Original Article

Evaluation of diagnostic indices of lectin antigen and anti-lectin antibodies in amebic liver abscess

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ABSTRACT

Objective: Diagnostic indices of Gal/Gal NAc lectin antigen and anti-lectin antibodies for amebic liver abscess were evaluated to see their usefulness.

Methodology: Forty (40) clinically suspected cases of liver abscess patients admitted in the Rajshahi Medical College Hospital (RMCH), Bangladesh during January to December 2007 were included. Liver abscess pus from all cases were tested for small subunit of ribosomal RNA (rRNA) gene of *Entamoeba histolytica* by Real Time PCR and only PCR-positive cases were further analyzed for detection of Gal/Gal NAc lectin antigen and anti-lectin antibodies in their liver abscess aspirates, plasma, saliva and urine using Enzyme-linked immunosorbent assay (ELISA) methods. Except liver abscess pus, all other samples were also tested for 20 patients suffering from diseases other than liver abscess, who served as controls for the study.

Results: Out of 40 patients, 39 were PCR-positive and considered as confirmed cases of amebic liver abscess. The rate of detection of lectin antigen and anti-lectin antibody in liver abscess pus was 12.82% and 56.41% respectively. Diagnostic sensitivities of lectin antigen in plasma, saliva and urine were 15.38% (95%CI 6-31%), 07.69% (95%CI 2-22%) and 00% respectively, while sensitivities of anti-lectin antibodies in all those samples were 100% (95%CI 88-100%), 87.17% (95%CI 72-95%) and 56.41% (95%CI 40-78%) respectively. Diagnostic specificities of lectin antigen was 100% in all specimens but for anti-lectin antibodies, specificities were 100% (95%CI 88-100%) in plasma, 50% (95%CI 28-78%) in saliva and 70% (95%CI 46-87%) in urine. Overwhelming majority of cases (94.87%) received Metronidazole therapy for variable period before sample collection, which is correlated with low rate of antigen detection.

Conclusion: Detection of lectin antigen for amebic liver abscess has very limited or no role where Metronidazole is used indiscriminately but detection of anti-lectin antibodies especially in plasma (100% sensitivity) and saliva (87.17% sensitivity) are excellent to satisfactory. Estimation of plasma IgG can be recommended as serodiagnostic tool for symptomatic amebic liver abscess.

KEY WORDS: Amebic liver abscess, Lectin antigen, Anti-lectin antibodies, Diagnostic indices.

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INTRODUCTION

Amebic liver abscess (ALA) is caused by *Entamoeba histolytica* (*E. histolytica*), the same organism that causes intestinal amebiasis. Extraintestinal amebiasis is the result of invasive intestinal infection with *E. histolytica* and liver is the most frequent part of the body to be infected by the organism.[1] Historically amebic infection has been estimated to be present in approximately 10% of the world population and in up to 50% in tropical

and subtropical regions.[2,3] Hepatic amebiasis is a world wide disease with high incidence in tropical countries.[4] Approximately 70,000 deaths occur annually mainly due to ALA, amebiasis is the third most common cause of death from parasitic disease (after schistosomiasis and malaria) according to recent World Health Organization report.[5] Incidence of hepatic amebiasis in Bangladesh is still difficult to figure out due to small number of sample are analyzed completely.

Although sensitive and specific test methods for intestinal amebiasis have been developed in recent years, which can differentiate the true pathogen *E. histolytica* from the identical-appearing commensal *Entamoeba dispar*[6], however, the use of these techniques for diagnosis of amebic liver abscess is mostly unexplored. Imaging techniques like ultrasound, computed tomography and magnetic resonance although are highly sensitive to detect liver abscesses of varied etiologies, but fail to distinguish ALA from that of pyogenic liver abscess.[7] The demonstration of *E. histolytica* trophozoite in liver abscess pus by microscopy confirms the diagnosis of ALA, but in best of the laboratories, the amoebic trophozoites can be demonstrated in only 15% of the liver pus.[8]

Further, due to presence of the trophozoites mainly in the periphery of the abscess, culture of the liver pus is also unsatisfactory.[9] Currently, detection of small subunit of ribosomal RNA (rRNA) gene of *E. histolytica* in the liver abscess pus by polymerase chain reaction (PCR) in particular Real Time PCR is considered as gold standard in the diagnosis of ALA.[10-12] Although nucleic acid detection has very high diagnostic sensitivity, but necessity of sophistication in the instruments, costing and highly skilled manpower are currently the limiting factors. Detection of Gal/Gal NAc lectin antigen and/or anti-lectin IgG or IgA antibodies is gaining importance as immunodiagnostics for amebic liver abscess in recent years.[13,14] Serum IgG and secretory IgA antibodies to lectin antigen, which mediates adherence to and contact-dependent cytolysis to human colonic mucosa have been detected in patients with amebiasis.[15,16]

The aim of the present study was to evaluate the diagnostic indices of *E. histolytica* lectin antigen and anti-lectin IgG and IgA antibodies in different clinical samples of hepatic amebiasis patients using improved antigen and antibody detection kits to find out reliable immunological markers.

METHODOLOGY

Subjects: Forty (40) clinically suspected amebic liver abscess patients and 20 diseased controls (suffering from diseases other that liver abscess) admitted in the Rajshahi Medical College Hospital (RMCH), Bangladesh during January to December, 2007 were included. Clinical diagnosis of ALA was based on (i) a space-occupying lesion suggestive of abscess in the liver diagnosed by ultrasonography (ii) clinical symptoms (fever, pain in the right hypochondrium often referred to the epigastrium, lower chest, back, or tip of the right shoulder), (iii) enlarged and/or tender liver, with or without jaundice, (iv) raised right dome of the diaphragm on chest radiograph.[17] Informed written consent was obtained from the patients or legal guardians. The Ethical Review Committee of the Rajshahi Medical College, Bangladesh reviewed and approved the study design.

Samples: Liver abscess pus: Following aseptic precautions and under sonographic guidance about 14 ml of pus was aspirated from clinically suspected ALA patients for Real Time PCR and ELISA detection of lectin antigen and anti-lectin antibodies.

Blood: Five ml venous blood was collected using disposable syringe from each patient and control into a sterile EDTA tube. Plasma was separated by centrifugation at 4,000 × *g* for 10 minutes for ELISA detection of both lectin antigen and anti-lectin antibodies.

Urine: Approximately 12ml of urine was collected from each patient and control in a sterile Falcon tube for ELISA detection of both lectin antigen and anti-lectin antibodies.

Saliva: About 2ml of saliva were collected in a sterile Falcon tube from each patient and control for ELISA detection of both lectin antigen and anti-lectin antibodies.

***ELISA for lectin antigen*:** The TechLab *E. histolytica* II test (designed to detect specifically *E. histolytica* lectin antigen) was performed on the liver abscess pus, serum, saliva and urine according to the manufacturer's instructions. For detection of antigen, 100µl of undiluted serum, saliva or urine was added to the coated microtiter well. Liver abscess pus was vortexed and centrifuged at 10,000 × *g* for 10 min, and 100 µl of the resulting undiluted supernatant was added to the microtiter well. A test was considered positive when the optical density reading of a sample was >0.15 at 450 nm.[13]

***ELISA for anti-E. histolytica lectin antibodies*:** The antilectin IgG enzyme-linked immunosorbent assay

procedure was modified from the procedure of Ravdin and colleagues.[18] Ninety-six-well microtiter plates were coated with purified *E. histolytica* Gal/GalNAc lectin (TechLab). Test samples (plasma/saliva/urine) were added at a 1:1,000 dilution in 0.9% PBS-0.05% Tween 20 (final volume, 100µl) for two hours at room temperature. Known serum samples were used as positive and negative controls. Wells were washed five times with PBS-Tween 20. The plates were incubated for one hour with 100µl of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-human IgG with 1% bovine serum albumin. The wells were again washed five times, followed by the addition of tetramethylbenzidine substrate (final volume, 100µl). After 10 min, 1 N H_2SO_4 was used as the stop solution. The optical density of the microtiter wells was measured at 450 nm with an ELISA plate reader. A sample was considered positive if the optical density reading was >0.5, as determined in previous studies.[13,18]

DNA extraction: Liver abscess pus specimens (0.2g) were washed twice with sterile PBS and centrifuged for 5 min at 14,000 rpm. DNA was isolated from these specimens using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany). After extraction of DNA from liver abscesses, the concentration and purity of all DNA samples were measured by spectrophotometry at 260/280 nm. The purity of the nucleic acid was considered sufficient if the ratio of the optical densities at 260 versus 280 nm was between 1.5 and 1.8.

Real-Time PCR assay: The primers and probes for *E. histolytica* (accession no. X64142) were designed for the small subunit rRNA gene. The amplified target was 134 bp. Primers and TaqMan probes used in this study were purchased from Eurogentec (Seraing, Belgium). The *E. histolytica*-specific primers consisted of the forward primer (Eh-f), 5′-AAC AGT AAT AGT TTC TTT GGT TAG TAA AA-3′, and the reverse primer (Eh-r), 5′-CTT AGA ATG TCA TTT CTC AAT TCAT-3′. The TaqMan probe used in this assay was a double-labeled probe, YYT, 5′-ATT AGT ACA AAC TGG CCA ATT CAT TCA-3′ (Eclipse). A 0.4-µmol/liter concentration of each primer (Eh-f and Eh-r primers), 0.08 µmol/liter of Eh-YYT), and 3.0 µl of the extracted DNA were used in each reaction mixture. Amplification reactions were performed in a volume of 25 µl with Bio-Rad IQ supermix (100 mM KCl, 40 mM Tris-HCl [pH 8.4], 1.6 mM deoxynucleoside triphosphates, i*Taq* DNA polymerase [50 U/ml], 2 mM $MgCl_2$) with an additional 3 mM $MgCl_2$ added. Amplification consisted of 40 cycles of 3 min at 95°C, 30 s at 60°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with the iCycler real-time detection system (Bio-Rad). Fluorescence was measured during the annealing step of each cycle. The ramping of the machine was 3.3°C/second in every step. Fluorescence was measured at 530nm.[11,17]

RESULTS

There was overwhelming male preponderance, history of treatment with Metronidazole and typical clinical manifestations of liver abscess. About 60% of patients have had regular habit of indigenous alcohol consumption and around 20% had concomitant diarrhoea or dysentery and jaundice as presentations (Table-I).

Out of 40 clinically suspected ALA patients, 39 were found PCR positive and considered as confirmed cases of ALA. Rate of detection of lectin antigen and anti-lectin antibodies in different clinical samples is shown in Table-II. Antigen detection rate in liver abscess pus, plasma, urine and saliva was 12.82%, 15.38%, 00% and 7.69% respectively. None of the controls was positive for lectin antigen in any of their samples tested. Anti-lectin IgG antibody was found positive in 22(56.41%) cases of liver abscess pus and all 39(100%) plasma samples of patients. All controls were negative for plasma antibody but anti-lectin IgG antibody was detected in urine samples of 22(56.41%) patients and 06(30.00%) controls and also anti-lectin IgA antibody in saliva

Table-I: Characteristics of clinically suspected ALA patients (n=40).

Characteristics	*n (%)*
Gender	Male: 36 (90%); Female: 04 (10%)
History of Metronidazole therapy	37 (94.87%)
Fever, Anorexia, Malaise, Right hypochondriac pain	33 (84.61%)
History of indigenous alcohol consumption habit	23 (58.97%)
Dysentery/Diarrhea	08 (20.51%)
Jaundice	07(17.94%)

Table-II: Rate of detection of lectin antigen and anti-lectin antibodies.

Specimens	*Subjects*	*Lectin Antigen*		*Anti-lectin antibodies*	
		Positive	*Negative*	*Positive*	*Negative*
Liver abscess pus	Patients (n=39)	05 (12.82)	34 (87.17)	22 (56.41)	17 (34.59)
	Controls (n=20)	Not aspirated			
Plasma	Patient	06 (15.38)	33 (84.61)	39 (100)	00
	Controls	00	20 (100)	00	20 (100)
Urine	Patient	00	39 (100)	22 (56.41)	17 (43.59)
	Controls	00	20 (100)	06 (30.00)	14 (70.00)
Saliva	Patient	03 (07.69)	36 (92.30)	34 (87.17)	05 (12.82)
	Controls	00	20 (100)	10 (50.00)	10 (50.00)

Figures in the parentheses indicate percentage

was detected in 34(87.17%) patients and 10(50.00%) controls.

Diagnostic sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of lectin antigen and anti-lectin antibodies are shown in Table-III. Diagnostic sensitivity of plasma IgG was found excellent (100%) and also salivary IgA was satisfactory (87.17%). Specificity for lectin antigen detection was 100% in all samples is but in case of urine IgG and salivary IgA, it was 70% and 50% respectively.

DISCUSSION

The noninvasive diagnosis of amebic liver abscess is challenging, as most patients at the time of diagnosis do not have a concurrent intestinal infection with *E. histolytica*. Therefore, stool microscopy or antigen detection in stool samples is not helpful for diagnosis: less than 10% of patients have identifiable amebae in stool.[19]

Unsatisfactory lectin antigen detection rate in different clinical samples of ALA patients can be correlated with history of Metronidazole therapy before sample collection noted in overwhelming majority (94.87%) of our patients. Anti-amebic drug like Metronidazole affects seriously on the rate of detection of lectin antigen in ALA reported by several investigators.[13,20] Serum antigen detection is more sensitive than antibody for ALA prior to treatment with Metronidazole because, antigenemia does not persist after treatment. Parija and Khairnar (2007) found that the probability of E. histolytica antigen detection in liver abscess pus by ELISA was 12 times more in patients who had not received prior treatment with Metronidazole.[20] In developing countries like Bangladesh where amoebiasis is endemic and antiamoebic drugs are used indiscriminately, diagnosis of ALA by antigen detection is speculated to be of low sensitivity.

Diagnostic accuracy of anti-lectin antibodies in plasma (100%), saliva (74.57%) and urine (61.01%) has been found excellent, moderate and good respectively. Similar or slightly lower serum antibody detection rates have also been reported by other investigators.[13,14,18,20] This detection rate can be correlated with the fact that anti-amebic drug does not affect antibody production. Secretory antilectin IgA in the saliva of invasive amebic patients was reported by a few researchers[15,16,21] with variable sensitivity and specificity and our results are consistent with a few reports.

Detection of *E. histolytica* DNA in urine of ALA patients by a few investigators[17,20] encouraged us to investigate lectin antigen and antibody in urine but both sensitivity and specificity were found not very satisfactory. Although serum anti-lectin antibody detection stands with excellent sensitivity and specificity found in several studies but diagnostic specificity of anti-lectin antibodies in other specimens is yet to be established. A drawback of serologic tests

Table-III: Diagnostic indices of lectin antigen and anti-lectin antibodies.

Specimens	*Diagnostic indices*									
	Sensitivity		*Specificity*		*PPV*		*NPV*		*Diagnostic accuracy*	
	Ag	*Ab*	*Ag*	*Ab*	*Ag*	*Ab*	*Ag*	*Ab*	*Ag*	*Ab*
Plasma	15.38%	100%	100%	100%	100%	100%	37.73%	100%	44.06%	100%
Saliva	07.69%	87.17%	100%	50%	100%	77.27%	35.71%	66.67%	38.98%	74.57%
Urine	00%	56.41%	100%	70%	-	78.57%	-	45.16%	-	61.01%

is that the serum antibody levels in people from areas of endemicity remain positive for years after infection with *E. histolytica,*[22] therefore, its presence should be cautiously interpretated in asymptomatic cases.

In conclusion, we would like to emphasize that detection of anti-amebic antibody in serum in a symptomatic patient of ALA could be a dependable non-invasive test especially in developing countries where there is indiscriminate use of anti-amebic drugs, while detection of secretory salivary IgA could be an absolute non-invasive alternative but urine antibody test needs further evaluation.

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