In the name of God

S.U.M.S.

Shiraz E-Medical Journal
Vol. 8, No. 3, July 2007

http://semj.sums.ac.ir/vol8/jul2007/amoebiasis.htm

Evaluation of Enzyme Linked Immunosorbent Assay (ELISA) and Dot ELISA for Diagnosis of Amoebiasis.

Hatam Gh*, Khorami HR**, Sahebani N†, Sarkari B§.

* Associate Professor, Department of Parasitology and Mycology, Faculty of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, ** Instructor in Parasitology, Department of Laboratory Sciences, Faculty of Gerash, Gerash, Iran, † Assistant Professor, Department of Microbiology, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran, §Assistant Professor, Department of Immunology, Faculty of Medicine, Yasuj University of Medical Sciences, Yasuj Iran.

Correspondence: Dr Bahador Sarkari, Department of Immunology, Yasuj University of Medical Sciences, Yasuj, Iran, Tel: +98(741) 333-7225, Fax: +98(741) 333-7493, E mail: sarkarib@sums.ac.ir.

Received for Publication: March 1, 2007, Accepted for Publication: June 11, 2007.

Abstract:

Introduction: This study was conducted to evaluate the usefulness of dot enzyme-linked immunosorbent assay (dot ELISA) and plate ELISA, using monoxenically-grown Entamoeba histolytica soluble crude antigen, for detecting antiamoebic antibodies in serum samples from suspected amoebic patients and healthy controls.

Materials and Methods: Sera from 18 suspected amoebic patients, 15 healthy subjects and 13 patients with other parasitic diseases (leishmaniasis, hydatidosis, ascariasis and trichostrongyloidiasis) were examined for the presence of specific Entamoeba histolytica antibodies by ELISA and dot-ELISA. Both dot and plate ELISA detected antiamoebic antibodies in 15 (out of 18) suspected amoebic.

Results: From 15 healthy controls, two sera were found to be positive by ELISA and one of these two was also positive by dot-ELISA. In both assays, no cross reaction was found with the sera from other parasitic diseases including leishmaniasis, hydatidosis, ascariasis and strongyloidiasis. Sensitivity of the ELISA system was determined as 83.3% (95% CI: 57.7-95.6%) and the specificity was found to be 92.5% (95% CI: 74.2-98.7%). Positive and negative predictive values of the system were 88% and 89% respectively. On the other hand, sensitivity of the dot-ELISA was determined as 83.3% (95% CI: 57.7-95.6%) and the specificity was found to be 96.3% (95% CI: 79-99.8%). Positive and negative predictive values of dot ELISA were 93.7% and 89.6% respectively.

Conclusion: While the sensitivity of both assays were equal (83.3%), dot-ELISA was found to be more specific (96.3%) in detecting antiamoebic antibodies compared to Plate ELISA (92.5%). Seeing that the dot ELISA is simpler, rapid, inexpensive, equally sensitive and more specific as compared to plate ELISA, its use in serodiagnosis of amoebiasis can be recommended.

Key Words: Amoebiasis, diagnosis, ELISA, dot ELISA.

Introduction:

An estimated 40 000–100 000 people die yearly from amoebiasis, making this disease the second leading cause of death from parasitic diseases [1]. The causative protozoan parasite, *Entamoeba histolytica*, is a potent pathogen [1]. Infection usually begins with the ingestion of the cysts in food or water that has been contaminated by human feces [1]. The diagnosis of amoebic colitis rests on the demonstration of *E. histolytica* in the stool or colonic mucosa of patients [2]. The diagnosis of amebiasis by microscopic identification of the parasite in stool is insensitive and unable to distinguish the invasive parasite *Entamoeba histolytica*, which can cause invasive intestinal and extraintestinal disease from the commensal parasite *E. dispar* [3]. Since this method cannot distinguish between these two parasites, it is unsuitable as a screening technique for epidemiological studies, and probably for patient care. In view of the high frequency of *E. dispar* in many areas, dysentery due to entities such as shigellosis or campylobacter infection will probably be misdiagnosed as amoebic colitis if microscopy is the only diagnostic criterion [4]. Alternatives to microscopy diagnosis are ELISA assays, with different range of sensitivity and specificity, which identify *E. histolytica* antigens in stool, or antibody in serum. However, the reported sensitivity and specificity of these tests are still need to be evaluated with independent studies. Today the diagnosis of invasive amebiasis is most commonly attempted by a combination of stool examination and serological testing [4]. Serum antibodies to *E. histolytica* can be detected in 75 to 85% of patients with symptomatic *E. histolytica* infection [4].

Serological techniques that have been used so far for immunodiagnosis of amoebiasis are IHA, counterimmunoelectrophoresis (CIE), amoebic gel diffusion test, complement fixation (CF), indirect fluorescence assay (IFA) latex agglutination and ELISA [5-8]. ELISA is among the most popular methods used in diagnostic laboratories throughout the world and the most studied technique for diagnosis of amoebiasis [9-12]. The sensitivity of detection of specific antibodies to *E. histolytica* in serum is reported to be higher than 95%. Serum antibodies could be present within 1 week after the onset of symptoms of patients with amebic colitis. In a study including 100 patients with amebic colitis, anti-lectin IgM and anti-lectin IgG were measured by ELISA, and their sensitivities for the first week were found to be 45.1 and 5.6% respectively. They increased to 79.3 and 93.1%, respectively, for period longer than 1 week [13].

The present study aimed to evaluate the usefulness of enzyme-linked immunosorbent assay (ELISA) and dot- ELISA for serodiagnosis of amoebiasis, using an antigen purified from the axenically-grown *E. histolytica.*

Materials and Methods:

Serum Samples: Serum samples from suspected cases of amoebiasis (n=18)) were obtained from patients referred to different university affiliated hospitals. Serum specimens were also obtained from patients infected with other para-

sites along with control samples (n=15) from the healthy subjects.

Preparation of Antigen: The amebic antigen was prepared from characterized strain of *E. histolytica* in Rabinson medium. The protein content of each antigen was measured by a Bio-Rad protein assay. The antigen was kept at -20°C until use.

Enzyme Linked Immunosorbent Assay (ELISA): ELISA was carried out in flat-bottom 96 microplates (Nunc, Nunc International, Roskilde, Denmark). The plates were coated with 5 µg/ml of purified Entamoeba histolytica antigen (100 µl/well) in coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) and incubated at 4ºC overnight. Excess antigen was removed by washing the plate five times in phosphate buffered saline-Tween 20 (PBST, pH 7.4 containing 0.05% Tween 20). Blocking was done with 3% skimmed milk in PBST for 2 hours. The wells were washed and 100 µl of serum samples (1/100 dilution in PBST) from suspected amoebiasis patients along with samples from healthy individuals as negative controls and samples from patients infected with other parasites were applied to the plates and incubated for 1.5 hour. The plates were washed as before and 100 µl of horseradish peroxidase conjugated polyclonal antibody against human immunglobulin (Dako) at a 1/3000 dilution in PBST was added to the plate and incubated for 1 hour at room temperature. After being washed as before, the plate was incubated with chromogen/substrate (100 µl/well of 0.040% OPD, 0.025% H2O2 in 0.1 M citrate buffer, pH 5) and the reaction terminated with 1mM sulphuric acid after 30 min. The absorbance at 490 nm was monitored with a microplate reader (LX800, Biotek).

Dot ELISA: Dot-ELISA was performed as described by Yamaura et al., with some modification [14]. A nitrocellulose membrane (Watmann, pore size, 0.45µm) was divided into 5 x 5 mm square. 5 ml of 50 mg/ml of E. histolytica antigen was dotted onto each square and the membrane was left in room temperature to dry. Unbound sites on the membrane was blocked with 5% skimmed milk in Tris buffered saline with 0.05% Tween 20 pH 7.4 (TBST) with 1% bovine serum albumin (BSA) for 1 hour at 37 °C. After blocking, serum sample from the patients (1:100 dilution in TBST) along with samples from healthy controls and samples from patients infected with other parasites were added to the membrane and incubated for 1 hour at 37 °C. The membrane was then washed three times each 5 min with TBST. Peroxidase-conjugated anti-human immunoglobulin G (1:3000 dilution in TBST +1% BSA) was added to the membrane and the membrane was incubated for 30 min at 37 °C. The membrane was washed three times as before and bound conjugated antibody was detected by adding freshly prepared substrate (0.1% hydrogen peroxide and 1 mg/ml DAB, 3,3'diaminobenzidine; Sigma Chemical Co., in substrate buffer (50 mM Tris-HCl, pH 7.6) and the membrane was incubated until the band development was optimal (about 5 min). The membrane was then rinsed with distilled water to stop the reaction. Brownish spots indicated a positive reaction.

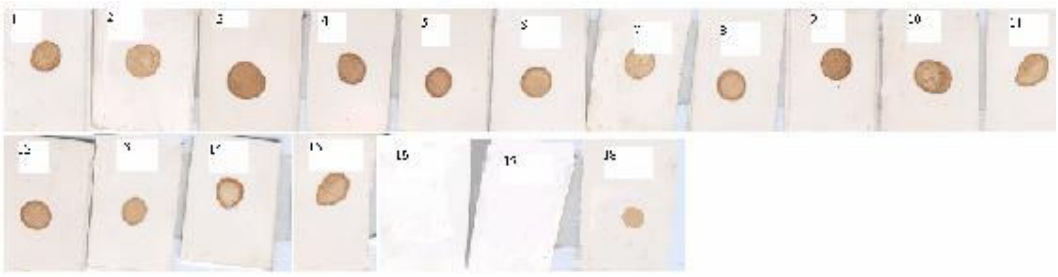
Results:

Serum samples obtained from 18 suspected cases of amoebiasis along with serum samples from patients with leishmaniasis (n=4), hydatidosis (n=3) ascariasis (n=3) and trichostrongyloidiasis (n=2) and samples from healthy individuals (n=15) were tested by ELISA and dot ELISA using antigen from axenically-grown *E. histolytica* trephozoites. The cut off point was set as 2SD above the mean of control samples for ELISA and brownish spots was considered as positive reaction for dot ELISA. Results of this study showed that 15 (out of 18) samples from suspected amoebiasis patients were positive by the ELISA and dot ELISA systems. It was found that 2 (out of 15) sera samples of healthy individual have a positive reaction with the antigen in ELISA system where only one of these samples was positive by dot ELISA assay. Hence, sensitivity of the ELISA system was determined as 83.3% (95% CI: 57.7-95.6%) and the specificity was found to be 92.5% (95% CI: 74.2-98.7%). Accordingly, positive and negative predictive values of the system were 88% and 89% respectively. No cross reaction was found with the sera from leishmaniasis, hydatidosis, ascariasis and trichostrongyloidiasis patients.

For dot-ELISA, in 15 sera from suspected amebiasis patients brownish spots were observed against diluted serum and also positive controls as shown in Fig. 1. Nonspecific reactions with the *E. histolytica* antigen were examined using sera from patients infected with parasites other than *E. histolytica* and healthy subjects. No positive spot against *E. histolytica* antigen was recognized in sera from other parasitic diseases but a spot was observed in a serum from one healthy subject.

Considering the above findings, sensitivity of the dot-ELISA was determined as 83.3% (95% CI: 57.7-95.6%) and the specificity was found to be 96.3% (95% CI: 79-99.8%). Therefore positive and negative predictive values of the assay were 93.7% and 89.6% respectively. While the sensitivity of both assays were equal (83.3%), dot-ELISA was found to be more specific (96.3%) in detecting antiamoebic antibodies compared to plate ELISA (92.5%).

Fig. 1 Results of dot-enzyme-linked immunosorbent assay (dot-ELISA) for serum samples form patients with intestinal amoebiasis (1-15: serum samples from amoebiasis patients, 16-18: control samples).



Discussion:

Diagnosis of amoebiasis is still a problem in many clinical laboratories. Microscopic diagnosis is unable to differentiate *E. histolytica* from E. *dispar* and its sensitivity is no higher than 60%. Moreover false-positive results due to misidentification of macrophages and nonpathogenic species of Entamoeba is another drawback of microscopical diagnosis [4]. Of all children with diarrhea diagnosed with amoebiasis by microscopy, only 40% were proven to have *E. histolytica* infection when specific methods (antigen detection and culture-isoenzyme analysis) were used, and of all children diagnosed with *E. histolytica* infection by specific methods, the majority was missed by microscopy [4]. Serological diagnosis seems to be a suitable alternative for diagnosis of amoebiasis. Several serological assays have been used for the diagnosis of amoebiasis and among them ELISA either for antibody detection or antigen detection has received the most attention [9-12]. The present study was conducted to evaluate the usefulness of dot enzyme-linked immunosorbent assay (dot ELISA) and plate ELISA, using axenically-grown *Entamoeba histolytica* soluble crude antigen, for detecting antiamoebic antibodies in serum samples from suspected amoebic patients and healthy controls. While the sensitivity of both assays were equal, dot-ELISA was found to be more specific in detecting antiamoebic antibodies compared to Plate ELISA.

The antibody titer measured by plate ELISA did not correlate with the intensity of the spots obtained by dot-ELISA in all cases and this has been reported in Haghighi study as well [10]. The dot ELISA technique has been used for serological diagnosis of many parasitic diseases and has several advantages over Plate ELISA [15-18].The results can be read with the naked eye and the antigen-dotted nitrocellulose membrane strips can be stored for 3 years at 4°C as demonstrated in Yamasaki and Araki study [14]. It has been also reported that antigen-dotted nitrocellulose membranes stored at room temperature is also stable for up to 3 months at room temperature or 37°C.

In the present study, the sensitivity of dot-ELISA was same as those of plate ELISA, but dot-ELISA was more specific that plate ELISA in detecting anti *E. histolytica* antibody since its specificity was found to be higher than plate ELISA. Considering the above mention benefits of this assay, it can be concluded that dot-ELISA is more appropriate than plate ELISA for the serological diagnosis of amoebiasis. Our finding is consistent with those reported by Gandi et al, where they employed dot ELISA for the diagnosis of amoebiasis [8]. They found that dot ELISA was positive in 93% of cases of amoebic liver abscess, 3% of healthy controls and none of the cyst passers. They reported 97% sensitivity and 93% specificity for the assay.

Taken together since dot ELISA had a higher specificity in comparison with Plate ELISA and sensitivity as high as plate ELISA and since it is a simple, non expensive and reliable test, its use can be recommended, as an appropriate serological test, for the diagnosis of amoebiasis.

Acknowledgment:

This study was finically supported by a grant from Vice Chancellor for Research, Shiraz University of Medical Sciences, Shiraz, Iran.

References:

1. Stanley SL: Amoebiasis. Lancet. 2003; 22; 361(9362):1025-34.

2. Stauffer W, Ravdin JI: Entamoeba histolytica: an update. Curr Opin Infect Dis 2003;16(5):479-85.

3. Nesbitt RA, Mosha FW, Katki HA, Ashraf M, Assenga C, Lee CM: Amebiasis and comparison of microscopy to ELISA technique in detection of Entamoeba histolytica and Entamoeba dispar. J Natl Med Assoc 2004; 96(5):671-7.

4. Tanyuksel M, Petri WA Jr: Laboratory diagnosis of amebiasis. Clin Microbiol Rev 2003;16(4):713-29.

5. Van Doorn HR, Hofwegen H, Koelewijn R, Gilis H, et al. Use of rapid dipstick and latex agglutination tests and enzyme-linked immunosorbent assay for serodiagnosis of amebic liver abscess, amebic Colitis, and Entamoeba histolytica Cyst Passage. J Clin Microbiol 2005; 43(9):4801-6.

6. Youssef FG, Mansour NS: Evaluation of enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination (IHA), counterimmunoelectrophoresis (CIEP), and immunodiffusion (ID) in the serodiagnosis of amebiasis. J Egypt Soc Parasitol 1988;18(2):373-81.

7. Baveja UK, Roy T, Kaur M, Kulpati DD, Aggarwal SK: Dot immunobinding assay versus sandwich ELISA in diagnosis of invasive amoebiasis. Indian J Med Res 1991; 93:161-5.

8. Gandhi BM: A dot immunobinding assay (DIA) on nitrocellulose membrane for the serological detection of antibodies to Entamoeba histolytica. Trans R Soc Trop Med Hyg 1986; 80(6):883-5.

9. Knappik M, Borner U, Jelinek T: Sensitivity and specificity of a new commercial enzyme-linked immunoassay kit for detecting Entamoeba histolytica IgG antibodies in serum samples. Eur J Clin Microbiol Infect Dis 2005; 24(10):701-3.

10. Haghighi A, Rezaeian M: Detection of serum antibody to Entameba histolytica in various population samples of amebic infection using an enzyme-linked immunosorbent assay. Parasitol Res 2005; 97(3):209-12.

11. Bernal R, Martinez LG, Zepeda B, Hernandez G, Baer GM: Determination of Entamoeba histolytica and Entamoeba dispar infection by enzyme-linked immunosorbent assay (ELISA) and its clinical correlation in pediatric patients. Arch Med Res 2000; 31:S55-6.

12. Delialioglu N, Aslan G, Sozen M, Babur C, Kanik A, Emekdas G: Detection of Entamoeba histolytica/Entamoeba dispar in stool specimens by using enzyme-linked immunosorbent assay. Mem Inst Oswaldo Cruz 2004; 99(7):769-72.

13. Abd-Alla, M. D., T. G. Jackson, and J. I. Ravdin: Serum IgM antibody response to the galactose-inhibitable adherence lectin of Entamoeba histolytica. Am J Trop Med Hyg 1998; 59:431-434.

14. Yamaura H, Araki K, Kikuchi K, Itoda I, Totsuka K, Kobayakawa T: Evaluation of dot-ELISA for serological diagnosis of amebiasis. J Infect Chemother 2003; 9(1):25-9.

15. Pappas MG, Hajkowski R, Hockmeyer WT: Dot enzyme-linked imunosorbent assay (dot-ELISA): a micro technique for the rapid diagnosis of visceral leishmaniasis. J Immunol Methods 1983; 64:205–14.

16. Araujo FG: A method for demonstration of antibodies to Trypanosoma cruzi by using antigen-coated nitrocellulose paper strips. Am J Trop Med Hyg 1985; 34:242–5.

17. Boctor FN, Stek MJ Jr, Peter JB, Kamal R: Simplification and standardization of dot-ELISA for human Schistosomiasis mansoni. J Parasitol 1987; 73:589–92.

18. Kraoul L, Adjmi H, Lavarde V, Pays JF, Tourte-Schaefer C, Hennequin C: Evaluation of a rapid enzyme immunoassay for diagnosis of hepatic amoebiasis. J Clin Microbiol 1997; 35(6):1530-2.

† Very sadly, Dr Sahebani passed away shortly before the preparation of this manuscript.