CO-AGGLUTINATION TEST FOR THE DETECTION OF CIRCULATING ANTIGEN IN AMEBIC LIVER ABSCESS

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Abstract. We report here a simple and economical slide agglutination test, the co-agglutination (Co-A) test, for the detection of circulating amebic antigen in sera for the diagnosis of amebic liver abscess. Fifty serum specimens from cases of amebic liver abscess, 25 from other individuals with parasitic and miscellaneous infections, and 25 from healthy controls were tested for the presence of serum antigen by the Co-A test. Forty-five (90%) amebic liver abscess sera were found to be amebic-antigen positive by the Co-A test. None of 25 sera from healthy controls were positive for the antigen. However, false-positive results were seen with two sera from those with other parasitic and miscellaneous infection controls. These results show that the Co-A test can be used as a sensitive and specific rapid slide agglutination test for the detection of amebic antigen in the sera for diagnosis of cases of amebic liver abscess in a routine parasitology laboratory.

Amebic liver abscess (ALA) is an extra-intestinal invasive form of amebiasis caused by the protozoan Entamoeba histolytica. This condition is being increasingly reported in India and is also quite common in Pondicherry in the southern part of India. More than 50 million cases of invasive amebic colitis or ALAs occur worldwide every year, resulting in 50,000–100,000 deaths annually. Since the clinical manifestations of ALA are protean and highly variable, the laboratory diagnostic methods, including serodiagnosis, play an important role in the diagnosis of this condition. Demonstration of trophozoites of E. histolytica in liver pus by microscopy establishes diagnosis of ALA. Unfortunately, the trophozoites can be demonstrated only in 15% of the cases with ALA, even in the best of laboratories. Thus, in the absence of a sensitive parasitic method, one has to depend greatly on serodiagnostic methods. The serologic tests traditionally demonstrate the presence of amebic antibodies in the serum. Recently, more emphasis has been placed on the detection of amebic antigen in the serum because it reflects more reliably than the antibody titers the viability and quantity of parasites in the infected host. The presence of antigen in the serum indicates active infection. Demonstration of the antigen, therefore, plays an useful role in monitoring course of the disease whether cured or still active.

In our laboratory, the co-agglutination (Co-A) test has been standardized and evaluated for the detection of hydatid antigen in serum and hydatid fluid for the diagnosis of hydatid disease. The Co-A test is a simple and rapid slide agglutination test that can be performed in a routine laboratory without any need for trained technical personnel, expensive reagents, or equipment. Therefore, in the present study, an attempt has been made to standardize and evaluate the Co-A test for the detection of amebic antigen in the serum for the diagnosis of ALA.

MATERIALS AND METHODS

Subjects. The subjects in the present study included 50 cases of ALA who attended the medical outpatient and inpatient clinics of the Jawaharlal Institute of Post Graduate Medical Education and Research Hospital in Pondicherry, India. All subjects were provided information on the nature of the study and their oral informed consent was obtained before participation. The study was reviewed and approved by the Director of the Jawaharlal Institute of Post Graduate Medical Education and Research. The cases of ALA were diagnosed on basis of three or more of the criteria as recommended by Chuttani and others with the following modifications: 1) needle aspiration from enlarged and tender liver; 2) imaging findings: a) radiographs of the abdomen showing raised and fixed right copula of the diaphragm; and b) ultrasonography of the liver showing no significant wall echoes, round or oval shape, less echogenicity than the normal liver parenchyma, with fine homogenous low level echoes through out at higher grain with particulate motion and a location contiguous to the liver capsules, and distal sonic enhancement titer; 3) positive serology by indirect hemagglutination (IHA), with an antibody ≥ 1:128 in the serum; and 4) positive response to anti-amebic therapy.

The control group included 25 cases of other parasitic infections and miscellaneous conditions (hydatid disease, 10 cases; filariasis, 10 cases; hookworm infestations, three cases, and lower respiratory tract infections, two cases) and 25 healthy controls (apparently healthy subjects from students, staff, and blood donors who did not have symptoms of amebiasis in the last six months).

Five milliliters of venous blood was collected from each of the subjects under aseptic precautions and was allowed to clot. Serum was separated, inactivated at 56°C for 30 min, preserved in 0.015 M sodium azide, and stored at −20°C until used.

Axenic antigen of E. histolytica. The sonicated antigen of axenically grown E. histolytica (NIH: 200) was prepared according to the method described by Sawney and others. Briefly, amebae from 48-hr cultures were washed three times in sterile physiologic saline, concentrated by centrifugation at 500 × g for 30 min, and sonicated at 4°C at 20 kilocycles in an ultrasonic disintegrator (MSE Instrumentation, London, United Kingdom) for 5 min with intermittent breaks for 2 min after continuous sonication every 1 min. The sonicated material was centrifuged at 10,000 × g for 30 min and the supernatant obtained was used as amebic antigen.
Hyperimmune antiserum. Axenic amebic antigen was emulsified with an equal volume of Freund's complete adjuvant. Adult rabbits (3–4 kg) were given 0.5 ml of this emulsion in all four limbs intramuscularly (IM). After five weeks, they were re-injected IM with 0.5 ml each in all four limbs with the same antigen in Freund’s incomplete adjuvant. After 10 days, serum samples were taken and monitored for amebic antibodies by the IHA test. The titer of the antibodies was 1:1,024. The hyperimmune serum was stored at −20°C.

The optimum sensitizing dose (OSD) of the hyperimmune serum was determined by checkerboard titration against the axenic amebic antigen. The highest dilution of the hyperimmune serum that showed the maximum hemagglutination with the antigen was considered as the OSD of the hyperimmune serum; the OSD was 1:20.

Co-agglutination test. The Co-A test was performed to detect amebic antigen in the serum as per the procedure described herein. It consists of the following steps.

Preparation of bacterial cells. *Staphylococcus aureus* (Cown’s strain I) bearing protein A (SAPA) were used. The cells were prepared as per the method described by Shariff and Parija. Cells were grown on Mueller-Hinton agar at 37°C for 18 hr, then harvested and centrifuged at 3,000 × g for 10 min and washed three times in phosphate buffered saline (PBS), pH 7.2, containing 0.05% sodium azide. The pellet was fixed in 10 volumes of 1.5% formaldehyde in PBS, pH 7.2, at room temperature for 90 min, washed three times in PBS, pH 7.2, resuspended in 10 volumes of buffer containing 0.05% sodium azide, and heated for 5 min at 80°C. The SAPA cells were washed twice in PBS, pH 7.2, and a 10% suspension in PBS, pH 7.2, containing 0.05% sodium azide was made.

Sensitization of SAPA cells. The SAPA cells were sensitized with the OSD hyperimmune antiserum immediately after the preparation of cells. One milliliter of 10% stabilized cells were added to 0.1 ml of hyperimmune antiserum (titer = 1:1,024 with an OSD of 1:20), mixed, and left at room temperature for 30 min. The cells were then washed in PBS, pH 7.2, and resuspended to a concentration of 2% in PBS, pH 7.2, containing 0.1% sodium azide. The sensitized reagent was stored at 4°C up to a period of one month. A 2% suspension of unsensitized cells was used as the cell control.

The Co-A test. A slide was marked with a glass marking pen into two halves. A drop of test serum was placed on each half of the slide. An equal volume of 2% sensitized SAPA cell suspension was added to the serum on one half. The same volume of a 2% suspension of unsensitized SAPA cells was added to the serum on the other half as a cell control. The slide was then rotated manually for 2 min and inspected. Agglutination with the sensitized cells and not with unsensitized cells was considered a positive result. Appropriate controls were examined in each test. Known positive and negative control sera were included every time the tests were performed. The antigen titers in serum were estimated by performing a quantitative test. This was performed by testing serum diluted from 1:2 to 1:128. The highest dilution of serum showing agglutination was considered as the Co-A titer.

Indirect hemagglutination test. Circulating amebic antibodies in serum were detected by the IHA test. This assay shows a sensitivity of 80–95% and a specificity of 85–95%. It was performed according to the procedure described by Parija and others. Double-aldehyde–stabilized (DAS) human type O red blood cells (RBCs) stabilized sequentially with pyruvic aldehyde, tannic acid, and glutaraldehyde were sensitized with the OSD of axenic amebic antigen. The amebic antigen–sensitized, double-aldehyde–stabilized cells were treated with serum to detect antibodies. The hemagglutination pattern of agglutinated RBCs was noted after overnight incubation at 4°C. The serum showing an antibody titer ≥ 1:128 was considered to be diagnostic of the disease.

Statistical analysis of the immunoassays. A database of all cases was made and analysis was carried out using the Epi-Info software package (World Health Organization, Geneva, Switzerland). The sensitivity and specificity were calculated according to the method of Park.

**RESULTS**

The results of the Co-A test with sera from cases of ALA and controls are summarized in Table 1. This test detected amebic antigen in 45 (90%) of 50 sera from cases of ALA. If the undiluted sera was positive for amebic antigen by the Co-A test, the test was then performed using the same sera diluted 1:2–1:128. The highest dilution showing agglutination was considered the Co-A titer. This titer ranged from 1:2 to 1:128. The mean ± SD titer was 13 ± 4 and the median was 16.

All 25 sera from healthy controls were negative for amebic antigen by the Co-A test. However, two (8%) sera from other parasitic and miscellaneous control cases gave false-positive results for amebic antigen, with titers ranging from 1:4 to 1:16. The mean ± SD titer was 1.1 ± 1.8, the median was 0, and the mode was 0.

The serum showing an antibody titer ≥ 1:128 was con-
Considered to be diagnostic for ALA. The IHA titers varied from 1:2 to 1:2,048. Hemagglutinating antibodies could be demonstrated among all the groups of patients with ALA and controls. Table 2 shows the comparison of the sensitivity and specificity of the Co-A and IHA tests in the diagnosis of ALA.

### Table 2

<table>
<thead>
<tr>
<th>Subject group</th>
<th>No. of positive sera (and cases)</th>
<th>Antigen by Co-A test</th>
<th>Antibody by IHA test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amebic liver abscess cases</td>
<td>45 (50)</td>
<td>41 (50)</td>
<td></td>
</tr>
<tr>
<td>Other parasitic and miscellaneous</td>
<td>2 (25)</td>
<td>3 (25)</td>
<td></td>
</tr>
<tr>
<td>infection controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0 (25)</td>
<td>0 (25)</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>90%</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>96%</td>
<td>94%</td>
<td></td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>95.7%</td>
<td>93.1%</td>
<td></td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>90.5%</td>
<td>83.9%</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Detection of amebic antigen in the serum confirms the diagnosis of ALA. An ELISA and a solid-phase radioimmunoassay (RIA) have been used to detect amebic antigen in serum and have showed variable sensitivity and specificity. The ELISA showed a sensitivity of 93%, while the RIA showed a sensitivity of 100%. However, neither method is practical in district or rural hospitals due to the lack of instruments, reagents, and kits. Furthermore, these hospitals do not have the trained technical personnel to perform these tests. Thus, there is a need for alternative, simple methods for the diagnosis of cases of ALA by antigen detection. Ideally, a test for use in these conditions should be simple, low-priced, sensitive, and reliable.

The results of our study shows that amebic antigen in the serum could be detected by the Co-A test. This test detected amebic antigen in the sera of 45 (90%) of 50 cases of ALA. It could discriminate between the geometric mean titer of antigens in the sera of cases of ALA and their controls. The Co-A test also detected amebic antigens in the serum of nine cases with ALA that tested negative for amebic antibodies by the IHA test. However, it failed to detect antigen in five cases of ALA that tested positive for amebic antibodies by the IHA test (Table 2).

The results of the present study show that the combined use of both the Co-A and IHA tests will optimize detection of cases of ALA. In this study, the IHA test showed a sensitivity of 82% in detecting the cases of ALA, which compares well with that of 80–95% reported by Pariaj and others and Mahajan and others. The IHA test showed a specificity of 94%, and false-positive results (12%) were observed with sera of control groups with other parasitic and miscellaneous diseases. The occurrence of false-positive results has also been reported in similar studies of amebiasis with the IHA test.

The Co-A test was also equally specific (96%). No false-positive reaction was observed with any healthy control serum. However, two (8%) sera from other parasitic and miscellaneous controls showed false-positive reactions. Amebic antigens were detected in these two sera with titers ranging from 1:4 to 1:16. These two sera were negative for amebic antibody by the IHA test. Conversely, three control sera from parasitic and miscellaneous controls that were positive for amebic antibodies by the IHA test were negative for serum antigen by the Co-A test.

The Co-A test is based on immunologic reaction between specific *E. histolytica* antibodies bound to SAPA cells and amebic antigens. In a test with a positive result, amebic antigens present in the serum combine with SAPA cells, resulting in visible clumping of the cells. The test is simple to perform and is reproducible. The assay is equally sensitive (90%) and specific (96%). The test could be performed and results obtained within 30–45 min of receipt of the sera. It does not require much technical skill and therefore is suitable for the detection of circulating amebic antigen in serum to establish the diagnosis of ALA in the field or in the poorly equipped routine clinical laboratory.

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