

An Improved ELISA Technique to Detect Amoebic Antibody in Blood

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Abstract

An Enzyme-Linked Immunosorbent Assay (ELISA) has been developed for the serologic analysis of IgG antibodies to *Entamoeba histolytica* using antigen coated polystyrene beads as the solid phase. The method was found to be highly specific and sensitive compared with the Indirect Haemagglutination Assay (IHA). This ELISA technique was further standardised to use blood collected on filter paper disc of 5 mm diameter by a finger prick. The blood soaked paper discs were found to be stable at room temperature (RT) for more than one month making the technique most suited for field studies. The ELISA system described here is recommended as a quick and simple test for serological diagnosis of Amoebic Liver Abscess (ALA).

Introduction

Amoebiasis is one of the major public-health problem in several tropical countries of the world. The confirmative diagnosis of invasive amoebiasis, particularly Amoebic Liver Abscess (ALA), has been highly assisted by the serologic detection of amoebic antibodies. Although, various sensitive immunoassays, such as, Immunofluorescence Assay (IFA), Indirect Haemagglutination, Assay (IHA) and ELISA technique, are already in use for detecting antibodies in serum (Cross and Wheeling, 1977 ; Lin *et al.*, 1981 ; Gandhi *et al.*, 1986; Grundy *et al.*, 1983), yet a simple, specific and sensitive method suitable for field studies, particularly for epidemiological studies, is needed. We have established an ELISA test to detect amoebic antibodies in blood directly without requiring the ice-cold conditions for storing and transporting the samples. Test can be established in routine hospital laboratories equipped with less sophisticated instruments.

Material and Methods

The sera samples from the following groups of subjects were selected to study amoebic antibody by ELISA technique using Horse Radish Peroxidase conjugated to protein-A from *Staphylococcus aureus* and rabbit anti-human IgG.

Sl. No.	Groups	Number tested
1.	Healthy controls	40
2.	Amoebic liver abscess	46
3.	<i>E. histolytica</i> cyst passers	10
4.	Acute viral hepatitis	15
5.	Cirrhosis of liver	15
6.	Hepatoma	15

Criteria for selection of subjects : The diagnosis of amoebic liver abscess and asymptomatic *E. histolytica* cyst passers was made as per WHO recommendations (WHO expert Committee, 1969), Normal healthy control group included males and females belonging to different socio-economic status, with no history of liver or bowel disease in recent past and negative IHA test for amoebic antibodies. The diagnosis of patients of acute viral hepatitis, cirrhosis of liver and hepatoma was based on standard clinical, biochemical and histological criteria.

Antigen : The *Entamoeba histolytica* (*E. h.*) associated soluble antigen was prepared according to the method of Diamond (1968). The protein content of antigen was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Coating of polystyrene beads with amoebic antigen : The polystyrene beads (6.4 mm diameter, Precision Plastic Ball Co. Chicago, IL) were activated with 0.25 % aqueous solution (1 ml/bead) of glutaraldehyde for one hour at room temperature (RT) and precoated with amoebic antigen in a concentration of 10 ug/ml in 0.1 M carbonate buffer, pH 9.6, standardised by checker board titrations. Post-coating was carried out by the treatment of beads with 1 % BSA in PBS (2 ml/bead) for overnight at RT to block the unoccupied sites on bead surface. Beads were washed with PBS-T and stored at 4°C until used.

Enzymatic tracers : Horse Radish Peroxidase (HRP, Sigma USA) coupled to staphylococcal protein-A, Sigma (PA-HRP tracer) and rabbit anti-human IgG, Dakopat (IgG-HRP tracer) by two step glutaraldehyde method (Engvall and Perlmann, 1972) was used in assay at the dilutions of 1:2000 and 1:6000, respectively in 0.1 M PBS, pH 7.2 after the optimum dilutions were determined by checker board titrations.

Collection of blood sample on filter paper : Whole blood was allowed to be absorbed on Whatman 3 MM paper, dried at RT and a round disc of 5 mm diameter from this area was punched off. The antibody from the disc was extracted in PBS by gentle stirring overnight with 1 ml PBS at RT. 200 ul aliquot was used in the assay.

ELISA Procedures : For performing the test, each serum sample was diluted 1:100 or more with PBS and 200 ul aliquot was incubated with an antigen coated bead at 37°C for 1 hour. The bead was washed with 5×2 ml PBS-T and then incubated with 200 ul of diluted PA-HRP or IgG-HRP tracer solution at 37°C for 1 hour. After washing, bead was transferred to a clean glass tube and incubated at RT (22±2°C) with 300 ul of enzyme substrate solution i.e., ortho-phenylene diamine (OPD) in citrate phosphate buffer containing H₂O₂ for 5 minutes. The enzyme activity was stopped by the addition of 1 ml 4N H₂SO₄ and the optical density of the solution was measured at 492 nm with Beckman double beam spectrophotometer (Model-25). Each test sample was tested in duplicate. Based on the optical density (OD) values obtained for sera of normal healthy controls, the value of mean ± 2 S.D. was calculated and this value (0.15 OD) was used arbitrarily as a cut off value. Any sample giving OD value greater than cut off point was considered positive and titrated further by using double dilution step.

Indirect hemagglutination assay (IHA) : Indirect haemagglutination assay was performed according to the method of Prakash (1974).

Results

Although ELISA described here has been standardised with two different tracers, PA-HRP and IgG-HRP, the data mentioned here are those obtained with PA-HRP only, as in each case the results are exactly the same.

Specificity of antigen-antibody binding : The specificity of antigen antibody binding in the present ELISA system was checked by incubating varying con-

centrations (5-80 ug/ml) of amoebic antigen with 200 ul aliquot of diluted serum (1:200), positive for amoebic antibody with a known titre of 1600, for 2 hours at 37°C and then measuring residual antibody activity by the ELISA system. The same serum without added antigen was used as control for 100 % antibody activity. The results obtained showed a decreasing antibody activity with increasing concentration of antigen. The concentration of 5, 15, 25, 40 and 80 ug antigen/ml showed the activity of 90, 82, 70, 62 and 50 % respectively as compared to control. This indicated a high specificity of antigen antibody binding in the present assay system.

Stability of Antigen Coated Beads : The antigen coated beads preserved at RT ($22 \pm 2^\circ\text{C}$) were used in the assay system every third day upto 60 days under identical conditions using same serum and the results obtained were compared. There was little change in OD values upto one month. However, a little decrease in OD was noted in next one month. This indicates the beads to be stable for more than one month at RT.

Frequency of Amoebic Antibody in Test Samples : The titre values of amoebic antibody observed in the sera samples of healthy controls and the patients have been shown in Fig. I. All the 40 sera from the healthy group were negative at 1 : 100 dilution. Out of 10 samples from cyst passers, 2 were positive at 1 : 100 dilution while one was positive at 1 : 200 dilution. The remaining 7 were negative even at 1 : 100 dilution. Out of 46 samples from ALA patients, 2 were positive at 1 : 100 dilution (4%), one was positive at 1 : 200 dilution (2%) and the rest 43 (94%) showed a titre of more than 200. Among the patients with viral hepatitis, cirrhosis and hepatoma, none had a titre of more than 200.

Comparison of ELISA and IHA : Sera from 40 patients with amoebic liver abscess (ALA) were assayed by both ELISA and IHA technique and the results compared. While ELISA technique showed positivity in 100% cases, IHA showed positivity in only 70% of these cases. Thus ELISA could detect antibody even in the sera samples negative by IHA.

Standardization of ELISA for blood absorbed on paper disc : Blood samples with known antibody titres of 1 : 200, 1 : 400, 1 : 800 and 1 : 1600 (Gandhi *et al.*, 1985) were allowed to be absorbed on Whatmann 3 MM filter paper separately, dried at RT and then circular discs of 5 mm diameter from each area were punched off. The discs in varying number (1-4) from each batch were extracted in 1 ml PBS and 200 μl aliquot from each extract was used in the present assay. The OD values obtained for each extract were plotted vs. number

of discs. The results showed OD values to be increasing with both titre as well as the discs number. Fig. 2 shows data only for discs with low titre blood (1 : 200). Using 0.15 OD as the cut off value, the minimum number of discs needed to give positive test for low titre (1 : 200) blood was found to be one disc. In order to find out the minimum volume of blood contained in one disc another experiment was designed. The varying volume (2-8 μ l) of this low titre blood was dissolved in 1 ml PBS as such and 200 μ l aliquot from each fraction was used in assay. OD values obtained were compared to those given by varying number of discs soaked with 200 titre blood and found that OD of 5 μ l blood was equivalent to that of one disc. Thus the volume of blood contained in one disc was 5 μ l. These blood soaked discs were preserved at RT and tested from time to time under identical conditions. The results showed no change in OD values upto one month.

Discussion

The ELISA test described here is very simple, quick, specific and highly sensitive. Although IHA (Elsdon, 1971; Krupp and Powell, 1971) was assumed to be the most sensitive technique, the comparison of present ELISA system with IHA documented the sensitivity of ELISA over IHA which has also been shown in our earlier study (Gandhi *et al.*, 1986).

The use of polystyrene beads as compared to other solid supports (Daugharty *et al.*, 1972) in the present ELISA method was found to be distinctly advantageous because of their easy handling and convenience of testing any number of samples without waste of materials. Antigen coated beads are stable at RT for more than one month and the total time of whole assay is 2.5 hours. Thus stability of coated beads and rapidity of assay is another advantage of the present ELISA technique. Similarly, standardization of assay with two tracers, PA-HRP and IgG-HRP, makes it easy to use any one depending on their availability with little difference in the results. The present test also shows high specificity of antigen antibody binding as indicated by a significant decrease in antibody activity after preincubating antibody positive serum with increasing concentration of antigen before assaying in the ELISA system.

The results as shown in Fig. 1 indicate that per cent positivity of amoebic antibody in different groups of subjects other than ALA was insignificant. At a dilution of 1 : 200 the per cent positivity of antibody found in control group, cyst passers, viral hepatitis, hepatoma and cirrhosis was 0%, 10%, 6.6%, 13% and 0%, respectively. None of the subjects in the above group was positive at more

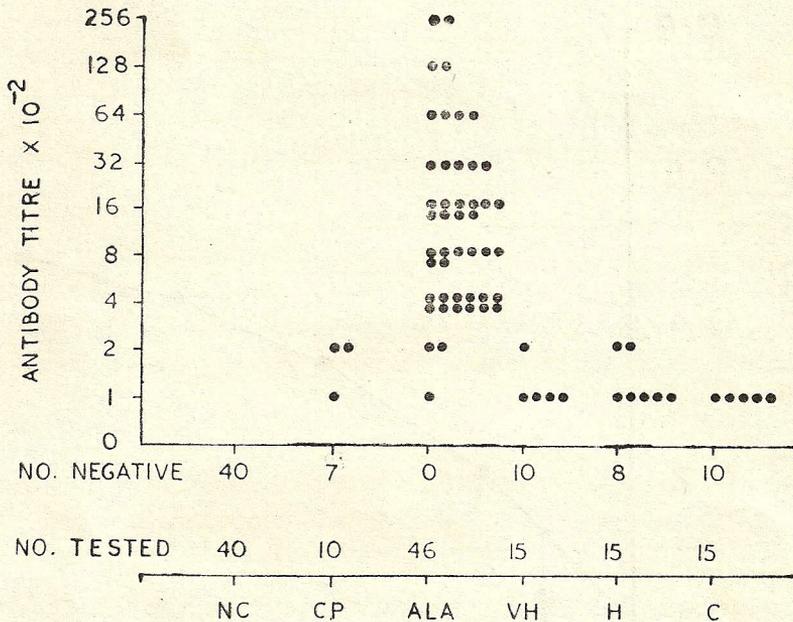


Fig. 1 : Comparative titre value of amoebic antibody in the sera samples of normal control (NC), *E. histolytica* cyst passers (CP) and the patients of Amoebic Liver Abscess (ALA), viral hepatitis (VH), hepatoma (H) and cirrhosis (C).

than 1 : 200 dilution. On the other hand, 94% samples of ALA group showed a titre value of more than 1 : 200. The titre value of 1 : 200, therefore, may be safely used for the confirmatory diagnosis of ALA. The low titre antibody detected in subjects other than ALA could be due to past or current sub-clinical amoebic infections.

One of the objective of standardising the above technique was to make it adapted to field study where collection, storing and transportation of test specimen is a problem. This goal was achieved by making use of whole blood rather than serum which could be collected on filter paper, in a very minute quantity (5µl), by a finger prick. Only a single paper disk (Fig. 2) with 5µl blood of low titre (1 : 200) was found to be enough to give positive test by this method. Besides, the stability of blood on filter paper is helpful for the preservation and transportation of sample at RT for sufficient time without any fear of antibody loss.

In conclusion, the ELISA method as described here is simple, rapid, sensitive and very suitable for epidemiological and hospital diagnostic study of

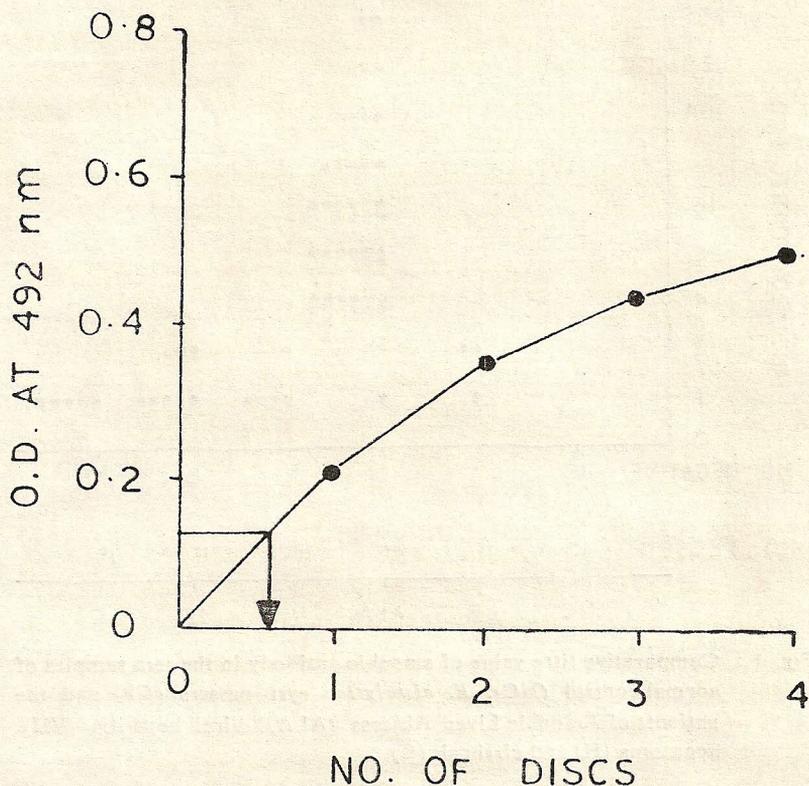


Fig. 2 : Fig. 2 shows the number of discs required to indicate the presence of amoebic antibody.

invasive amoebiasis. It is recommended as serological test of choice for these purposes in countries where amoebiasis is endemic.

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