samples from 91 healthy people and from 22 patients with several parasitic diseases such as: strongyloidiasis, fascioliasis, leishmaniasis, paragonimiasis, *Plasmodium vivax* malaria, toxocariasis, giardiasis and other diseases such as hepatitis, hepatic carcinoma, cirrhosis, pyogenic liver abscess, non-identified collagen disease, biliary atresia, lupus erythematosus and jaundice were processed.

Antigen preparation – An HMI clone from an *E. histolytica* strain supplied by Centro de Investigaciones y Estudios Avanzados del Instituto Politécnico Nacional de México was used.

The antigen was obtained from trophozoites incubated in BI-S-33 culture medium (Diamond et al. 1978) supplemented with vitamin mixture 107 (Evans et al. 1956). The procedure followed for the preparation of the antigen was modified from the method described by Gandhi et al. (1988).

Trophozoites were harvested from cultures by centrifuging at 700 g for 5 min at 4°C and then washed six times with a duration of 5 min for each washing with sterile physiological saline solution, pH 7.2, at 250 g, and 4 °C. The parasites were submitted to a process of freezing at –196 °C, then thawed at 4 °C and finally fragmented by sonication (Biosonik II A) in chilled water for 30 sec at 20 kilocycles, the process being repeated five times. The suspension was centrifuged at 1000 g for 10 min at 4 °C and the protein concentration of the supernatant (antigen) was determined by the Lowry method (Lowry et al. 1951). The antigen was distributed in 200 µl aliquots and stored at –20 °C.

ELISA standardization – One positive serum sample from a patient with clinical, immunological and tomographical confirmation of amoebic liver abscess and one negative serum sample from a healthy subject with no history of amoebic disentery or amoebic liver abscess were used during the standardization in order to determine the optimal dilution of serum and conjugate and the optimal concentration of antigen.

"Dynatech Immulon I" polystyrene plates were used for the following procedure: the wells were coated with 100 µl of antigen diluted in carbonate buffer (0.05M, pH 9.6) in serial concentrations ranging from 0.5 to 80 µg/ml and each was added in triplicate. After 3 hr of incubation in a humid chamber at room temperature, each microplate was washed three consecutive times for a duration of 5 min with phosphate buffer solution (PBS) 0.15M, pH 7.4, containing 0.05% Tween 20.

One hundred µl of positive, as well as negative sera, in serial 1:100-1:6400 dilutions, were added in triplicate to the microplate. The antigen-antibody reaction was carried out at room temperature (20 °C) for 2 hr and then washed with PBS.

The conjugate (Sigma Chemical Co. AO 287 – anti-human IgG gamma-chain specific linked to alkaline phosphatase) in serial dilutions from 1:1500 to 1:6000, was added in triplicate in 100 µl aliquots to each well of the microplate and incubated for 18 hr at 4 °C. A final washing was performed with PBS, as described previously.

The enzyme-substrate reaction was developed by the addition of 100 µl of p-nitro phenyl phosphate at a 1 mg/ml concentration to each well and incubated at room temperature for 30 min. The reaction was stopped by adding 25 µl of 3M NaOH to each well. The presence of antibodies was determined by absorbance reading at 405 nm from a "Uniskan I" spectrophotometer.

Patient follow-up – The antibody titers of three patients with ALA diagnosis were determined in serum samples before and after treatment. Blood samples were collected at different intervals between four and 20 weeks after the end of treatment. All patients were found to be clinically cured at each follow-up visit.

Statistical analysis – (1) Determination of the cut-off point: the absorbance value which differentiates between positive and negative samples was established by calculating the mean (0.10) plus two standard deviations (S.D.= 0.12) ($p < 0.05$) of the absorbance values of samples of healthy patients or those of patients with diagnosis other than ALA; (2) Determination of test parameters: sensitivity (S), specificity (E), positive predictive value (PPV) and negative predictive value (NPV) of ELISA were determined using 2 x 2 contingency tables.

RESULTS

ELISA standardization – The optimum concentration of *E. histolytica* antigen was 1.25 µg/ml (Fig. 1) and optimum dilutions of serum and anti-human IgG – alkaline phosphatase conjugate were 1: 400 (Fig.2) and 1:4000 (Fig. 3) respectively.

ELISA evaluation – The mean and standard deviation values of the samples of normal subjects and patients with a diagnosis different from ALA were 0.10 and 0.12, respectively. Those of the samples from patients with confirmed diagnosis of ALA were 0.72 and 0.26 respectively. The cut-off point of the ELISA in this study was 0.34. Samples were considered positive if the absorbance value readings were equal or greater than this value.

The sensitivity of the ELISA ranged from 85.7 to 100% and NPV varied between 98.1 and 100% ($p < 0.05$). These variations are due to the fact that three different criteria were used for ALA diagnosis definition (Tables I - III).

Antibody titers after treatment – The behaviour of antibody titers in response to treatment showed a slow decline with time (Table IV).

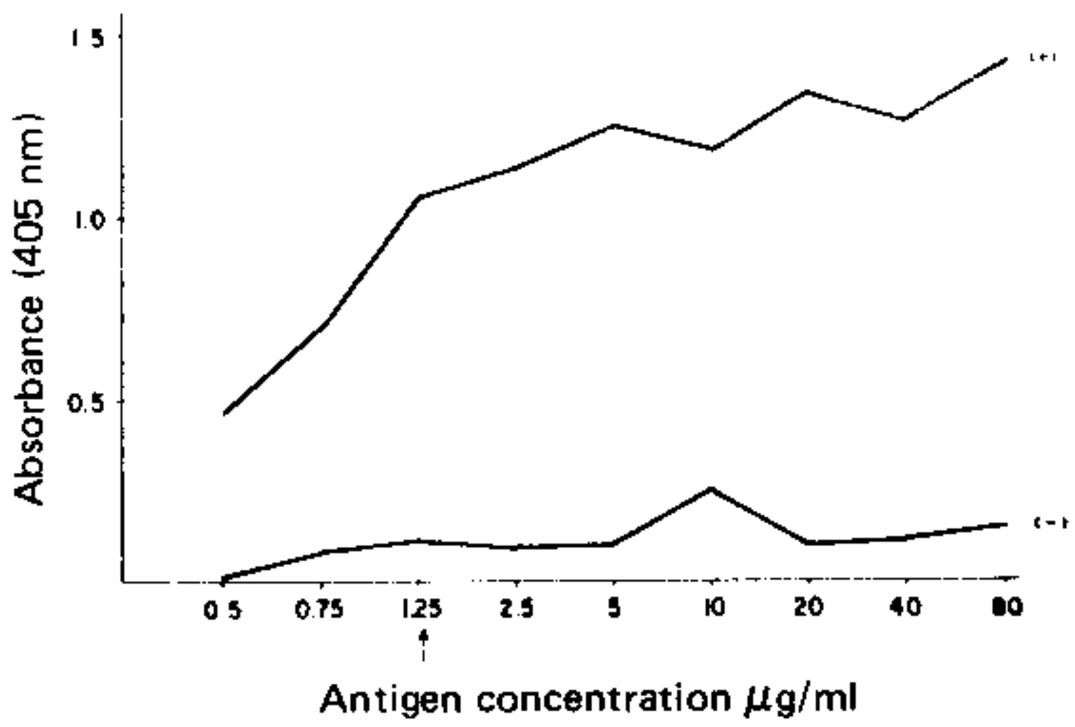

Fig. 1: amoebic liver abscess. Optimum concentration of antigen of *Entamoeba histolytica*. X axis: antigen concentration µg/ml. Y axis: absorbance (405 nm). + : positive samples. – : negative samples.

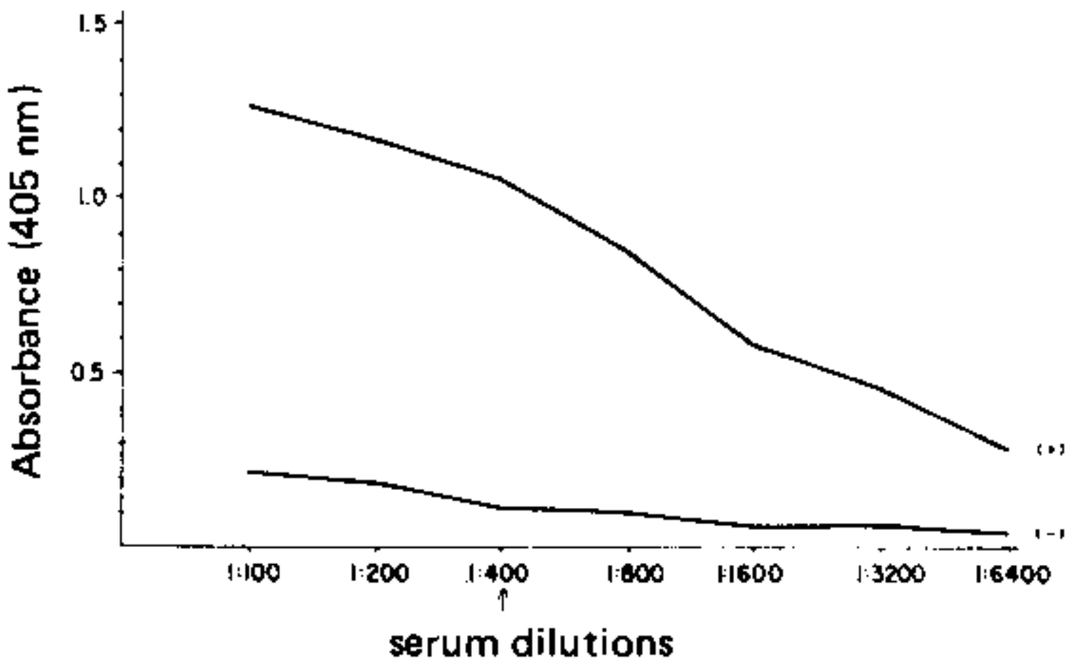

Fig. 2: amoebic liver abscess. Optimum dilution of serum samples. X axis: serum dilutions. Y axis: absorbance (405 nm). + : positive samples. – : negative samples.

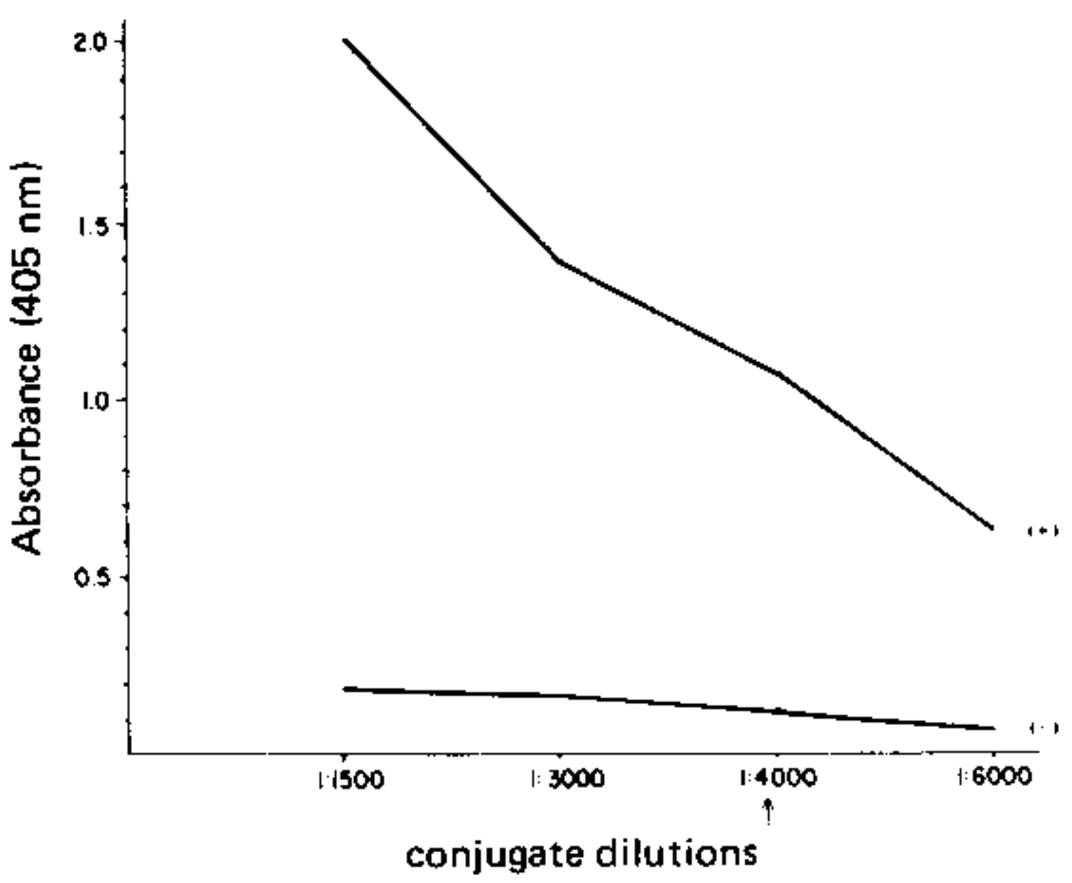

Fig. 3: amoebic liver abscess. Optimum dilution of anti-human IgG-alkaline phosphatase conjugate. X axis: conjugate dilution. Y axis: absorbance (405 nm). + : positive samples. – : negative samples.

TABLE I

Evaluation of the ELISA test for immunodiagnosis of amoebic liver abscess confirmed by parasitological diagnosis

		Amoebic liver abscess		
		(+)	(–)	
ELISA	> 0.34 (+)	3	0	3
	< 0.33 (–)	0	107	107
		3	107	110

($p < 0.05$); > 0.34 (absorbance value at 405 nm); < 0.33 (absorbance value at 405 nm).
Sensitivity: 100%; Specificity: 100%; PPV: 100%; NPV: 100%.

TABLE II

Evaluation of the ELISA test for immunodiagnosis of amoebic liver abscess confirmed by ultrasonographical diagnosis

		Amoebic liver abscess		
		(+)	(–)	
ELISA	> 0.34 (+)	36	0	36
	< 0.33 (–)	1	107	108
		37	107	144

($p < 0.05$); > 0.34 (absorbance value at 405 nm); < 0.33 (absorbance value at 405 nm).
Sensitivity: 97.3%; Specificity: 100.0%; PPV: 100.0%; NPV: 99.1%.

TABLE III

Evaluation of the ELISA test for immunodiagnosis of amoebic liver abscess confirmed by clinical and immunodiffusion diagnosis

		Amoebic liver abscess		
		(+)	(–)	
ELISA	> 0.34 (+)	6	0	6
	< 0.33 (–)	1	107	108
		7	107	114

($p < 0.05$); > 0.34 (absorbance value at 405 nm); < 0.33 (absorbance value at 405 nm).
Sensitivity: 85.7%; Specificity: 100.0%; PPV: 100.0%; NPV: 99.1%.

TABLE IV

Amoebic liver abscess levels of antibodies ELISA test (absorbance values at 405 nm)

Patient	Pre-treatment	Patient follow-up weeks post-treatment					
No.		1	4	8	12	16	20
1	0.735			0.529		0.459	
2	0.650		0.662		0.651		0.491
3	1.018	0.996					0.663

DISCUSSION

ALA is a clinical entity whose etiological diagnosis can be confirmed by the detection of antibodies against *E. histolytica* using ELISA. The standardization of the ELISA test carried out in this study, not only allows its daily application but also grants an accurate diagnosis when coupled to clinical supposition and ultrasound evaluation.

ELISA has been used in endemic areas for the determination of IgG, IgM, IgA and IgG antibody subtypes in sera from patients infected by *E. histolytica* (Shetty et al. 1990). Sera from patients with amoebic colitis and ALA show ELISA values for all antibody subtypes higher than those of healthy controls.

The sensitivity of the test for the detection of IgG, IgM and IgA in patients with amoebiasis has been reported as being 97.4%, 43.6% and 79.5%, respectively (Hock et al. 1989a).

The predominant IgG subtypes in amoebiasis are IgG1 and IgG4 (Hock et al. 1989b). The sensitivity of the test in patients with ALA, ranges between 74.7% and 100% and specificity between 91% and 91.7% (Samrejrongroj & Tharavanij 1985, Gandhi et al. 1987, Sathar et al. 1988, 1990, Hock et al. 1989b, Nagano et al. 1990).

The sensitivity results obtained in our study are similar to those reported by other authors, while the specificity is 100%, higher than that reported in similar studies. The ELISA test as standardized and evaluated in this study allows the detection of IgG antibodies and is an easy, fast and reliable test, with a 95.7% sensitivity and a 100% specificity, a PPV of 100% and NPV of 98.2% in patients suspected of having ALA.

The specificity of the test is confirmed by the absence of cross-reaction in healthy individuals as well as in patients with other parasitic diseases whose signs and symptoms are similar to those of ALA. Paragonimiasis and strongyloidiasis were included as differential diagnoses since pleuropulmonary manifestations are frequent in ALA (Lyche et al. 1990).

When we analyzed the behaviour of antibody titers in sera from patients with ALA, we observed their slow and progressive decline over a period of time following treatment. This behaviour is corroborated by other studies in which antibody titers against *E. histolytica* detected by indirect haemagglutination, decrease slowly, with positive titers still present at 15 to 21 months after clinical and parasitological healing of an attack of invasive amoebiasis (Healy 1986).

However, the persistence of positive titers is indeed useful for seroepidemiological studies of point prevalence, since they are indicators of invasive disease in a given population. It is also likely that if a greater number of patients with ALA is studied and regularly followed-up for an extended period of time (one to two years), a cut-off point that could allow the differentiation between acute phase and clinical healing could be determined. Thus, we can assert that the test is useful for the follow-up of the response to treatment.

The conclusion of this study is that the ELISA test is of great use as a diagnostic tool to establish amoebic etiology in patients with clinical supposition of ALA.

The test can also be used for seroepidemiological surveys of the prevalence of invasive amoebiasis in a given population and contributes to epidemiological surveillance since it allows the processing of a greater number of samples at a lesser cost than other serological tests.

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