A dot immunobinding assay (DIA) on nitrocellulose membrane for the serological detection of antibodies to *Entamoeba histolytica*

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Abstract

A rapid, cheap, simple and specific serological test of adequate sensitivity for detecting IgG antibodies against *Entamoeba histolytica* antigen is described. Axenically cultured amoebic antigen was used to precoat the nitrocellulose membrane. The strips were incubated with test samples and later with horseradish peroxidase (HRPO) labelled protein-A conjugate. A dark blue spot was obtained by treatment with peroxidase substrate, 4-chloro-1-naphthol, in positive samples. Serum samples from 32 healthy controls, 45 patients with acute amoebic liver abscess and 10 asymptomatic *E. histolytica* cyst passers were tested. This test was positive in 93% of cases of amoebic liver abscess, 3% of healthy controls and none of the cyst passers; its sensitivity (97%) and specificity (93%) were as good as that of the ELISA test and, because it is simple, quick and cheap it is recommended as a serological test of choice for the diagnosis of invasive amoebiasis.

Introduction

Various tests have been developed for detecting circulating antibodies to amoebic antigen in amoebiasis. Indirect haemagglutination assay (IHA) (PRAKASH *et al.*, 1969) and enzyme linked immunosorbent assay (ELISA) (LIN *et al.*, 1981; YANG & KENNEDY, 1979) are considered highly sensitive and specific tests. The enzyme linked protein-A ELISA system, an improvement over the standard ELISA test, for detecting amoebic antibody in patients with amoebic liver abscess, has been recommended as the method of choice for serological diagnosis of amoebic liver abscess (GANDHI *et al.*, 1987).

Several solid phase antigen carriers have been used for different techniques. These include 96-well microtitre plates, polystyrene beads, polyvenylene and polystyrene or glass tubes. Recently nitrocellulose membrane (BEYER, 1984; OGATA *et al.*, 1983) and diazobenzyloxymethyl (DBM) membrane (HERBRINK *et al.*, 1982) have been used for immunobinding of proteins. In this paper we describe an assay for detecting IgG antibodies to amoebic antigen by the use of protein-A conjugate using a nitrocellulose membrane as carrier for the amoebic antigen. The test has been compared with the enzyme-linked protein-A ELISA system which we have reported earlier as comparable in its sensitivity and specificity to IHA (GANDHI *et al.*, 1987).

Materials and Methods

Dot immunobinding assay (DIA) to detect the presence of circulating antibodies to amoebic antigen was tested in normal healthy controls (32 subjects), patients with amoebic liver abscess (45 subjects) and asymptomatic *Entamoeba histolytica* cyst passers (10 subjects). The normal healthy control group included males and females of different socio-economic status. None had a recent or past history of amoebic disease. Stools were negative for *E. histolytica*. Amoebic liver abscess patients were admitted to the wards of the Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India. The diagnosis of amoebic liver abscess was confirmed according to the WHO recommendations (WHO Expert Committee, 1969). All these patients showed space-occupying lesions in liver scan and ultrasound examinations. "Anchovy sauce" pus from the liver abscess, which was sterile for bacterial culture, was aspirated from all the patients. Further, all the patients responded very well to anti-amoebic therapy. Cyst passers were all asymptomatic but two consecutive stool samples were positive for *E. histolytica* cysts.

Blood samples were drawn, serum was separated after centrifugation and the samples stored at 4°C. In 15 control subjects, whole blood from a finger prick was also directly applied to the strips to run the assay.

Nitrocellulose membrane sheets BA 83, 0·2 μm pore size, were obtained from Schleicher and Schull, Dassel, West Germany. The sheets were cut into strips of convenient size, e.g., 90 × 7 mm. *E. histolytica* antigen was prepared from cultures of *E. histolytica*, strain NIH-200, grown axenically in Diamond's TPS-1 medium (DIAMOND, 1968). Harvested trophozoites were washed with saline and stored frozen. The thawed amoebae were sonicated and centrifuged at 10 000*g* for 30 min. at 4°C. The clear supernatant was used as antigen solution and the protein concentration was determined by the method of LOWRY *et al.* (1951). The antigen was stored frozen in small aliquots of 0·5 ml each.

Protein-A from *Staphylococcus aureus* (Sigma P-6650) was labelled with horseradish peroxidase (HRPO) obtained from Sigma (P-8375) by the technique of ENGVALL (1980) using a two-step procedure. Glutaraldehyde (Sigma G-5882) was used to activate the HRPO. The conjugate was stored in an equal amount of glycerol in aliquots (B. M. GANDHI).

The peroxidase substrate, 4-chloro-1-naphthol, obtained from Sigma (C-8890), was prepared fresh just before use. 30 mg of 4-chloro-1-naphthol were dissolved in 10 ml of methanol. This solution was mixed with 50 ml of Tris-saline buffer pH 7·5 (20 mM Tris base, 500 mM NaCl, pH adjusted with HCl) containing hydrogen peroxide at a final concentration of 0·015%.

Preparation of the strips

Nitrocellulose strips were marked with waterproof ink for identification. The strips were soaked for 5 min in Tris-saline buffer (TS) and activated in 0·25% glutaraldehyde by incubation for one hour in a 15 ml screw-cap tube laid horizontally, containing 5 ml of solution. The strips were handled with tweezers and all the incubations were carried out at room temperature. The strips were rinsed separately

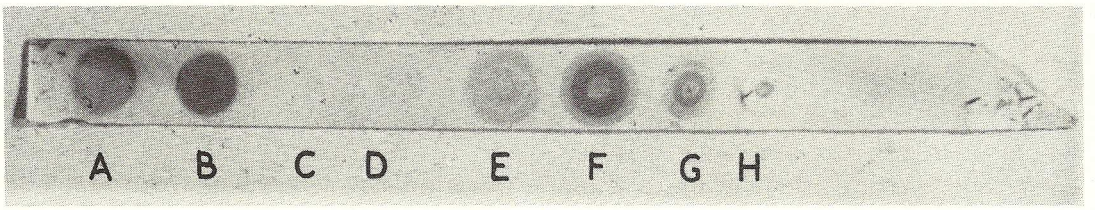

Fig. 1. IgG antibodies against *E. histolytica*. Immunochemical detection of IgG antibodies against *Entamoeba histolytica* by dot immuno-binding assay (DIA) on nitrocellulose membrane. Dots shown correspond to amoebic liver abscess (A, B, E & F), normal healthy controls (C and D) and asymptomatic *E. histolytica* cyst-passers (G and H).

Table 1—Comparative values of dot immunobinding assay and micro-ELISA for different groups

Groups	Dot immunobinding assay		Micro-ELISA	
Normal controls	1/32	(3)*	1/32	(3)
Amoebic liver abscess	42/45	(93)**	41/45	(91)**
E. histolytica cyst-passers	0/10	(0)	0/10	(0)

*Values in parentheses show the percentage positivity.
**Means not significantly different using the test of proportions.

for 5 min in about 100 ml of water and then together in two changes of 100 ml TS buffer for 30 min. After soaking the strips on filter paper, they were transferred to separate tubes containing 10 μg/ml solution of axenically prepared amoebic antigen in Tris-saline buffer and the tubes were incubated in the horizontal position for one hour for pre-coating with antigen. The strips were washed as above and incubated with 1% bovine serum albumin (Sigma A-9647) for overnight blocking and washed. The strips were stored in TS buffer at 4°C until used for running the assay. The solutions in stoppered tubes, i.e., amoebic antigen and bovine serum albumin, were stored frozen. We found that they could be reused several times over a few days without significant loss of the activity, although this is not recommended by all research workers.

Assay

The pre-coated strips were soaked between two filter papers (always handled with tweezers), laid horizontally, dried for 5 min and 1·0 to 2 μl of the test samples, a positive and a negative control, were applied as small spots to each strip. The spots were allowed to dry and the strips were washed for 30 min in 100 ml TS buffer. The strips were then incubated with 1:2000 dilution of protein-A peroxidase solution in TS buffer for 30 min, in different stoppered tubes laid horizontally, each containing 5 ml of solution. This solution was also saved at 4°C for re-use. The strips were rinsed together for 5 min in 100 ml water and twice in 100 ml TS buffer for 10 min each time. The substrate solution was prepared afresh and the strips were transferred into the solution and incubated for 10 to 30 min. To stop the enzymatic reaction, the strips were transferred into water and photographed while wet. The colour was stable for one to two weeks if kept wet in water. On drying, the colour faded. The deep coloured spot was considered as positive. No colour or a light colour were considered as negative tests (Fig. 1).

Results

Results are presented in Table 1. Only one of the 32 healthy controls gave a positive dot test. The remaining 31 included 28 samples which were completely negative and gave no colour on dot test and three samples which gave mild blue discoloration which were considered negative for the purposes of this study. There was no difference when, in 15 cases, the test was done with serum and blood simultaneously. On washing with TS buffer, there was complete removal of red blood cells and no interference with the intensity of the final colour product. 42 of the 45 samples from patients with amoebic liver abscess gave deep blue colour spots (positive test) whereas in the remaining three there was only slight blue discoloration and these samples were taken as negative. None of the 10 patients who were asymptomatic *E. histolytica* cyst passers showed a positive blue spot but six showed a mild discoloration and were also taken as negative.

All these blood samples were run simultaneously in the protein-A ELISA technique described earlier (GANDHI *et al.*, 1987). The results are presented in Table 1. A test of proportions was applied and no difference in sensitivity and specificity for serological diagnosis of amoebic liver abscess was noted between the dot immunobinding technique on nitrocellulose membrane and the ELISA test on micro-ELISA plates.

Discussion

Nitrocellulose membrane has been shown to have a high binding capacity as a solid phase antigen carrier (BEYER, 1984; OGATA *et al.*, 1983). It binds proteins rapidly and quantitively with high capacity. Initial activation of the nitrocellulose membrane with glutaraldehyde has further increased the binding capacity of the amoebic antigen as pre-coat, and bovine serum albumin as blocking reagent. The coating was complete with a clear background after the final reaction and this enabled us to detect even the smallest amount of antibody.

42 of the 45 samples from amoebic liver abscess

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were positive by the dot test and three were negative. Only one of the 32 healthy controls and none of the *E. histolytica* cyst-passers gave positive reactions. DIA had a sensitivity of 93% and specificity of 97% for the serological diagnosis of amoebic liver abscess compared to healthy controls. Corresponding data for the micro-ELISA technique on the same subjects were 91% and 97%. Both the tests are thus equally sensitive and specific in detecting amoebic antibodies. Three of the amoebic liver abscess (7%), three of the healthy controls (9%) and six of the 10 *E. histolytica* cyst-passers showed light blue discoloration on the dot-test. For the purpose of this report development of light discoloration on DIA has been considered negative but this may suggest presence of circulating amoebic antibodies in very low titres in these subjects. A similar problem has been observed with other serological tests such as IHA. A cut-off point for titre for positive interpretation must be agreed (MITHAL *et al.*, 1978). It is recommended that for diagnosis only strong blue coloration of the dot by this technique should be considered and further evaluated by other investigators.

The advantages of the dot test over the indirect haemagglutination assay, radioimmunoassay or enzyme linked immunosorbent assay (all known to be as specific and sensitive as the dot test in the present series) are as follows. (a) DIA is quite simple and can be carried out in most of the routine clinical service laboratories in tropical countries with a high prevalence of amoebic liver disease. It can be done at room temperature and does not require special microtitre plates or an ELISA reader. The use of a nitrocellulose membrane permits the reaction to be viewed against a white background. The discriminatory power is greater by this assay than in a microtitre plate. It becomes easy to detect positive or negative reactions. The same reagents can be used repeatedly and very little serum or blood sample is required. (b) The DIA test is much quicker, results being obtained within one hour, compared with the IHA test which requires six hours and the ELISA test which requires five hours. The other quick test, counter immunoelectrophoresis (CIEP) is very much less sensitive and specific than the dot test (c) The DIA test is significantly cheaper as the same reagents can be used repeatedly.

The preliminary observations suggest that it may be possible to carry out the dot test directly from finger prick with whole blood. This will make the dot test most suitable for field studies of amoebiasis.

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