Anti-Amebic Antibody Activity in Patients, Determined with Antigens Prepared from Virulent Parasites (Indirect Hemagglutination Assay and Enzyme-Linked Immunosorbent Assay)

Eitan Israeli MMed.Sc[1], Batya Talis PhD[3], Nehama Peled MSc[2], Rachella Snier[1] and Joseph El-On PhD[1]

[1]Department of Microbiology and Immunology and [2]Clinical Microbiology Laboratory, Soroka University Medical Center and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel
[3]Zamenhoff Central Laboratories, Tel Aviv, Israel

Key words: E*ntamoeba histolytica*, axenic cultures, xenic cultures, indirect hemagglutination assay, enzyme-linked immunosorbent assay

Abstract

Background: The serology of amebiasis is affected by low sensitivity and specificity.

Objectives: To evaluate the advantage of the indirect hemagglutination assay and enzyme-linked immunosorbent assay in the diagnosis of amebiasis, using E*ntamoeba histolytica* soluble antigen (macerated amebic antigens) prepared from four different virulent isolates, continuously cultivated in the presence of the original enteric bacteria.

Methods: Using IHA and ELISA with MAA antigen we examined 147 sera samples from patients with gastrointestinal symptoms, and 11 sera from amebiasis cases (confirmed by microscopy and copro-antigen ELISA).

Results: Of 104 of the 147 (70.7%) symptomatic cases that were amebiasis positive by IHA, 81 (55.1%) were positive by MAA-ELISA. In addition, of 11 amebiasis cases confirmed by microscopy and copro-antigen ELISA, 7 (64%) were amebiasis positive by both tests. Four species of bacteria were isolated from the ameba cultures: *Escherichia coli, Morganella morganii, Proteus mirabilis*, and *Streptococcus lactis*. Elimination of the bacteria from the cultures by an antibiotics cocktail containing gentamicin, imipenem, piperacillin-tazobactam and vancomycin was the preferred method. Absorption of patients' sera to bacterial antigen prior to serological analysis had only a marginal effect.

Conclusions: These results indicate a correlation of 61% between the ELISA developed in this study and the IHA tests in the diagnosis of amebiasis.

IMAJ 2007;9:663–667

Amebiasis caused by E*ntamoeba histolytica* is a protozoan disease affecting approximately 12% of the world population; 50% of cases inhabit tropical and subtropical regions [1]. In most cases (~90%) the disease is asymptomatic. Symptomatic cases may suffer from either intestinal or extra-intestinal infections, manifested as local tissue destruction by the ameba trophozoites. In endemic areas, prevalence of the disease may be very high, affecting approximately 55% of the population, with 4% of school-age children suffering from amebic colitis [2]. During the 1950s the disease affected 0.6–16% of the Israeli population [3]. Today, the intestinal disease is less prevalent, and extra-intestinal infections are very rare, probably due to improvement in nutrition and hygienic conditions and the availability of effective treatment [4].

In most cases, the diagnosis of amebiasis is based on microscopic detection of the parasite in both fresh stool samples and in culture at 37°C. However, due to morphological similarities between E. *histolytica* and the non-pathogenic E. *dispar* these tests may be misleading [5]. During the last decade, copro-antigen ELISA tests were further developed for routine diagnosis of the disease. One of them (TechLab, Blacksburg, VA, USA), which is based on the detection of the amebic Gal/GalNAc lectin, allows a differentiation between E. *histolytica* and E. *dispar* with a sensitivity and specificity of 87% and > 90%, respectively [6].

Amebic infection induces a specific humoral immune response in approximately 90% of patients suffering from invasive disease [7]. However, routine serological examinations are only partially reliable since they are limited to invasive extra-intestinal infection and cannot differentiate between past and current infections [8]. It is for this reason that several tests (microscopy, copro-antigen ELISA, serology) are generally used jointly for the detection of the disease. Several commercial kits are currently available for serological examination. Both indirect hemagglutination assay (Behring Diagnostic, Marburg, Germany and Fumouze Diagnostic, Levallois-Perret, France) and enzyme-linked immunosorbent assay (LMD Laboratories Inc., Carlsbad California, USA; Melotec, S.A, Barcelona, Spain; NovaTec Immundiagnostica GmbH, Dietzenbach, Germany and R-biopharm, Darmstadt, Germany) are limited to extra-intestinal amebiasis, with moderate sensitivity and specificity, and are very expensive. The polymerase chain reaction technique was found to be similarly sensitive and specific for the diagnosis and differentiation of E. *histolytica* and E. *dispar* [9].

To date, most of the commercially available serological kits use amebic antigens (crude material, Gal/GalNac lectin) prepared from axenic E. *histolytica* cultures [10]. The interaction of E. *histolytica* with specific bacteria was shown to affect the morphology, virulence and probably the antigenicity [11]. However, no such difference was demonstrated by other researchers [12,13]. The present study examines the usefulness of E. *histolytica* soluble antigen, prepared from four different isolates continuously cultivated in the presence of the original enteric bacteria in the diagnosis of the disease, using IHA and ELISA.

IHA = indirect hemagglutination assay
ELISA = enzyme-linked immunosorbent assay
MAA = macerated amoebic antigens

Patients and Methods

Patient sera

A total of 158 sera samples from symptomatic cases were collected: 147 at the Zamenhoff Central Laboratories (Tel Aviv, Israel) and 11 at the Soroka University Medical Center (Beer Sheva). We also examined control sera from five healthy people and eight sera samples from patients with other diseases (toxoplasmosis in six patients, H*ymenolepis nana in* one and C*ampylobacter* spp. in one). In the first group (147 cases) the disease was confirmed by an IHA test currently used at the Zamenhoff Central Laboratories for the diagnosis of the disease [14], and in the other groups (11 cases) by both microscopic stool examination with copro-antigen ELISA (TechLab) and serology using an ELISA commercial kit (R-biopharm).

Preparation of amebic antigens

Four virulent E. *histolytica* isolates (Y, 785, 1456 and 2118 strains) [14] were collected from symptomatic amebiasis patients suffering from severe amebic dysentery. The isolated parasites together with the accompanied enteric bacteria were grown in Boeck and Drbohlav diphasic culture medium at 37°C [9]. Subcultures were made every 3 days. The parasites were characterized by hematoxylin/eosin staining and copro-antigen ELISA (TechLab), and virulence was further confirmed by the ability to engulf sheep red blood cells [15]. In addition, an axenic culture of E. *histolytica* (HM1 strain) (kindly supplied by Prof. D. Gold, Tel Aviv University, Israel) was continuously grown in TYI-S-33 Diamond's medium [9] and sub-cultured every 3 days. Antigen prepared from this parasite was further used in the serological tests.

Homogenate of E. *histolytica* from xenic culture was prepared as described previously [14]. Briefly, trophozoites of each isolate (Y, 785, 1456, 2118) were cultivated separately in Boeck and Drbohlav's medium, in 10 ml glass tubes containing 3 ml of solid phase and 2 ml of liquid phase at 37°C. On the third day of cultivation, the culture of each strain was filtered through double gauze and washed twice with phosphate-buffered saline (pH 7.2). The filtrate was centrifuged for 10 minutes at 200 x g at room temperature, the supernatant was removed and the pellet was resuspended in PBS and centrifuged again as described above. The pellet that contained only a minimal number of the accompanied bacteria was resuspended in a small amount of PBS and exposed to three cycles of freezing and thawing, followed by three cycles (10 seconds each) of sonication (Misonix model XL-2015, Heat System Inc.; power setting "3"). Thiomersal (1%) was added to the homogenate. Parasite homogenates (1 mg/ml) from the four isolated strains were mixed (vol/vol) macerated amebic antigens and stored at 4°C until used. A homogenate from the axenic culture of the HM1 strain (HMA) was further prepared. Trophozoites were collected on the third day of cultivation, washed three times with PBS (pH 7.2), and centrifuged at 11,000 x g for 30 min at 4°C. The last pellet was resuspended in a minimal amount of PBS and exposed to freezing and thawing, followed by sonication and centrifugation as above. The supernatant was collected, and after the addition of 5 mM N-N-L-3-transcarboxirane-2-carbonyl-L-leucyl-agmatine (E-64, Sigma), it was kept at -70°C until used. Before each test, the homogenates were centrifuged at 11,000 x g for 30 min at 4°C, and the soluble fractions were collected and used for the serological studies. Protein concentration of each antigen was determined according to the Bradford method as described in the manufacture's instructions (BioRad, Rehovot, Israel).

PBS = phosphate-buffered saline

Isolation and characterization of bacteria from xenic amebic cultures

Samples of amebic culture containing enteric bacteria were first cultivated overnight in thioglycollate medium (Hylabs, Rehovot, Israel), at 37°C. Following Gram staining, the bacteria were planted on blood and MacConkey agars (Hylabs) and cultivated under the same conditions. A single colony of each type was collected and further cultivated in differential substrates, including Enterotest, Simmons citrate and Aesculin bile agars (Hylabs). Both the enzymatic test, API 20 strep (bioMerieux, Missouri, USA), and bacterial characterization were performed according to the manufacturer's instructions.

Resistance and susceptibility of isolated bacteria to antibiotics

In order to eliminate the bacteria from the ameba xenic culture, the susceptibility of the isolated bacteria to antibiotics was tested by the Kirby-Bauer disk diffusion method. Seventeen different types of antibiotics were used including: ampicillin, cefuroxime, ceftriaxone, ceftazidime, gentamicin, tetracycline, trimethoprim/sulfamethoxazole (Resprim®), amoxicillin/clavulanate (Augmentin®), mezlocillin, amikacin, tobramycin, ciprofloxacin, aztreonam (Azactam®), imipenem, piperacillin-tazobactam (Tazocin®) and vancomycin.

Preparation of bacterial antigens

A suspension of whole intact bacteria and their homogenates was prepared. Each isolated bacteria colony was first cultivated overnight in Luria-Bertani medium at 37°C. The culture was centrifuged (10 min at 5000 x g) and the pellet was washed twice in PBS and finally resuspended in PBS and used either as whole intact undisrupted bacterium or as a whole bacterial homogenate. The homogenate was prepared similarly to the amebic antigen and stored at -20°C until used.

Absorption of sera to bacterial antigens

In certain cases, sera (100 µl) were absorbed to either whole intact or a bacterial dry homogenate (300 µg) prior to their serological examination. Serum was mixed (vol/vol) with the bacteria preparation. After 1 hour incubation at 37°C followed by overnight incubation at 4°C, the mixture was centrifuged for 30 min at 11,000 x g and the supernatant was collected and tested for anti-amebic antibody activity.

Diagnostic procedures

- **Microscopic examinations**

Stool samples and ameba cultures were examined microscopically as a wet smear and after hematoxylin and lugol staining [9].

Virulence of the parasites was determined by erythrophagocytosis of fresh sheep red blood cells [15].

- **Serological examinations**

Indirect hemagglutination: The IHA procedure was performed as described by Stein et al. [16]. MAA, at 1 mg/ml in PBS (pH 6.4) was used for coating fresh sheep red blood cells. Formalized and tanned RBCs were mixed vol/vol. with MAA (5 µg/ml in PBS). Coated RBCs were finally resuspended to 2.5% in a bovine serum albumin buffer [0.125% BSA (Merck) made in PBS, pH 7.2]. Thiomersal (1:10,000 v/v) was added to the RBC suspension that was kept at 4°C (up to 30 days) until used for the IHA test. The test was performed in glass tubes, each containing 500 µl of patient's serum diluted 1:40 in BSA buffer and 50 µl of coated RBCs. Results were recorded after 24 hours incubation at room temperature.

ELISA: Coating with MAA was carried out in an eight-well flat bottomed microtiter strip with "high binding capacity" (Maxisorp plates, Nunc, Roskilde, Denmark). To each well, 100 µl of either MAA or HMA (250 µg) antigen in bicarbonate buffer (pH 9.6) was added. After incubation overnight at 4°C, the wells were washed three times with phosphate buffer, pH 7.4, containing 0.3% Tween-80 (Sigma). The wells were blocked with 100 µl of 5% milk (Ault foods, Ontario, Canada) in PBST, and after additional three washings with PBST, 100 µl of serum diluted 1:100 in PBST were added. After incubation for 1 hour at 37°C and three washings with PBST, 100 µl of goat anti-human polyvalent immunoglobulin (IgA, IgM) conjugate (1:2,000) (Sigma) was added to each well. After an additional incubation for an hour at 37°C, the wells were washed three times with PBST and 100 µl of the substrate, phenylenediamine-dihydrochloride (Sigma) (8 mg in 15 ml citric acid pH 5, and 10 µl 10% H_2O_2) was added. The reaction was arrested by 2 M sulfuric acid and the absorbance was determined at 450 nm.

Results

Isolation and characterization of enteric bacteria

Four species of bacteria were isolated from the E. *histolytica* cultures, including E*scherichia coli*, M*organella morganii*, P*roteus mirabilis* and S*treptococcus lactis*. The first three bacteria were highly susceptible to a mixture containing gentamicin (25 µg/ml), imipenem (1.5 µg/ml) and piperacillin-tazobactam (25 µg/ml), and S. *lactis* was susceptible to a similar cocktail combined with vancomycin (5000 µg/ml). Although these cocktails eliminated all the bacteria from the cultures, they only allowed a limited growth of the parasite. However, the ameba developed very well in monoxenic cultures containing either S. *lactis* (monoS*l*) or E. *coli* (monoE*c*). In addition, partial purification of the parasites was obtained by a differential centrifugation and by a fractionation on a Urografin stepwise gradient [17]. Centrifugation of the ameba culture for 15 min at 1000 x g on a 20/40% (5/5 ml) stepwise gradient removed most of the bacteria from the ameba fraction at a concentration of 40%.

RBCs = red blood cells
BSA = bovine serum albumin
PBST = PBS Tween 20

Anti-amebic antibody activity determined by ELISA and IHA

In the group of 147 cases with gastrointestinal symptoms, 104 sera samples (70.7%) were IHA positive (titer ≥ 800) vs. 81 (55.1%) positive in the MAA-ELISA [Table 1]. Also, in this group of 66 patients who were highly positive (titer ≥ 3200) by the IHA test, 39 (59%) were positive by the MAA-ELISA test [11 (17%) highly positive and 27 (41%) moderately positive]. In addition, in the IHA-moderately positive (800–1600) group of 38 patients, 25 (65.7%) were positive in the MAA-ELISA test [10 (26%) highly positive and 15 (40%) moderately positive]. In the 43 IHA-negative group (titer < 800), 26 (60%) were negative, 11 (26%) were highly positive and 6 (14%) were moderately positive in the MAA-ELISA test [Table 1]. These results indicate a correlation of 61% between the IHA and the ELISA tests, and 71% sensitivity and 60% specificity of the ELISA test.

In the group of 11 amebiasis patients, confirmed by microscopy and copro-antigen ELISA, 7 (63.6%) were positive by both IHA and MAA-ELISA, 6 (54.5%) by HMA-ELISA, and only 2 (18.2%) by the ELISA commercial kit [Table 2]. In addition, of the five healthy individuals who were negative by microscopic examination, copro-antigen ELISA and ELISA commercial kit, two were moderately positive by the MAA-ELISA and three by the HMA-ELISA tests [Table 3]. Also, of the eight patients suffering from various diseases (toxoplasmosis in six patients, H*ymenolepis nana* in one, C*ampylobacter* sp. in one), four toxoplasmosis patients were moderately positive by MAA-ELISA.

Anti-amebic antibody activity of absorbed sera determined by ELISA

Absorption of serologically positive patient sera to either whole intact or bacterial homogenate reduced the non-specific activity

Table 1. Anti-E*ntamoeba histolytica* antibody activity in 147 sera samples from patients with gastrointestinal symptoms, determined by IHA and ELISA, using xenic culture antigen (MAA)

	IHA		
ELISA	**Positive (%)**	**Negative (%)**	**Total (%)**
Positive	64 (43.6)	17 (11.5)	81(55.1)
Negative	40 (27.2)	26 (17.7)	66 (44.9)
Total	104 (70.8)	43 (29.2)	147 (100)

Table 2. Anti-amebic antibody activity in sera of 11 amebiasis patients (confirmed by microscopy and copro-antigen ELISA), determined by ELISA using xenic (MAA) and axenic (HMA) amebic antigens

	Microscopy		Commercial kits		IHA	ELISA	
	Wet smear and H&E staining	Culture	Copro-antigen (TechLab)	ELISA (R-biopharm)	MAA	MAA	HMA
Total positive	11/11	11/11	11/11	2/11	7/11	7/11	6/11
(%) positive	100	100	100	18.2	63.6	63.6	54.5

Table 3. Anti-amebic antibody activity in sera from 11 amebiasis patients (confirmed by microscopy and copro-antigen ELISA), before and after absorption to homologous bacterial antigens, determined by ELISA using polyxenic (MAA), monoxenic (E.*coli*-mono*Ec* and S.*lactis*-mono*Sl*) and axenic (HMA) cultures as antigens

Group	No. of cases	Sera absorption	ELISA-positive				Microscopic examination		Copro-antigen (Techlab)	ELISA (R-biopharm)
			MAA	monoSl	monoEc	HMA	Wet smear	Culture		
Infected	11	–	7/11	7/11	7/11	6/11	11/11	11/11	11/11	2/11
		+	7/11	7/11	7/11	6/11				
Control	5	–	5/2	4/5	3/5	5/3	5/0	5/0	0/5	0/5
		+	5/2	2/5	2/5	5/3				

by 75% and 90%, respectively. However, following absorption, these sera reacted similarly with the polyxenic (MAA) and the monoxenic homologous (monoE*c*, monoS*l*) antigens [Table 3]. Furthermore, in the amebiasis-infected group, confirmed by microscopy and copro-antigen ELISA, no difference between absorbed and non-absorbed sera against both the MAA and the HMA antigens was demonstrated by ELISA.

Discussion

In Israel, the IHA test using MAA antigen is considered the test of choice for the diagnosis of both intestinal and extra-intestinal amebiasis [14]. The MAA antigen was prepared from a mixture of four virulent E. *histolytica* isolates collected from different symptomatic amebiasis patients and grown in xenic cultures in the presence of the original intestinal bacteria. In this study, an ELISA test using a similar antigen was developed and its usefulness in the diagnosis of the disease was evaluated. Four species of bacteria were isolated from the ameba cultures, including: E. *coli*, M. *morganii*, P. *mirabilis* and S. *lactis*. A total elimination of the bacteria was achieved, using a cocktail of: gentamicin, imipenem and piperacillin-tazobactam, either alone against E. *coli*, M. *morganii* and P. *mirabilis*, or combined with vancomycin against S. *lactis*. However, the purified parasites grew very slowly in the axenic culture, and a supplement of either E. *coli or* S. *lactis* (monoxenic cultures) was required to achieve a continuous growth of the parasites.

Serological examinations of both intestinal and extra-intestinal amebiasis continue to be characterized by low sensitivity and specificity [18]. Several serological tests are currently commercially available for the diagnosis of invasive amebiasis. However, in all cases the antigen used was prepared from axenic cultures of the parasites. A total homogenate [19], partially purified antigens [20] and recombinant antigens [21] have been used as antigens for the diagnosis of the amebiasis. In the present study, the MAA-ELISA test developed was only partially successful as a diagnostic tool. In the group of 147 patients suffering from gastrointestinal symptoms, a correlation of 61% was observed between the IHA and the ELISA tests, indicating 71% sensitivity and 60% specificity of the ELISA test. In the group of 11 amebiasis patients confirmed by microscopy and copro-antigen ELISA, 54% (6 patients) and 64% (7 patients) were positive by HMA-ELISA and MAA-ELISA, respectively. These results are in contrast to those reported by Kraoul et al. [22] and Sánchez-Guillén et al. [23], who found a higher correlation (> 95%) between the two tests. These differences might be the result of different techniques, antigens, and secondary conjugated antibodies used in these specific studies.

It was previously suggested that amebic antigens prepared from xenic E. *histolytica* culture might be superior to that prepared from axenic culture in the serological diagnosis of the disease [24]. Further studies demonstrated the presence of a 30 kDa antigen only in the xenic strain [25], while others did not report any difference between the two isolates [12,13]. In the present study, only a marginal difference between axenic, monoxenic and polyxenic antigens in the ELISA test was demonstrated. This may be due to the number of isolates used in each antigen preparation, four in the MAA vs. only one in the HMA antigen, rather than a difference in their virulence.

Amebiasis is still considered an important disease, causing morbidity and mortality in humans worldwide. The present study was an attempt to develop an ELISA test, using antigen prepared from virulent E. *histolytica* isolates growing in xenic culture (MAA) for the diagnosis of the disease. The development of an accurate test for the diagnosis of amebiasis before and after treatment is still required. Further studies using highly purified and specific amebic antigens from xenic E. *histolytica* cultures are necessary for determining any superiority of these antigens in the diagnosis of the disease.

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Correspondence: Dr. J. El-On, Dept. of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer Sheva 84105, Israel.
Phone: (972-8) 640-0899
Fax: (972-8) 647-7626
email: jelon@bgu.ac.il