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Original Article

Microbiology Section

Indirect Haemagglutination Test in Comparison with ELISA for Detection of Antibodies against Invasive Amoebiasis

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ABSTRACT

Introduction: Diagnosis of amoebiasis is based on combination of tests like microscopy, imaging, serology and molecular methods. In absence of molecular techniques, serology can be used as an alternative aid. Various serological techniques were reported with different sensitivity and specificity. The diagnostic efficiency of these assays mainly depends on the characteristics of antigen that is being used and various conditions of performance.

Aim: To evaluate the efficiency of recombinant calcium binding domain containing protein by Indirect Haemagglutination Assay (IHA) against a commercial ELISA among amoebic liver abscess cases and control group.

Materials and Methods: The study was carried out during the period of 2011-2015 and blood samples were collected from suspected amoebiasis cases who were attending the clinics of Medicine and Paediatrics department, JIPMER. A total of 200 sera samples which included 100 Amoebic Liver Abscess (ALA), 50 cases of other parasitic infections and liver diseases and 50 presumed healthy controls were examined by IHA and commercial ELISA. In brief, chick cells were stabilized by Double Aldehyde Sensitization (DAS) method. Optimum Sensitizing Dose (OSD) of the antigen was determined. The test was performed in a U-bottomed microtiter plate with recombinant amoebic antigen (12.5µg/ml), incubated at Room Temperature (RT) for 2 hours. RIDASCREEN *Entamoeba* IgG ELISA kit which is commercially available was used to evaluate the samples as per manufacturer's instruction.

Results: The overall sensitivity and specificity of the IHA was 62% and 96%, respectively when compared to ELISA having sensitivity and specificity of 69% and 90%, respectively. The positive predictive value of the IHA was 91% while negative predictive value was 79%. Similarly, the positive predictive value of the ELISA was 87% while negative predictive value was 74%.

Conclusion: As serology heavily suffers due to lack of a standardised test system employing the native antigen, there arises need to identify alternative source of recombinant antigen which could effectively improvise the existing lacunae in the current system. Serology acts as an adjunct in clinical decision making if properly interpreted. This is an important consideration in endemic region where health services resources are limited.

Keywords: Amoebic Liver Abscess, Double aldehyde sensitized cells, Recombinant antigen, Seroepidemiology

INTRODUCTION

Amoebiasis caused by the eukaryotic protozoan parasite *Entamoeba histolytica* manifests as non-invasive intestinal form as amoebic dysentery while extra-intestinal invasive form as Amoebic Liver Abscess (ALA). Worldwide about 34-50 million cases suffer from invasive form of the disease and the annual reported mortality due to ALA ranged from 50,000-100,000 [1,2]. Invasive form of this disease is increasingly reported in less developed nations like India where socio economic conditions and sanitary facilities are limited [3,4]. The diagnosis of ALA is challenging because of their varied clinical manifestations. Often it is diagnosed by combination of techniques such as microscopy, imaging, serology, molecular methods along with the clinical presentation. In endemic nation like India where molecular techniques cannot be employed routinely, serology comes as an alternative aid [5,6]. Also, there is a need to design and evaluate standard serological tests that is not only sensitive and specific, but also easy to operate and cost-effective.

Various serological tests such as immunoprecipitation, Counter Immunoelectrophoresis (CIEP), Latex Agglutination (LA), Indirect Haemagglutination (IHA), Immunofluorescence, Enzyme Linked Immunosorbent Assay (ELISA) and Radio Immunoassay (RIA) are commonly used to detect amoebic antibodies from ALA cases [7]. However, the drawback with the usage of these above said serology techniques is the non-availability of the standard amoebic antigen. Polyxenic antigen prepared along with bacteria often results in contradictory and variable results [8]. Even though axenic antigen seems to have higher efficacy, their preparation is laborious and cannot be carried out in routine diagnostic laboratory [9]. Thus, usage of standard amoebic antigen serves as a critical factor in serodiagnosis and seroepidemiology of invasive amoebiasis [10,11].

An immunodominant 170kDa lectin antigen is responsible for invasion of host tissue and resistance of parasite towards host's immune response [12]. This protein is widely used as diagnostic marker in many commercial as well as in house serology tests for detection of amoebic antibodies [13,14]. Though the above mentioned antigen has been widely used and evaluated, there could be other discrete parasitic antigens of clinical importance which needs to be identified and evaluated further. Some of the other recombinant antigens like serine rich protein, 170kDa subunit galactose specific adhesin, cysteine proteinase, putative alcohol dehydrogenase, phosphoglucomutase and pyruvate phosphate dikinase were used as alternative antigenic targets in amoebic serology tests in the recent years [15-20]. Therefore, this study was carried out to evaluate the potency of recombinant calcium binding domain containing protein (27.8kDa) to diagnose ALA by IHA.

MATERIALS AND METHODS

Serum was collected from suspected amoebiasis cases who were attending the clinics of Medicine and Paediatrics, JIPMER, from 2011–2015. The Institute Human Ethics Committee approval was obtained (EC/2011/3/4 dated 03/08/2011).

A total number of 200 sera samples were subjected to this study. Sera from 100 patients diagnosed with Amoebic Liver Abscess (ALA) based on clinical symptoms such as fever and pain in the epigastrium, enlarged tender liver, febrile-associated toxaemia, with bacteriologically sterile abscess aspirate and abscess demonstrated by ultrasound were included in the study. Additionally sera from filaria (19), hydatid (7), neurocysticercosis (4), toxoplasmosis (6), malaria (1), chronic liver disease (3), alcoholic liver disease (1), hepatitis B (1), jaundice (4), cirrhosis (2), hepatoma (2) patients and presumed healthy controls (50) were also included. Informed consent was obtained from all the subjects participated in the study.

Approximately 5ml venous blood was drawn from ALA and control group. It was centrifuged to obtain serum samples and stored at –80°C until the tests were performed.

ELISA: All the 200 serum samples were evaluated for the presence of IgG antibodies against *E. histolytica* using a commercial Enzyme Linked Immunosorbent Assay (ELISA) kit (RIDASCREEN *E. histolytica* IgG, R-Biopharm, Germany, K-1721). The plates were coated with purified antigens of *E. histolytica*. All the serum samples were diluted to 1:50 using diluent as per the manufacturer's instruction. Diluted samples (100µl) were added into the micro titre plate along with the positive and negative controls. After 15 minutes incubation at RT and washing, 100µl of protein A conjugate was again added and incubated. After washing 100µl of substrate added and incubated. Finally, 50µl stop solution was added and optical density was measured at 450 nm.

The sample index was calculated from the average absorbance value of the negative control. The cut-off value of the test was obtained by adding 0.15 to the average absorbance. Sample absorbance divided by cut-off value yielded the sample index.

Antigen: Recombinant Calcium binding domain containing protein (27.8 kDa) prepared in our lab was used as antigen in the study (Data yet to be published).

Preparation and sensitization of Chick cells by Double Aldehyde Stabilization (DAS): Blood from chicken was collected into Alsever's solution and cells were washed thrice in phosphate-buffered saline (PBS; pH 7.2). The RBCs were stabilised sequentially with pyruvic aldehyde, tannic acid and glutaraldehyde as described by Parija and Ananthakrishnan [7]. Optimum Sensitizing Dose (OSD) of the antigen was determined by chequer board using positive and negative control. In brief, one volume of packed DAS cells was added to 10 volumes of OSD of the recombinant calcium binding domain containing protein and incubated at 50°C in a water bath for 5 minutes, followed by overnight storage at 4°C. Next day, the cells were again incubated at 50°C for 10 minutes. The sensitized cells were washed thrice with PBS (pH 7.2) and made into 1% suspension using 0.1% Bovine Serum Albumin (BSA).

The test sera were inactivated by incubating at 56°C for 30mins. In a U-bottomed microtiter plate 25µl of diluent (PBS with 1% BSA) was added to all the wells to which 25µl of the test sera added to the first well of appropriate row. Serial dilution was performed up to eleventh well leaving the last well as control. To all the wells, 25µl of OSD recombinant amoebic antigen was added, agitated gently for few minutes and incubated at RT. Reading was taken every 30mins up to 2hours. The serum showing an antibody titer value of ≥1:128 were considered positive for ALA.

RESULTS

IHA: The IHA titer values ranged from 1:2 to 1:2048 dilutions.

For IHA, 48 cases were positive while 52 cases were negative in amoebic liver abscess group [Table/Fig-1]. Among the other parasitic and liver disease control group, 2 were positive while 48 were negative. In presumed healthy control group 4 control samples were positive while 46 controls were negative.

The sensitivity and specificity of the IHA test was 62% and 95% respectively [Table/Fig-2]. The positive predictive value was 91% while negative predictive value was 79%.

ELISA: Among the total 100 cases of ALA, 69 cases were found to be positive while 31 cases were negative [Table/Fig-1]. In other parasitic and liver disease control group, 10/50 controls were positive while 40/50 controls were negative. Of the 50 presumed healthy controls, all the 50 controls were negative.

The sensitivity and specificity of ELISA was 69% and 90%, respectively. The positive predictive value was 87% while negative predictive value was 74% [Table/Fig-2].

Subject Group	Total No of Cases	IHA		ELISA	
		Positive	Negative	Positive	Negative
Amoebic Liver Abscess Cases	100	48	52	69	31
Other Parasitic and liver disease group	50	2	48	10	40
Presumed Healthy controls	50	4	46	0	50

[Table/Fig-1]: Comparison of IHA and ELISA in ALA and other groups.

	IHA	ELISA
Sensitivity (%)	62	69
Specificity (%)	96	90
Positive Predictive Value (%)	91	87
Negative Predictive Value (%)	79	74

[Table/Fig-2]: Sensitivity and Specificity of IHA and ELISA.

DISCUSSION

The most common clinical manifestation of extra-intestinal amoebiasis which is the ALA needs accurate diagnosis from the non pathogenic form to avoid unnecessary treatment and development of resistance against amoebiasis [21]. Morbidity often occurs due to delay in diagnosis of this disease and early diagnosis plays a pivotal role in treatment of infection. The various modes of diagnosis include microscopy, culture methods, iso-enzyme analysis, serology based techniques including antigen and antibody based methods. The main confirmatory tests include molecular techniques like conventional PCR, nested and real time PCR [7] Though the above mentioned tests such as PCR and iso-enzyme analysis accurately distinguish between species, their usage is not practical in developing nations where *E. histolytica* infection is endemic [7]. India is a highly endemic region for ALA and also resource limited nation where molecular techniques cannot be routinely employed. Thus, in absence of a confirmatory test, serology is recommended as the reliable diagnostic tool [5,6].

Serodiagnosis plays a key role in determining the presence of acute form of the disease [22,23]. Also it acts as an adjunct with other tests and often used for epidemiological studies of amoebiasis [24]. In cases of suspected amoebiasis, serology acts as rapid diagnostic tool in clinical decision making to minimize the hospital stay thereby reducing the cost of additional treatment [25]. Various serological tests have been employed with different sensitivity, specificity and reproducibility. Among the serological tests, ELISA has been reported to have high sensitivity and specificity [26]. However the high cost associated with a commercial ELISA is a major challenge in resource limited countries [27]. The role of serology is restricted in diagnosis of amoebiasis because of the poorly characterised amoebic antigen obtained from *E. histolytica* trophozoites grown axenically [9]. The trophozoites often grown along with the enteric bacteria changes the morphology, virulence and antigenicity [8]. Even their growth is affected by minor changes in temperature, pH and concentration of medium [28]. The serological tests using native antigen usually leads to cross

reaction with other parasites since they share epitopes common to eukaryotic cells. Therefore, serological tests employing native antigens lack sensitivity, specificity and reproducibility [29]. Hence, the challenges associated with the diagnosis of amoebiasis in an endemic as well as a resource limited nation such as India leads to the necessity to develop appropriate in-house serological tests employing either standard native/recombinant diagnostic antigens.

All the individuals who are affected by *E. histolytica* do not progress to develop invasive disease. The factor that contributes for transforming commensal to virulent organism is not yet clear. However, from various studies it is understood that calcium (Ca^{2+}) is involved in pathogenesis [30]. Pathogenesis involves penetration of the organism into human tissues, attachment to the host leading to cytolysis and phagocytosis of erythrocytes, bacteria and epithelial cells. The major route of food intake of *E. histolytica* is through phagocytosis which attributes for virulence [31]. Calcium and calcium binding proteins (CaBP) modulates the cytopathic properties of the parasite thereby plays a key role in virulence and pathogenesis [32]. Therefore, our study was conducted to immunologically characterize the recombinant calcium binding domain containing protein of *E. histolytica*. Though various serological tests like RIA, CFT, IE, Gel precipitation, ELISA are often used, our study employed IHA owing to its simplicity, cost effectiveness and specificity [33]. This is a very simple technique which can be carried out even by lab personnel without any equipment using minimal samples.

The sensitivity of IHA employing the recombinant calcium binding domain containing protein was reported to be 62% while specificity 96% for diagnosis of ALA. However, the study conducted by Hira et al., reported to have a sensitivity of 99% and specificity of 99.8% employing the commercial IHA test [26]. The same study using a commercial ELISA reported to have sensitivity of 97.9% and specificity of 94.8%. However, our study reported to have a sensitivity of 69% and specificity of 90% for ELISA. The commercial ELISA employed in our study was different from the ELISA used in the above mentioned study. Cross reactions were mostly observed in the other parasitic and other liver disease group. This contributes to the fact that these subjects may be exposed to the infection but remain asymptomatic since this is a highly endemic region for ALA. The study conducted by Mohammed et al., 2009 reported to have a sensitivity of 70% and specificity of 88.7% using the cellognost commercial IHA kit [34]. In spite of using a commercial test, almost similar low sensitivity has been reported like our study. In 95% cases of amoebic colitis and ALA, serum IgG antibody appears within one week after the onset of symptoms [35]. The absence of antibody in many of the cases probably indicates the invasion is yet to occur. Also various other factors like circulating immune complexes and other inhibitory factors in sera may be responsible for false negative reactions. The above mentioned facts may have contributed to the low sensitivity rate reported in our study. However, the sensitivity of the test can be increased several fold by modification of the IHA technique as per the method of Parija and Ananthakrishnan [36]. Even though the commercial ELISA had higher sensitivity when compared to IHA, it can be employed where minimal facilities are available for measuring antibodies and in the field to conduct epidemiological surveys.

LIMITATION

The limitation of this study is that the serology tests IHA and ELISA cannot distinguish the past from current infection as India is a highly endemic nation where infections due to *E. histolytica* is common. Thus, definitive diagnosis by serology is highly difficult.

CONCLUSION

As serology heavily suffers due to lack of a standardised test system employing the native antigen, there arises need to identify alternative source of recombinant antigen which could effectively improvise the existing lacunae in the current system. Serology acts as an adjunct in clinical decision making if properly interpreted. This is an important consideration in endemic region where health services resources are limited.

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