PUBLICATION AND PRODUCT
A simple & rapid Dot-ELISA dipstick technique for detection of antibodies to *Entamoeba histolytica* in amoebic liver abscess

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A rapid dipstick Dot-ELISA has been developed for detection of antibodies against *E. histolytica* employing soluble fraction or the sonicate prepared from axenically grown *E. histolytica* NIH : 200. The antigen was immobilized on nitrocellulose paper fixed to a plastic strip, at a concentration of 2.5 µg (protein content) which gave optimal results. The assay developed was usable with test serum diluted 1:200. 5 µl of test serum was adequate for the assay. Most of the antiamoebic antibodies in the sera of patients with amoebic liver abscess (ALA) were found to be of IgG type. Protein-A conjugated to HRPO was employed for the test and 4-chloro-1-naphthol plus H₂O₂ was used as substrate. A clear blue dot against white background indicated the presence of antiamoebic antibodies in the test serum. The test could be completed in 35 min. This dipstick Dot-ELISA was evaluated in another independent laboratory on 101 serum samples of which 40 sera were from microbiologically and clinically confirmed patients of ALA. Thirty nine of these samples were diagnosed correctly by the present technique. None of the 25 healthy controls were positive in the assay. The test was also negative in subjects with giardiasis, hepatomegaly, peptic ulcers and pyrexia of unknown origin. It was positive only in 8 of 25 patients of amoebic colitis. Its comparative merits with previously available lab assays for the diagnosis of ALA are discussed.

Nearly 150 million Indians are believed to suffer from amoebiasis. The parasite *Entamoeba histolytica* usually colonizes the caecum or colon but often spreads to the liver, which is the major site of metastasis, leading to amoebic liver abscess (ALA). It is essential to discriminate ALA from pyogenic liver abscess, hepatic neoplasm and hydatid disease. Serological investigations are required in ALA since stool examination may be negative when extra-intestinal sites are involved. Several serological methods to detect anti-amoebic antibodies in the serum have been developed, such as indirect haemagglutination (IHA), bentonite flocculation, counter-current immunoelectrophoresis, immunodiffusion, indirect fluorescent antibody, enzyme linked immunosorbant assay (ELISA). IHA is the most commonly used test. However,
Dot ELISA dipstick for detection of antibodies to *E. histolytica*

This test is time consuming and requires considerable technical skill.

We reported previously an immunodot EIA for ALA detection. In the present paper, the principle described earlier has been exploited with additional developments and refinements to evolve a 'dipstick' procedure which can be performed within 35 min. The method has been simplified to enable its being handled by an average paramedical personnel. The present investigation was undertaken to determine whether this dipstick procedure could reliably diagnose ALA. The dipsicks developed at the National Institute of Immunology were evaluated at the Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi, on samples from those patients of ALA in whom clinical and microbiological confirmatory evidence was available. The second objective was to determine whether the assay could be used to distinguish healthy control subjects at the cut-off point.

**Material & Methods**

**Sera:** The serum samples were collected from patients hospitalised in the AIIMS, New Delhi. Diagnosis of amoebic liver abscess was based on history of pain in the right hypochondrium, enlarged tender liver, fever, ultra sound investigation, absence of bacteria in aspirated pus, good response to antiamoebic therapy and IHA titers of more than 1:128. Patients of intestinal amoebiasis complained of pain in abdomen without splenomegaly and presence of cysts in the stool. Patients with abdominal pain, chronic or persistent diarrhoea with or without malabsorption showing *Giardia* in the stool were also investigated. Sera of 25 apparently healthy individuals who had no clinical history of amoebiasis and were found to be negative for intestinal parasites as per stool examination, were also examined.

**Antigen:** Axenically grown trophozoites of *E. histolytica* (NIH : 200) were harvested from 48 h culture tubes, washed twice with phosphate buffer saline (PBS, 50 mM, pH 7.4) and subjected to ultrasonic disintegration. The soluble fraction after centrifugation at 10,000 xg for 30 min in cold (4°C) was used as antigen.

**Preparation of 'dipstick':** Dipstick is a 7.6 x 0.7 mm plastic strip to which two 0.7 mm sq. nitrocellulose pads are fixed (Fig. 1). Antigen at varying concentrations (0.312 to 15 pg) was spotted in 1 µl with the help of a micro-dispenser (Hamilton, Switzerland) on the lower pad and allowed to dry in air at room temperature for 30 min. The upper pad without antigen served as reagent control. Untreated sites were blocked with 1 per cent bovine serum albumin (BSA) in PBS for 1h at 37°C. Dipsticks were then washed with PBS and stored refrigerated in a solution of 10 per cent sucrose containing 0.01 per cent sodium azide. Dipsticks thus prepared could be used up to six months without loss of activity. Precoated dipsicks can also be stored in dry state sealed in polyethylene aluminium foils. Such dipsicks withstood storage at temperatures up to 45°C for many months.

**Standardization and development of dipstick EIA:** All steps were carried out at room
Sharma et al

Fig. 1. Dipsticks with two nitrocellulose pads attached at one end. Blue dot on the lower pad shows positive reaction.

temperature and all reagents except substrate were prepared in PBS containing 1 per cent milk (Lactogen, Hindustan Lever). Positive and negative sera were included in each run to monitor the assay. Checkerboard titrations were carried out with various concentrations of antigen and antiserum dilutions to arrive at the optimal assay conditions. The dilution of test serum was fixed at 1:200 to eliminate the low level signal in healthy subjects living in areas where exposure to such infections could occur. Previous studies had shown high titres of antibodies in ALA patients with active infection. The concentrations of protein-A-HRPO and substrate were also fixed after due experimentation during initial standardization. The enzyme conjugate was prepared in the laboratory and diluted 1:1000 for use in the assay.

A quantity of 5 µl serum was adequate for the assay. It was diluted to 1 ml (200 times) with PBS containing 1 per cent milk. It was possible to pre-mark the tube so that dilution could be made to this mark without necessitating a second measurement of the diluent. When one drop of serum is employed (50 µl) the dilution would be to 10 ml.

The assay involves a three step process. In the first step, the antigen coated dipstick is washed with PBS and incubated with the diluted serum sample for 15 min at room temperature. It is then washed with running tap water and incubated with protein-A-HRPO solution for 15 min at room temperature. It is again washed with running tap water and immersed in substrate solution of 4-chloro-1-naphthol (3mg dissolved in 1 ml methanol and diluted 1:6 with PBS). Just before use, 6 µl H₂O₂ is added to the above mixture. Incubation is carried out for 5 min followed by washing with tap water to stop the reaction. A positive result is indicated by a clear blue dot against white background (Fig. 1).

Results

Fig. 2 gives the results of checker board titration of E. histolytica antigen and antibodies from pooled sera of 5 known amoebic liver abscess patients. All known ALA patients sera gave positive reaction with
Dot ELISA dipstick for detection of antibodies to *E. histolytica*

Fig. 2. Checker board titration of *E. histolytica* antigen and pooled sera from five ALA patients.

$> 2.5 \mu g$ antigen per dot when sera were used at dilutions up to 1:3200. In a separate experiment it was noted that negative control serum dilutions lower than 1:200 gave false positive reaction with high concentrations of the antigen but at and above 1:200 no false reaction was observed with the antigen at 2.5 $\mu g$ per dot (data not shown). Therefore 2.5 $\mu g$ antigen per dot and serum dilution of 1:200 were selected for this assay.

As this test titrates antibodies, the class of antibody in the patients' serum which reacted with the amoebic antigens used in the assay, was determined. The predominant class of antibodies present in the sera of ALA patients was of IgG class (Fig. 3). A double blind study was conducted on coded samples, to determine the sensitivity and specificity of the 'Dipstick' assay. A total of 102 well defined clinical samples were analysed. The present dipstick Dot ELISA could diagnose correctly 39 of the 40 (97.5%) confirmed ALA patients (Table I) as compared to 35 (87.5%) and 39 (97.5%) detected by IHA and plate ELISA respectively. However, only 8 (32%), 2 (8%) and 18 (72%) of 25 patients of amoebic colitis were found to be positive by Dot ELISA, IHA and plate ELISA respectively. All patients of pyrexia of unknown origin (PUO), hepatomegaly, peptic ulcers and giardiasis were found to be negative by this dipstick Dot ELISA whereas one of twelve patients gave false positivity in IHA.

Discussion

In any antibody based test, it is desirable
Table I. Sensitivity and specificity of Dot ELISA for the diagnosis of amoebic liver abscess

<table>
<thead>
<tr>
<th>Subject</th>
<th>DotEIA+ PEIA+ IHA+</th>
<th>DotEIA+ PEIA+ IHA-</th>
<th>DotEIA- PEIA- IHA+</th>
<th>DotEIA- PEIA- IHA-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmed ALA</td>
<td>40</td>
<td>35</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Amoebic colitis</td>
<td>25</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>PUO, hepatomegely, peptic ulcer</td>
<td>8</td>
<td></td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Giardiasis</td>
<td>4</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>25</td>
<td></td>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>

Dot EIA, Dot-ELISA; PEIA, plate ELISA; PUO, pyrexia of unknown origin; ALA, amoebic liver abscess

to distinguish between active infection and past exposure to the pathogen. The IgM class of antibodies is usually indicative of current infection while IgG can persist, indicating chronic infection. Our findings showed that the antibodies against E. histolytica in patients suffering from ALA were primarily of IgG type. These results concur with those of other studies carried out in areas endemic for E. histolytica, where levels of IgG were found to be elevated, whereas those of IgA and IgM were less affected. Thus, protein-A-HRPO could be used as the revealing agent. Protein-A-HRPO has an added advantage of being cost effective as compared to anti-human IgG coupled to the enzyme. Of the various substrates tested, 4-chloro-l-naphthol was found to be suitable since it gave clear blue dot with minimum background.

The present dipstick Dot-EIA was found to have a high degree of sensitivity and specificity for ALA. However, all patients of intestinal amoebiasis and cyst passers could not be detected by this test. It is generally assumed that serum antibodies to E. histolytica appear only after invasion by the parasite and acute invasive amoebiasis provokes high serum titers as compared to those in asymptomatic patients. It is therefore, not surprising that Dot ELISA missed some of the cyst passers who are not likely to produce significant amounts of antibodies while it detected almost all patients of ALA.

The test is novel in that it is simple, rapid, easy to perform and not requiring sophisticated equipment. Most tests used for detection of ALA are cumbersome and time consuming (Table II). Dipstick Dot-ELISA which is based on the principle of ELISA, while retaining the sensitivity and specificity of microplate ELISA, has a number of operational advantages over the latter technique. In plate ELISA the actual concentration of antigen adsorbed to the micro-titre plate is generally not known and different proteins have different binding characteristics. This may be crucial if weakly binding antigens are important from the
Dot ELISA dipstick for detection of antibodies to *E. histolytica*

**Table II. Comparison of dipstick Dot EIA with conventional serological tests for the diagnosis of amoebic liver abscess**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity, %</th>
<th>Time consumed, h</th>
<th>Expertise required</th>
<th>Whether field based</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHA</td>
<td>90—95</td>
<td>6—16</td>
<td>Skilled</td>
<td>No</td>
</tr>
<tr>
<td>CIEP</td>
<td>70—80</td>
<td>2</td>
<td>Skilled</td>
<td>No</td>
</tr>
<tr>
<td>Plate ELISA</td>
<td>95—98</td>
<td>6</td>
<td>Skilled</td>
<td>No</td>
</tr>
<tr>
<td>Dipstick Dot EIA</td>
<td>95—98</td>
<td>35 min</td>
<td>Semi-skilled</td>
<td>Yes</td>
</tr>
</tbody>
</table>

IHA, indirect haemagglutination; CIEP, counter current immunoelectrophoresis

Diagnostic point of view. Moreover, adsorption of antigen may vary from plate to plate or from well to well in the same plate. Unlike plate ELISA technique which requires a large volume of antigen, dipstick Dot-ELISA requires only one μl antigen per dot, all of which gets absorbed to the nitrocellulose pad. Similarly 5 μl serum is sufficient per test. The assay is rapid and can be read in 35 min using pre-coated dipsticks. Dipsticks can be coated and stored after blocking unreacted sites, for over 6 months in a solution of sucrose containing sodium azide or in the dry state, for over six months at temperatures ranging up to 45°C when packed suitably.

Although antigen detection is theoretically a better index of active infection and attempts are being made to develop antigen based tests, the EIA developed so far for determination of *E. histolytica* antigen gives a sensitivity of about 80 per cent only and further improvements are required. Pillai and Mohimen have, however, reported an RIA based antigen detection test with acceptable degree of sensitivity. This test requires prior precipitation of immune complexes from patient serum and involves the use of radioactive material which makes it unsuitable for field usage.

Dipsticks used in the present Dot EIA are readily available from indigenous sources (Advanced Microdevices, Ambala) and the cost is Re 0.50 per stick. The total reagent cost including overheads is an additional Re. 0.50 (total cost Re 1.0). Dipstick Dot EIA is not only less expensive than other currently available tests such as IHA, CIE and plate ELISA, but is also easy to perform, and has a fairly high degree of sensitivity and specificity.

In continuing product development efforts, it is observed that pre-coated dipsticks can also be stored dry in suitable packs at temperatures ranging from 4°C to 45°C for several months without loss of activity. Third party validation trials are in progress in different centres in India to confirm independently the sensitivity and utility of this test.

**References**


Reprint requests: Shri Manoj Sharma, National Institute of Immunology, Saheed Jeet Singh Marg JNU Complex, New Delhi 110067
Biotechnology transfer from NII to Indian Industry

Dipstick Dot-ELISA for detection of anti-\textit{E. histolytica} antibodies for diagnosis of invasive amoebiasis

Transferred to

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