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A study on the usefulness of Techlab *Entamoeba histolytica* II antigen detection ELISA in the diagnosis of amoebic liver abscess (ALA) at Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia

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Abstract. Amoebic serodiagnosis at Hospital Universiti Sains Malaysia (HUSM), Kelantan employs an indirect haemagglutination assay (IHA) which detects anti-*Entamoeba histolytica* antibodies in patients' serum samples. In an amoebiasis endemic area such as Kelantan, interpretation of a positive IHA result can be problematic due to the high background antibody levels. The TechLab *E. histolytica* II ELISA is a commercial kit for detection of specific Gal/GalNAc lectin antigen in stool samples, and has been reported to be able to detect the antigen in serum samples from patients with amoebic liver abscess (ALA). Thus in this study we investigated the usefulness of TechLab *E. histolytica* II ELISA for diagnosis of ALA by comparing it with IHA. This is a cross sectional study involving 58 suspected ALA patients who were admitted to the surgical ward, HUSM, Kelantan. The diagnosis of ALA was established based on clinical symptoms and signs, ultrasound and/or CT scan results. The serum specimens obtained from the patients were tested with IHA (Dade Behring Diagnostics, Marburg, Germany) and TechLab *E. histolytica* II ELISA (Techlab, Blacksburg, Virginia, USA) according to the manufacturers' instructions. Of the 58 patients, 72.4% (42) were positive by IHA and only 8.6% (5) were positive by the TechLab *E. histolytica* II ELISA. Agreement between the IHA and ELISA was poor (kappa value 0.019, p=0.691). There was also no correlation between ELISA results and IHA antibody titers. The TechLab *E. histolytica* II ELISA was not sensitive in detecting amoebic antigen in samples from ALA patients. In addition the results of the test did not correlate with the IHA anti-*E. histolytica* antibody titres. Therefore, the TechLab *E. histolytica* II ELISA was found not to be useful for serological diagnosis of ALA at HUSM.

INTRODUCTION

Amoebiasis is the most aggressive protozoan disease caused by *Entamoeba histolytica*. It is a common worldwide disease, and 100 000 people are estimated to die each year from amoebic colitis and amoebic liver abscess (ALA). Estimated worldwide prevalence of ALA is about 50 million infections per year (WHO, 1997). It is considered the third leading cause of death amongst the parasitic diseases, surpassed only by malaria and schistosomiasis.

The expression of disease varies with geographic location. For example, in Egypt the predominant presentation is amoebic colitis, whereas in South Africa there is an excessive rate of ALA (Ravdin & Stauffer, 2005). In an endemic area in Vietnam, the incidence of ALA was noted to be at least 21 per 100,000 inhabitants per year (Blessmann *et al.*, 2002). In Malaysia, a number of

hospital based studies on ALA have been carried out. It was reported to be positive in 44.1% of liver abscess patients (Goh *et al.*, 1987) and 39% of patients with amoebiasis (Jamaiah & Shekhar, 1999).

The definitive confirmation of ALA is based on the demonstration of *E. histolytica* trophozoites in the aspirated pus, or more frequently from the necrotic material obtained by needle biopsy of the edge or the bottom of the lesion. Nevertheless, the amoebas usually are found in only a small percentage of cases. Most patients with ALA do not have coexistent amoebic colitis. Therefore, stool microscopy or antigen detection in stool samples are not helpful for diagnosis; less than ten percent of patients have identifiable amoebae in stool (Haque *et al.*, 2000).

Serology has become a valuable tool for diagnosis of ALA and detects specific circulating antibodies against the invasive form of the parasite. Among the current commercially available serological tests, indirect haemagglutination assay (IHA) is widely employed. It is a very sensitive assay, being positive in 90% to 100% of patients with ALA (Salles *et al.*, 2003). This test is being used at the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia to detect anti-amoebic antibody. It is a good and useful test but the interpretation of the results are often difficult particularly in endemic areas where there are high background levels of seropositivity for amoebiasis. It is not clearly known what levels of endemicity exist in Kelantan, thus interpretation of amoebic serology can be uncertain especially if the antibody titer is not high. In 2003, the number of requests for amoebic serology was 108 and 68 (62.9%) cases were detected as positive by IHA, using a cut-off titer of 1:256.

Several groups have reported the detection of amoebic antigen in the serum of liver abscess patients. For example, Abd-Alla and colleagues (1993) detected the Gal/GalNAc lectin in the sera of 75% of South African patients with ALA. A commercially available antigen detection test, the TechLab *E. histolytica* test, Inc. (Blacksburg, Va.), detects the Gal/GalNAc lectin in stool samples and has been proven to be a sensitive and specific means of diagnosis of colitis (Haque *et al.*, 1995). A second-generation kit that uses an improved capture antibody has been developed by TechLab. Haque *et al.* (2000) has evaluated this kit for the detection of lectin antigen in the serum of ALA patients in Dhaka, Bangladesh and found that 96% of them were positive for Gal/GalNAc lectin when tested prior to treatment with metronidazole.

Therefore, in our effort to improve diagnosis of ALA at HUSM, we compared the ELISA (TechLab *E. histolytica* II kit) with the current IHA test that is being used at HUSM for serological diagnosis of ALA patients.

MATERIALS AND METHODS

A cross sectional study was performed whereby all clinical or suspected ALA patients who were admitted to the HUSM surgical ward within the study period were included (January 2005 till June 2007). Patients who were found to have positive bacterial culture of the pus aspirates from the liver and/or blood were excluded from this study. Both IHA and the ELISA were performed on the patients' serum samples and the results compared. The patients' clinical data were collected from the hospital files.

The diagnosis of ALA was established based on clinical symptoms of fever, abdominal pain (usually in the right hypochondrium or epigastrium), clinical signs of hepatomegaly and/or tender liver with or without jaundice, ultrasound and/or CT scan results (such as space occupying and hypoechoic lesion in the liver highly suggestive of abscess), and an improvement seen after treatment with metronidazole. Written informed consents were obtained from all patients who participated in this study.

The blood samples were collected in plain tubes and sent to the serology laboratory, HUSM for amoebic serology. In the laboratory, the blood samples were

centrifuged; the sera were separated and stored in 4 to 8ºC or -20ºC until used.

The sample size for this study was determined by using STATA software version 8.0 with additional module for sample size calculation of kappa. The minimum required sample size calculated by the software was 53 (95% confidence interval, 0.15 absolute precision and estimated kappa value of 0.9). This study had been approved by the university's research ethics committee.

Amoebic serology

Each serum sample was tested with IHA for antibody detection. The patients were grouped into IHA positive and IHA negative patients. In each group, ELISA for antigen detection was also carried out.

Antibody detection by IHA

The IHA was performed according to manufacturer's instructions (Dade Behring Diagnostics, Marburg, Germany). Each patient's serum sample was mixed with human group O erythrocytes sensitized with soluble, purified *E.histolytica* (HK9 strain) antigen in V-shaped microtiter wells. The specific antibodies present in the serum sample cross-link with the sensitized erythrocytes and the agglutinated erythrocytes will settle down in the well as a carpet formation. For qualitative or screening test, the amoebiasis IHA reagent was diluted before the test with Tris buffer solution, pH 8.0 in a 1+2 ratio. Five microlitre of amoebiasis control serum positive was dispensed into well A1, 5 µl negative control serum into well A2, and beginning with well A3, 5 µl of the samples were pipetted into the remaining wells of the microtitration plate. A volume of 100 µl diluted amoebiasis IHA reagent was added to each well containing the serum. The microtitre plate was placed on a shaker for 15 to 20 seconds, at 900 to 1100 rpm, then covered with polystyrene cover and subsequently incubated at room temperature without agitation for 2 to 24 hours.

For quantitative test, 175 µl Tris buffer solution, pH 8.0 was dispensed into the first column of wells of the microtitration plate (A1 through H1) and 50 µl of the buffer was dispensed into the remaining wells except for A12. Then, 25 µl amoebiasis control serum positive was added to the buffer in well A1 and mixed well. Fifty microlitre of negative control serum was dispensed and diluted 1 + 15 in Tris buffer solution, pH 8.0 into well A 12. Twenty-five microlitre of the samples to be tested were dispensed into wells B1 through H1 and mixed well with the buffer. Then 50 µl was transferred from well 1 (A1 through H1) to well 2 (B2 through H2), and the serial dilutions were continued from rows A1 through A11; finally 50µl from the last well was discarded. Subsequently, 25 µl amoebiasis IHA reagent was dispensed into the wells of rows 2 to 12, this corresponded to a starting dilution of 1: 16. The microtitre plate was then placed on a shaker for 15 to 20 seconds, then incubated without agitation for 2 to 24 hours.

For both qualitative and quantitative tests, the reaction was read by comparing the test samples to the controls using a reading mirror for microtitre plate. The test results were interpreted as positive whenever complete agglutination of the cells (carpet formation) were observed. A negative result were interpreted whenever the cells form a sediment (button formation)

Antigen detection by ELISA

The TechLab *E.histolytica* II test (TechLab Inc, Blacksburg, VA) was employed for the antigen detection assay. The microtiter wells were coated with immobilized polyclonal antibody that binds adhesin of *E.histolytica/dispar*. The conjugate is a monoclonal antibody-peroxidase conjugate specific for *E.histolytica* adhesin. If adhesin is present in the specimen, it binds to the conjugate and immobilized polyclonal antibody. The *E. histolytica* II test detects approximately 0.2 to 0.4 ng per well of adhesion.

According to the manufacturer's instructions, the test is intended for use on fresh fecal specimens without preservatives. However, in this study we used the test for the detection of adhesin in serum samples. Therefore, we have optimized several steps in the test procedure according to previous recommendations (Haque *et al.*, 2000). One drop (50 µl) of the conjugate was added to

the positive control well, negative control well and patient sample well. One drop (50 µl) of the positive control reagent was added to the positive control well and 100 µl of the negative control (i.e.diluent) to the negative control well. Patient's sera samples (100 µl/ sample) were transferred to the test wells, and then covered with adhesive plastic sheet, followed by incubation at room temperature for two hours.

After a washing step, two drops (100 µl) of substrate were added to each well and incubated at room temperature for 10 minutes. Then, one drop (50 µl) of stop solution was added to each well, and after two minutes, the optical density was read at 450 nm on a microplate ELISA reader. The results of the microplate ELISA reader was set to blank on the negative control well. The test was considered positive for adhesin if the optical density reading of the sample at 450 nm was 0.050 or higher.

Statistical analysis

Data entry and data analysis were done using Statistical Package for Social Science (SPSS) software version 12.0.1. Descriptive analysis was performed for demographic findings. Results were expressed as number and percentage for categorical variables whereas mean ± standard deviation (SD) and median (IQR) were used for numerical variables. Fisher's Exact Test and Pearson Chi-square were used to determine association between concurrent medical illness and IHA result, association between time of blood sampling and ELISA result and association between IHA with different antibody titer and ELISA. Kappa statistics and Fisher's Exact Test were applied to determine the agreement of ELISA technique used for assessing IHA and its significance respectively. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Of 58 cases of clinical or suspected ALA, 56 patients (96.6%) were males and two patients (3.4%) were females (male to female ratio was 28:1). Majority of the patients were adult, aged 20 to 59 years old. IHA positive (titer more than or equal to 1:256) results were observed in 42 (72.4%) patients and 16 (27.6%) patients were IHA negative (titer less than 1:256). Table 1 shows the distribution of IHA positive with different antibody titers among clinical or suspected ALA.

From the patients' hospital files, we identified 32 (55.2%) patients whose blood samples were collected within 48 hours of intravenous metronidazole administration. The association between ELISA results and time of blood sampling was not significant as shown in table 2 below.

Table 3 shows agreement between IHA and ELISA among the suspected ALA patients. There was one patient who was

Table 1. Distribution of IHA positive with different antibody titers among the suspected ALA patients (n = 58)

IHA titer	n	%
1:128 or less	16	27.6
1:256 to 1: 2048	29	50.0
1:4096 to 1:8192	11	19.0
1: 16 384	2	3.4

Table 2. Association between ELISA and time of blood sampling (n = 58) using Fisher's Exact test

	ELISA		
	negative	positive	P value
Blood sampling within 48 hours of treatment			
No	24 (92.1%)	2 (7.7%)	> 0.95
Yes	29 (90.6%)	3 (9.4%)	

Table 3. Agreement between IHA and ELISA among suspected ALA patients (n = 58)

	IHA		Kappa	P value
	negative	positive		
ELISA				
negative	15 (28.3%)	38 (71.7%)	0.019	0.691
positive	1 (20.0%)	4 (80.0%)		

Table 4. Association between ELISA and IHA of different antibody titer (n=58)

	IHA				P value
	1:128 or less	1:256 to 1: 2048	1:4096 to 1: 8192	1:16 384	
ELISA					
Negative	15 (28.3%)	26 (49.1%)	10 (18.9%)	2 (3.8%)	0.276
Positive	1 (20.0%)	3 (60.0%)	1 (20.0%)	0 (0.0%)	

positive by ELISA among the fifteen patients in the IHA negative group. Of 42 patients in IHA positive group, 38 patients were ELISA negative while only four patients had positive ELISA. The agreement between IHA and ELISA was poor when tested using Kappa statistic. Table 4 shows that the association between different antibody titers of IHA and ELISA was also not significant when tested using Pearson Chi-Square.

DISCUSSION

The diagnosis of ALA is sometimes difficult since its clinical manifestations are highly variable. In endemic areas, ALA should always be suspected in a patient with fever, weight loss, and right upper quadrant abdominal pain and tenderness. Imaging techniques such as ultrasonography, computerized tomography, and magnetic resonance imaging have excellent sensitivity for the detection of liver abscess arising from any cause but cannot distinguish amoebic abscesses from pyogenic abscesses or necrotic tumors. Once suspected clinically, ALA requires specific treatment without delay, thus this necessitates a reliable laboratory diagnostic test.

Serology tests have an important role in supporting the diagnosis of ALA. It is well recognized that in *E. histolytica* infection, the antibody response varies with the individual patient and the type of infection. The antibody response is greatest in ALA, less in intestinal amebiasis, and least in asymptomatic cyst passers (Hira *et al.*, 2001). The serum anti-amoebic antibodies become detectable at 7 to 10 days after the onset of symptoms. Such antibodies are present in up to 99% (Ravdin *et al.*, 1990) to 100% (Shetty *et al.*, 1988) of ALA patients and therefore become one of the definitive studies in the diagnosis of ALA.

The drawbacks of antibody detection are that the antibody may not be detectable in the early phase of the infection. According to one report, in patients with an acute presentation of less than seven days, serologic studies may be negative (Hira *et al.*, 2001). On the other hand, antibodies to *E. histolytica* are known to persist for many months following a resolution of acute ALA. Thus the presence of an antibody may or may not signify acute infection, hence reducing the value of serological diagnostic test among patients with acute disease.

Amoebiasis serology currently employed in our hospital is an antibody-based detection method by indirect haemagglutination assay (IHA, Dade Behring Diagnostics, Marburg, Germany) which has a high sensitivity and specificity. Because of difficulty in interpreting the result of antibody level in Kelantan, an amoebiasis endemic area, and to assist in serological diagnosis of ALA patients particularly in the early stage of the disease, we compared IHA method with a commercially available TechLab *E. histolytica* II ELISA test kit in diagnosis of ALA.

In this study, the cut off titer for positive antibody detection by IHA was 1:256. The IHA was found to be positive in 72.4% of patients (42/58) while 27.6% were IHA negative. Among IHA positive group, 50.0% (29 patients) had titer ranging from 1:256 to 1:2048, 19.0% (11 patients) were in the range of 1:4096 to 1:8192 and 3.4% (2 patients) had very strong antibody response indicated by titer of 1:16384. Among 42 patients in the IHA

positive group, only 4 patients were positive for antigen detection by TechLab *E. histolytica* II ELISA while another 38 patients had negative ELISA results. Among 15 patients in the IHA negative group, one patient was detected positive for ELISA. Thus in our study, only 5 patients had positive ELISA whereas 91.2% of patients were ELISA negative. The agreement between IHA and ELISA was measured using Kappa analysis which showed that the agreement (0.019) between the two tests was poor. There was no significant association between ELISA seropositivity and IHA of different antibody titers in this study. Thus, the presence of lectin antigens in the patients' sera did not correspond with the presence of antibody in ALA patients.

Therefore, the results of our study showed that the TechLab *E. histolytica* II test was not useful for detection of ALA. This is in contrast with the results reported by Haque *et al.* (2000) in which *E. histolytica* Gal/GalNAc lectin was detectable in the sera of 96% ALA patients in Dhaka, Bangladesh prior to treatment with metronidazole. In the study, ALA patients were divided into two groups; one group of patients had blood collection before metronidazole treatment and another group of patients had metronidazole treatment initiated from a few days to several weeks before collection of the blood samples (Haque *et al.*, 2000). The lectin antigen was detectable only in 15% of ALA patients after treatment with metronidazole and zero percent in controls. It was reported in the same study that after one week of treatment with metronidazole, 82% of patients became serum lectin antigen negative. In our study, among 58 serum specimens, 32 (55.2%) were collected and sent to our serology laboratory within 48 hours of intravenous metronidazole administration. Therefore, most of the patients were on treatment when the blood samples were collected. Among 32 blood samples that were taken within 48 hours of treatment, only 3 (9.4%) patients had positive ELISA test, while 90.6% were negative. Thus, there was no significant correlation between the antigen detection and time of blood sampling.

The effect of metronidazole in clearing antigenemia has been shown in an animal model (Thammapalerd *et al.*, 1996). According to the animal study, circulating amoebic antigens was determined by using a sandwich ELISA with specific monoclonal antibody in the sera of hamsters. They found that 100% of the hamsters were positive after the animals were infected 15 days onwards while reduction of antigenemia was shown by another group of infected hamsters in which metronidazole treatment was given. However, our study did not show any significant correlation between the results of the antigen detection test and time of blood sampling.

In developing countries where ALA is endemic, anti-amoebic drugs and antibiotics may be used indiscriminately, where patients might have seen doctors in private medical centers or clinics for initial treatment. Some patients even had self prescription of antibiotics by buying the medication over the counter. Similarly in this study, it was difficult to obtain an accurate treatment history and verification from all patients as to whether they have taken any medicine (medication name, dosage and duration) prior to the admission. This reason could partially explain the negative findings of the antigen test in our study. TechLab *E. histolytica* II ELISA has been employed in our laboratory for detection of *E. histolytica* in stool samples with good results (unpublished), thus strain variation is probably not a reason for the insensitivity of the test for diagnosis of ALA at HUSM.

The study by Haque *et al.* (2000) has shown that antigen detection test may become a promising method of detection in serum to diagnose ALA in future. The test was particularly useful for acute non-treated ALA patients who had not yet developed a significant detectable antibody. However, most of the patients in our study have already developed a significant level of antibodies in the serum as being detected by IHA method. Therefore, the formation of antigen-antibody complexes may have occurred resulting in negative ELISA because of reduced amount or undetectable level of antigen left in the circulation. This could be

another reasonable explanation for the findings in our study.

In conclusion, the results of this study showed that 72.4% (42/58) of the patients were positive by IHA, while only 8.6% (5/58) were positive for the lectin antigen by TechLab *E. histolytica* II ELISA. The statistical agreement between the IHA and ELISA tests was poor, and there was no significant correlation between ELISA results and IHA antibody titers. Thus, the presence of lectin antigens did not correspond with the presence of anti-*E. histolytica* antibody in the patients' sera. Therefore, this study demonstrated that TechLab *E. histolytica* II ELISA is not useful for serological diagnosis of ALA in our setting.

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