

PROTEIN A-ANTIBODY MEDIATED HEMAGGLUTINATION ASSAY FOR SERODIAGNOSIS OF AMEBIC LIVER ABSCESS

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Abstract. A successful modification of the indirect hemagglutination test to demonstrate antibodies for serodiagnosis of amebic liver abscess has been described in the present study. In the modified test, the protein A-IMA, *Staphylococcus aureus* (Cowan's strain I) bearing protein A (SAPA) cells were used to enhance hemagglutination of sensitized red cells. Use of SAPA cells markedly enhanced sensitivity of the test and greatly increased the titers obtained with most of the sera. At a diagnostic antibody titer of 1:128 and above, the protein A-IHA could detect 72 (100%) of amebic liver abscess (ALA) cases. Amongst the controls, no false positive reaction was observed in non-amebic liver disease controls. However 1(2%) of sera demonstrated false positive reactions from healthy controls. The protein A-IHA was highly sensitive when compared with that of the indirect hemagglutination (IHA) for serodiagnosis of amebic liver abscess. The novel immunoassay is simple, inexpensive and requires little technical skill. It has the potential for wide application in serodiagnosis of amebic liver abscess.

INTRODUCTION

A number of serological tests to detect amebic antibodies have been developed for serodiagnosis of amebic liver abscess. The tests that are most commonly used include indirect hemagglutination, immunoprecipitation, immunofluorescence, counter-immunoelectrophoresis, latex agglutination and recently, enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) (WHO, 1983). In spite of the high sensitivity and specificity shown by some of these tests, there is still a need for reliable and less sophisticated tests for screening sera from suspected cases of amebic liver abscess.

The indirect hemagglutination (IHA), by virtue of its simplicity and specificity is widely used in serodiagnosis of amebic liver abscess (Dutta and Sharma, 1979). However the IHA is not as sensitive as ELISA and RIA. We have recently developed a method to enhance sensitivity of the IHA by use of *Staphylococcus aureus* bearing protein A (SAPA) in the test. The modified immunoassay, the protein A-antibody mediated hemagglutination (Protein A-IHA) proved to be a highly sensitive and technically simple assay for detection of specific antio-

dies in serodiagnosis of hydatid disease (Parija and Rao, 1986) and filariasis (Parija *et al*, 1988). We report here application of this assay for detection of specific antibodies in serodiagnosis of amebic liver abscess.

MATERIALS AND METHODS

Subjects

The subjects of the present study included clinical cases of amebic liver abscess and appropriate control groups, who attended the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Pondicherry. The study included sera from 72 cases of amebic liver abscess diagnosed as per the criteria recommended by Chuttani *et al* (1963) with few modifications. This included sera from 50 cases of pyogenic liver abscess, hydatid liver, other pathological lesions of the liver and hepatitis of various etiology other than amebic, as non-amebic liver disease controls and sera from 50 apparently healthy subjects from staff, students and blood donors who were apparently in good health and had not suffered from symptoms attributable to amebiasis during last six

months as healthy controls. The sera were stored at -20°C until used.

The protein-A antibody mediated hemagglutination (Protein A-IHA) test

Reagents

(a) *Amebic antigen*: The soluble axenic amebic antigen was prepared from a sonicated extract of axenically grown *Entamoeba histolytica* (NIH : 200) prepared according to the method described by Sawhney *et al* (1980). Briefly, amebae from 48 hours old cultures were washed thrice in sterile physiological saline, concentrated by centrifugation at 500g for 10 minutes and sonicated in cold at 20 Kc in an MSE ultrasonic disintegrator for 5 minutes with intermittent breaks of 2 minutes after continuous sonication for every one minute. The sonicated material was centrifuged at 10,000 g for 30 minutes and the supernatant was labeled as axenic amebic antigen. This antigen was stored in aliquots at -20°C and used for sensitization of red blood cells (RBCs) for use in the protein A-IHA assay.

(b) *Bacterial suspensions: Staphylococcus aureus* (Cowan's strain I) cells bearing protein A (SAPA) : The SAPA cells were grown on Mueller-Hinton agar at 37°C, fixed with formalin and heat and used in the test as per the method described by us earlier with few modification (Parija and Rao, 1986). After incubation for 18 hours under aeration, SAPA cells were harvested at 3000g for 10 minutes and washed thrice in phosphate buffer saline (PBS) (pH 7.2) containing 0.05% sodium azide (NaN₃). The pellet was fixed in 10 volumes of 1.5% formaldehyde in PBS at room temperature for 90 minutes washed thrice in buffer, resuspended in 10 volumes of the buffer containing 0.05% NaN₃ and heated for 5 minutes at 80°C. The cocci were again harvested and washed twice, a 10% suspension of cocci in PBS with 0.1% NaN₃ was prepared and stored in small volumes of different aliquets at -20°C until use in the test. The optimal co-hemagglutinating dose of SAPA cells was determined by chess-board titration with different dilutions of bacteria (0.1% to 0.3% in diluent usually sufficed) against antigen sensitized red cells in a microtiter system as described below. The lowest concentration of bacterial cells which showed the maximum co-hemagglutination (hemagglutination mediated by the cocci) titer with the standard sera was considered to be optimum co-hemagglutinating dose of SAPA cells valid for that batch of the cells. Protein

A-negative *S. aureus* (Weed 46 strain) (PANSA). The cocci were cultured on nutrient agar and harvested after formalin and heat treatment as for SAPA cells. They were stored as 10% suspension in PBS with preservation at 4°C for use in the test.

(c) *Antigen sensitized RBCs*: The human '0' RBCs collected in Alsever's solution were used in the test. The RBCs were stabilized by double aldehyde method of Parija and Ananthkrishna (1985) by treating the cells sequentially with pyruvic aldehyde, tannic acid and glutaraldehyde. The double aldehyde stabilized (DAS) '0' cells were sensitized with optimum sensitizing dose of axenic amebic antigen (Parija *et al*, 1989). Briefly the DAS red cells were washed twice with PBS, packed cells were suspended in 10 volumes of PBS (pH 6.4) containing an appropriate OSD of antigen and incubated in a water bath at 50°C for 5 minutes. The suspension was then kept over night at 4°C before the reincubation at 50°C for 3 minutes. The sensitized RBCs were washed twice with PBS and stored in small volumes as 10% suspension in PBS containing 0.1% NaN₃ as preservative at 4°C up to a minimum period of 45 days for use in the test (Parija *et al*, 1989). Before use in the test, the stored sensitized cells were washed once with diluent (PBS containing 0.1% bovine serum albumin) and reconstituted to 1% in the diluent.

Test

The test was performed in U bottomed microtiter plates according to the procedure described by Parija and Rao (1986). Briefly, a 25 µl volume of the diluent was dispensed in all the rows of the microtiter plate. A 25 µl volume of serum was then serially diluted in the fold steps up to the tenth wells. A 25 µl volume of SAPA at their optimal concentration was dispensed into each well. The plates were gently agitated at 70 oscillation/minute on a VDRL shaker for 20 minutes at room temperature. Subsequently a 25 µl volume of sensitized DAS '0' cells was then added to each well. The plates were gently agitated for 2 minutes and incubated at room temperature for 1 hour followed by overnight incubation at 4°C. The co-hemagglutination patterns were scored as for the IHA according to the method of Stavitsky (1954).

Appropriate controls were included in each test. SAPA cells plus sensitized DAS '0' cells only

PROTEIN A-IHA IN AMEBIC LIVER ABSCESS

in 11th wells and the diluent plus sensitized DAS cells in 12th wells, in each row served as the controls in the test. As a control of the SAPA cells, the tests were repeated with PANSA cells instead of SAPA cells following the same protocol mentioned above.

The SAPA suspensions were stored at -20°C, 4°C and room temperature and the tests were repeated at 15 days intervals of storage with a batch of known negative and positive amebic sera to determine effect of storage on reactivity of the SAPA suspensions.

The indirect hemagglutination (IHA) was performed on the same batch of sera using axenic antigen according to the method described elsewhere (Dutta and Sharma, 1979). This was done to compare sensitivity of protein A-IHA with that of standard assay.

RESULTS

The addition of SAPA cells prior to that of sensitized RBCs resulted in enhancement of sensitivity of the protein A-IHA, while that of protein A-negative PANSA cells, failed to enhance sensitivity of the test. Usually 0.2%-0.3% of SAPA cells were found to be the optimum cocci-mediated hemagglutination dose of the cells for use in the test. Minor variations in the concentration of SAPA cells were not found to affect the assay procedure.

The cocci-mediated hemagglutinating antibody (CHA) could be demonstrated amongst amebic liver abscess cases and their controls

examined by the test. All the CHA titers obtained in the presence of SAPA cells were at least double those observed by IHA in general. In most cases, much greater increase in titers were obtained and in a few cases there were 4-7 fold increase in titers when compared to that of IHA. In contrast, in the test using PANSA cells instead of SAPA cells, antibody titers were identical to those of the IHA test. The serum free controls revealed no hemagglutination by SAPA suspensions alone. These observations indicate that co-hemagglutination is mediated by protein A of SAPA cells only, in presence of specific antibodies.

The results of protein A-IHA on sera from amebic liver abscess and controls have been summarized in the Table 1. The sera sharing a CHA titer of 1:128 (mean of normal \pm 2 SD) and above was considered to be positive by this test. At this diagnostic titer 72 of 72 (100%) amebic liver abscess sera were positive with titers ranging from 1:128 to 1:137,072. Amongst the controls, none of the non-amebic liver disease controls showed any positive diagnostic titer, whereas only 1 (2%) healthy control serum showed a positive titer by the assay.

Table 2 shows comparison of sensitivity and specificity of protein A-IHA with that of IHA. The IHA detected 62 out of 72 (86.11%) cases and no false positive cases were seen amongst the non-amebic liver disease control sera, but 2 (4%) of healthy controls showed false positive reactions.

The SAPA cells after preparation could be stored at -20°C for one year, 4°C for six months and room temperature for 2 months without any loss of their antibody binding capacity.

Table 1
Results of protein A-IHA on sera from amebic liver abscess and controls.

Clinical diagnosis	No. of cases	Reciprocal antibody titer														No. positive at \geq 128	
		131072	65536	32768	16384	8192	4096	2048	1024	512	256	128	64	32	16		16
Amebic liver abscess	72	3	3	2	3	8	3	9	11	13	17	0	0	0	0	0	72
Non-amebic liver disease control	50	0	0	0	0	0	0	0	0	0	0	0	6	27	16	1	0
Healthy control	50	0	0	0	0	0	0	1	0	0	0	0	11	21	16	1	1

Table 2
Comparison of sensitivity and specificity of protein A-IHA
with IHA for diagnosis of amebic liver abscess cases.

Clinical diagnosis	No. of cases	No. of positive sera detected by	
		protein A-IHA	IHA
Amebic liver abscess	72	72 (100%)	62 (86.11%)
Non-amebic liver disease control	50	0 (0%)	0 (0%)
Healthy control	50	1 (2%)	2 (4%)

Figure in the parenthesis = percentage

The diagnostic titers by protein A-IHA = 1 : 128 and IHA = 1 : 128.

DISCUSSION

The use of *Staphylococcus aureus* (Cowan's strain I) bearing protein A (SAPA) as an anti-Ig reagent in immunological technique has been extended in recent years as a tool in both quantitative and qualitative immunological techniques (Kronvall, 1973; Langine, 1982). The protein A has the ability to interact with immunoglobulins, predominantly IgG, from mammalian species including humans, without affecting their immunological activity. Interactions between antibody bound SAPA cells have been demonstrated in a study in which it was observed that specific antibody coated SAPA cells agglutinated corresponding antigen sensitized but not unsensitized red cells (Bril *et al.*, 1979). Here SAPA bearing protein A was used as a substitute for the Coombs sera in order to bind the Fc portion of IgG and subsequently agglutinate antigen sensitized RBCs (Parija and Rao, 1986; Parija *et al.*, 1988; Jagannath *et al.*, 1984).

The method developed in this study, derived its use from the principle that in a fluid system containing both specific and non-specific antibodies, SAPA would bind specifically with IgG antibody with high affinity. The distribution of specific IgG antibodies on the surface of SAPA cells would simulate a particulate sphere with multiple antigen binding sites. On subsequent incubation with antigen sensitized red cells, antigen will bind to specific IgG, fixed on surface of the SAPA cells.

The test system demonstrated that use of SAPA cells bearing protein A greatly enhanced

sensitivity of IHA in serodiagnosis of amebiasis. Since the PANSAs strain which lacked protein A did not alter sensitivity of the IHA and since SAPA cells bearing protein A did not agglutinate sensitized DAS cells in the absence of antibody, the co-hemagglutination appeared to be mediated through protein A exclusively and occurred in the presence of specific antibodies only. The co-hemagglutinating antibodies could be demonstrated in amebic liver abscess sera and its control (Table 1). The protein A-IHA was highly specific and more sensitive than that of IHA (Table 2). A 4 to 7 fold increase in titer of amebic antibodies in amebic liver abscess sera was demonstrated by protein A-IHA in comparison to the IHA test. The protein A-IHA could detect 72 (100%) of ALA when compared with IHA 62 (86.11%) cases (Table 2). No false positivity was observed amongst the non-amebic liver disease sera by protein A-IHA. A false positive of 1 (2%) sera of normal healthy controls was observed by this assay.

The protein A-IHA procedure appears to have several advantages. The assay is highly sensitive. The test could be performed within 2 hours, if stored antigen sensitized RBCs and SAPA suspensions were used in the test. These reagents are stable, easily available and inexpensive. The amebic antigen sensitized RBCs could be stored upto 45 days and 15 days of storage at 4°C and room temperature respectively without losing their reactivity (Parija *et al.*, 1989). The SAPA cells as demonstrated in the study could be stored at -20°C for one year at 4°C for six months and room temperature for 2 months without any loss of their antibody binding capacity. This is also supported by results of other similar studies (Jagannath *et al.*, 1981; Parija *et al.*, 1986). Besides stability of the reagents and sensitivity, protein A-IHA has got an advantage over ELISA by not requiring sophisticated equipment such as an ELISA reader or too much technical skill (Jagannath *et al.*, 1984; Parija and Rao, 1986; Paraja *et al.*, 1988).

The protein A-IHA is relatively simple, highly sensitive, specific and requires minimum technical skill. Hence the assay deserves further evaluation for serodiagnosis of amebic liver abscess and other clinical manifestations of amebiasis.

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PROTEIN A-IHA IN AMEBIC LIVER ABSCESS

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