**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 livers with macroscopic characteristic (anchovy sauce) pus, bacteriologically sterile pus, with or without demonstration of trophozoites of *E. histolytica* by direct microscopy and/or culture; 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 titer of 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 Sawhney 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.
and Parija. (Cowans’ strain I) bearing protein A (SAPA) were used. The detect amebic antigen in the serum as per the procedure de-

with the antigen was considered as the OSD of the hyper-

immune serum that showed the maximum hemagglutination 

axonemal amebic antigen. The highest dilution of the hyper-

serum was determined by checkerboard titration against the 

antigen was stored at 4°C, pH 7.2, containing 0.1% sodium azide. The sensitized re-

suspended to a concentration of 2% in PBS, 

and resuspended in 10 volumes of buffer 

PBS, pH 7.2, at room temperature for 90 min, washed three 

pellet was fixed in 10 volumes of 1.5% formaldehyde in 

saline (PBS), pH 7.2, containing 0.05% sodium azide. The 

for 10 min and washed three times in phosphate buffered 

PBS, pH 7.2, and resuspended to a concentration of 2% in PBS, 

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.

Hyperimmune antiserum. Axenic amebic antigen was 

emulsified with an equal volume of Freund’s complete ad-

juvant. 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 adju-

vant. After 10 days, serum samples were taken and moni-

tored 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 hyper-

immune serum that showed the maximum hemagglutination 

with the antigen was considered as the OSD of the hyper-

immune 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 de-

scribed herein. It consists of the following steps. 

Preparation of bacterial cells. Staphylococcus aureus 

(Cowans’ strain I) bearing protein A (SAPA) were used. The 

cells were prepared as per the method described by Shariff 

and Parija.4 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 sensi-

tized with the OSD hyperimmune antiserum immediately af-

fter the preparation of cells. One milliliter of 10% stabilized 

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. Ap-

propriate controls were examined in each test. Known pos-

itive and negative control sera were included every time the 

tests were performed. The antigen titer in serum were es-

imated by performing a quantitative test. This was per-

formed by testing serum diluted from 1:2 to 1:128. The high-

est dilution of serum showing agglutination was considered 

as the Co-A titer.

Indirect hemagglutination test. Circulating amebic an-

tibodies 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.9 Double-aldehyde–stabilized (DAS) hu-

man 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 ame-

bic antigen–sensitized, double-aldehyde–stabilized cells 

were treated with serum to detect antibodies. The hemagglu-

tination pattern of agglutinated RBCs was noted after over-

night 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 were made and analysis was carried out using the 

Epi-Info software package (World Health Organization, Ge-

nova, Switzerland). The sensitivity and specificity were cal-

culated according to the method of Park.10

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 agglutina-

tion 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 ame-

bic 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-

TABLE 1

Distribution of amoebic antigen titers in serum of amoebic liver abscess cases and controls by Co-A

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>No. of cases</th>
<th>No. of sera positive for amoebic antigen dilution</th>
<th>Number negative</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amebic liver abscess cases</td>
<td>50</td>
<td>45</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Other parasitic and miscellaneous infection controls</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

The mean ± SD titer was 13 ± 4 and the median was 16.

The serum showing an antibody titer ≥ 1:128 was con-
negative for amebic antibodies by the IHA test were negative for serum antigen by the Co-A 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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