Immune-detection of *Entamoeba histolytica* in symptomatic and asymptomatic infection

Fayez Muhammad Shaldoum[1], NahlaMezeid[2] and Adnan I. Al-Hindi[3]

[1]Department of Zoology, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt
[2]Department of Zoology, Faculty of Science (Girls), Al-Azhar University, Cairo, Egypt
[3]Department of Zoology, Faculty of Science, Islamic University, Gaza, Palestine
fshaldoum@gmail.com

Abstract: High prevalence of intestinal amoebiasis is commonly reported by microscopy in Gaza.To determine the misdiagnosis of intestinal amoebiasis associated with microscopic examination of faces, two tests were applied: 1-a non-specific antigen *Entamoeba* (ELISA) to differentiate *Entamoeba histolytica/Entamoeba dispar* complex from other non-pathogenic intestinal amoebae.2- An enzyme-linked immunosorbent assay (ELISA) for detecting antibody against *E. histolytica*, 53 stool and serum samples were used in this study: 32 (60.38%) samples were symptomatic (diarrheal) patients and 21 (39.62%) were asymptomatic. Data from these two immunological diagnostic tests were compared with those obtained by microscopic examination. A routine microscopic examination detected 7(13.25) prevalence of *Giardia lamblia*, 21(39.6%) prevalence of *Entamoeba spp.* 13(40.6%; X^2=0.578; *P*-value= 0.749) in symptomatic stool samples and 8(38.1%) in asymptomatic stool samples. ELISA antigen detected only 2(6.2%; X^2=1.364; P-value= 0.243) prevalence of *E. histolytica/ dispar* complex in symptomatic samples and none in asymptomatic samples. The two positive symptomatic samples by ELISA/Ag were also positive by microscopy, and no negative subject by microscopy was positive by ELISA. ELISA antibody detected 4 (7.5%; X^2=0.195; *P*-value= 0.659) prevalence of *E. histolytica* in all subjects: 2(6.2%) in symptomatic samples which were positive by both microscopy and ELISA antigen and 2(9.5%) in asymptomatic samples, that were negative by both microscopy and ELISA antigen. The ratio of *E. histolytica/ dispar* was very low (3.7%) suggesting that the vast majority of *Entamoeba* infections in this area were nonpathogenic. The microscopic examination is less sensitive than both ELISA/Ag and ELISA/IgG tests.

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1. Introduction

Gaza Strip is one of the most over populated areas in the world. The population density in Gaza Strip is estimated at 3.867/km^2 out of the total area of Gaza Strip 364km^2 (Human Development Report, 1997).

Various microscope investigations have been carried out to determine the prevalence of intestinal parasite infections in Gaza. Most of the data of prevalence were obtained from school and pre-school children; Pre-school children from Al-Shatei refugee camp children (48%); children from Gaza (27.6%); children from Deir El-Balah (36.3%); children from Beit-lahia (72.9%), (Al-Wahaidi, 1997; Yassin *et al.,* 1999; Al-Hindi, 2002; AL-Zain and Al-Hindi, 2005). Just one study was carried out using molecular PCR (Adnan, 2005) revealed (69%) infection with *Entamoeba histolytica* to be considered the most common parasitic causes of acute diarrhea in Gaza.

Enteric parasites are the most common causes of parasitic diseases, and they cause significant morbidity and mortality, particularly in endemic areas (WHO, 2004). Children and young adults are the most affected group, particularly in regions with limited resources and in areas which have low hygienic measures (Guerrant *et al.*, 2005).

Amoebiasis is a significant health problem worldwide, especially in developing countries. It is presently one of the three most common causes of death. An estimated 40,000-100,000 people die of invasive amoebiasis annually (Stanley, 2003). It has also been estimated that, approximately 500 million individuals are infected with *E. histolytica* each year and only about 10% experience symptomatic disease (Trol *et al.,* 1997 and Haque *et al.*, 1998).

Although the distribution of the parasite is worldwide, the preponderance of morbidity and mortality is experienced in Central and South America, Africa, and India (Parija *et al.,* 2014). Amoebiasis is defined as asymptomatic, invasive intestinal or extraintestinal disease due to *E. histolytica* infection. Asymptomatic cyst is the most frequent manifestation of intestinal *Entamoeba* infection and 90% of *E. histolytica* infections are asymptomatic (WHO, 1997).

The genus *Entamoeba* contains many species, some of which (i.e. *E. histolytica, E. dispar, E. moshkovskii, E. polecki, E. coli and E. hartmanni*) can

be found in human stools (Clark *et al.,* 1991 and Debnath *et al.,* 2004). *E. histolytica*, thus far, is the only species associated with disease (WHO 1997; Petri and Singh, 1999 and Haque, 2006). It is generally accepted that, *E. histolytica* actually comprises two genetically distinct but morphologically indistinguishable species. *E. dispar* has never been documented to cause colitis or liver abscess, but is responsible for many cases of asymptomatic infection. Identification and differentiation of *E. histolytica* and *E. dispar* in stool samples by microscopy is imprecise. False-positive results occur as a result of both pathogenic/non-pathogenic indiscrimination and identification of leukocytes, macrophages and other amoebas as *E. histolytica* in stool (Kebede *et al*., 2004).

Compared to the sensitivities of Enzyme-linked immunosorbent assay (ELISA) antigen in stool and the traditional PCR, real-time PCR has proven to be the most sensitive test for the detection of *E. histolytica*in stool (Braga *et al.,* 1998; Haque *et al.,* 1998; Leo *et al*., 2006 and Uyar *et al.*, 2009). Real-time PCR is not easy for routine diagnosis because it requires expensive equipment and specialized personnel for analysis of the results. For this reason, antigen and antibody detection by ELISA is becoming the standard method for diagnosis of *E. histolytica* infection (Haque *et al.,* 2006).

The main objective of our study is to measure the specificity and sensitivity of ELISA technique for detection of amoebic antigen *E. histolytica/ dispar* in stool samples and IgG antibody *E. histolytica* in serum compared with microscopic diagnosis in both symptomatic and asymptomatic individual in Gaza Strip.

2. Subjects and Methods

Population:

The study was conducted using random sampling among outpatients at hospitals in Gaza Strip. A total of 53 stool samples were collected from patients aged between 5 and 34 years old. 32 patients visiting the hospital for reasons of diarrhea or other gastrointestinal symptoms and 21 subjects had no diarrhea illness (asymptomatic).

Ethical consideration:

1-An approval was obtained from the Ministry of Health dated before the commencement of the study**.** 2-Informed consent, Patients were asked to participate voluntarily after clear explanation.

Collection of Stool Samples:

From each subject approximately 10 g of fresh stool was taken using a wooden spatula ensuring that the sample was not contaminated with urine or water in a clean sterile screw disposable plastic container labeled clearly with child name, gender, age, address and date of collection. The collected stool samples were divided into two parts: The first part of the specimen was preserved at -20ºC to be used later, while the second part was processed immediately for microscopic examination.

Blood Samples:

Blood samples were collected from subjects; a sample of blood consisting of 2 milliliters was obtained from anticubital and/or jugular vein by a sterile disposable syringe from each subject. The blood sample was poured into clean test tube without anticoagulant and left for 2-3 minutes in water bath (37ºC), then centrifuged at 3000 rpm for 6-10 minutes. The serum was separated and transferred to label multiple clean Eppendrof tubes with subject full information then stored at -20ºC.

Parasitological methods

1- Microscopic Examination:

The stool samples were collected from patients in wide-mouthed, screw-capped, labeled containers. Primary detection of cysts and ova were made by the examination of a wet preparation taken from fresh stool. The formalin ether sedimentation technique was used (WHO, 1994).

2-Enzyme-linked immunosorbent assay (ELISA):

1- ELISA for detection of *E. histolytica/ dispar* antigen in stools. The RIDASCREEN® *Entamoeba* (R-Biopharm AG, Darmstadt, Germany, C1701) commercial kit, designed to detect *Entamoeba* sensu lato antigen qualitatively in stool samples, was used for antigen detection in stool samples according to the manufacturer's instructions. 2- ELISA for detection of IgG antibodies against *E. histolytica* in human serum. The RIDASCREEN® *E. histolytica* IgG (R-Biopharm AG, Darmstadt, Germany, K1721) test is an enzyme immunoassay for the qualitative determination of IgG antibodies against *E. histolytica* in human serum. Test was also performed according to manufacturer's instructions.

Statistical Analysis

The data was analyzed using Statistical Package for Social Sciences for Windows, version 17 (SPSS Inc., Chicago. IL, USA). Chi-square analyses were used to investigate the association between dependent and independent variables. Differences were considered significant at $p>0.05$.

The validity indications used for the detection of antibody in sera and antigens in stool samples were sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV). The indicators were calculated according to following author (Kanchanaraksa, 2008).

3. Results

Fecal and serum samples of 53 patients were collected from a Gaza Strip. About 32 (60.38%) samples were found symptomatic (diarrheal) patients and 21 (39.62%) were asymptomatic.

Of the symptomatic group, the microscopic examination of the fecal sample has revealed: 14 (43.8%) clean subjects, 13(40.6%) infected with *Entamoeba* species and 5(15.6%) infected with *Gardia lamblia*. The detection of *E. histolytica/ dispar* antigens (Ag) in the fecal samples by ELISA has shown 30(93.8%) samples negative to Ag of *E. histolytica/ dispar* and 2(6.2%) positive samples. The examination of the serum samples for IgG of *E. histolytica* using ELISA has also resulted in 30(93.8%) negative and 2(6.2%) positive samples for *E. histolytica* IgG (Table 1).

The asymptomatic group, the microscopic examination of the fecal sample has revealed: 11 (52.40%) clean subjects, 8 (38.10%) infected with *E. spp*., and 2 (9.50%) infected with *G. lamblia*. The detection of Ag of *E. histolytica/ dispar* in the fecal samples by ELISA has shown that all subjects were negative. The examination of the serum samples for IgG of *E. histolytica* using ELISA has also resulted in 19 (90.50%) negative and 2 (9.50%) positive subjects for *E. histolytica* IgG (Table 1).

In total 53 samples were examined; 21(39.6%) were positive for *Entamoeba s*pp. and 7(13.2%) for *G. lamblia* by microscopy. ELISA/Ag test confirmed 2 (3.7%) stool samples positive for *E. histolytica / dispar*, while ELISA/IgG detected 4 (7.5%) positive samples (Tables 2, 3).

The **microscopic** examination, the detection of ELISA/**Ag** of *E. histolytica/ dispar* complex **in stool** samples and the detection of *E. histolytica* **IgG in serum** samples regarding *E. histolytica* infection were compared in both symptomatic and asymptomatic subjects. In microscopic examination of symptomatic patients: 13(40.6%) were infected with *Entamoeba spp.*, 5 (15.6%) with *G. lamblia* and 14 (43.8%) were clean, while in asymptomatic subjects 8 (38.1%) were infected with *E. sp.,* 2 (9.5%) with *G. lamblia* and 11 (52.4%) were clean (**X^2= 0.578 and *P*-value= 0.749).** In ELISA/Ag test of symptomatic patients 30 (93.8%) were negative and 2 (6.2%) were positive, while all asymptomatic subjects were negative (**X^2= 1.364 and *P*-value= 0.243)**. For ELISA/IgG test, 30 (93.8%) were negative and 2 (6.2%) were positive in symptomatic patients, while 19 (90.5%) were negative and 2 (9.5%) were positive in asymptomatic clean subjects (**X^2= 0.195 and *P*-value= 0.659), Table 1 and Figure 1**.

The sensitivity and specificity of test, microscopy has 9.5% sensitivity and 100% specificity in comparison with ELISA/Ag, the positive predictive value (PPV) was 100% and negative predictive value (NPV) was 62%. ELISA/Ag revealed a sensitivity of 100% and the specificity of 62.7% compared with microscopy. The PPV was 9.5% and NPV was 100%. ELISA/ IgG test revealed a sensitivity of 50%, the specificity of 61.2%, the PPV was 6.2% and a NPV was 93% compared with microscopy, Tables 2 and 3.

Table 1: Comparison between symptomatic and asymptomatic group regarding; microscopy, ELISA/Ag in stool and ELISA/IgG in serum test results.

Parameters		**Symptomatic patients**		**Asymptomatic patients**		**Chi-square test**	
		No.	**%**	**No.**	**%**	**X^2**	**P-value**
Microscopy	*Entamoeba spp.*	13	40.60%	8	38.10%	0.578	0.749
	G. lamblia	5	15.60%	2	9.50%		
	-ve	14	43.80%	11	52.40%		
ELISA /Ag	-ve	30	93.80%	21	100.00%	1.364	0.243
	+ve	2	6.20%	0	0.00%		
ELISA /IgG	-ve	30	93.80%	19	90.50%	0.195	0.659
	+ve	2	6.20%	2	9.50%		

Table 2: Comparison of all *Entamoeba histolytica*/dispar samples detected using microscopy and ELISA/Ag stool samples.

	ELISA/Ag +	**ELISA/ Ag -**	**Total**
Microscopy +	2 (100%)	19 (37.3%)	21 (39.6%)
Microscopy -	0 (0%)	32 (62.7)%	32 (60.4%)
Total	2 (100%)	51 (100%)	53 (100%)

Table 3: *Entamoeba histolytica* samples detected by microscopy and ELISA/IgG serum sample.

	ELISA/ IgG +	**ELISA/ IgG -**	**Total**
Microscopy +	2 (50%)	19 (38.8%)	21 (39.6%)
Microscopy -	2 (50%)	30 (61.2%)	32 (60.4%)
Total	4 (100%)	49 (100%)	53 (100%)

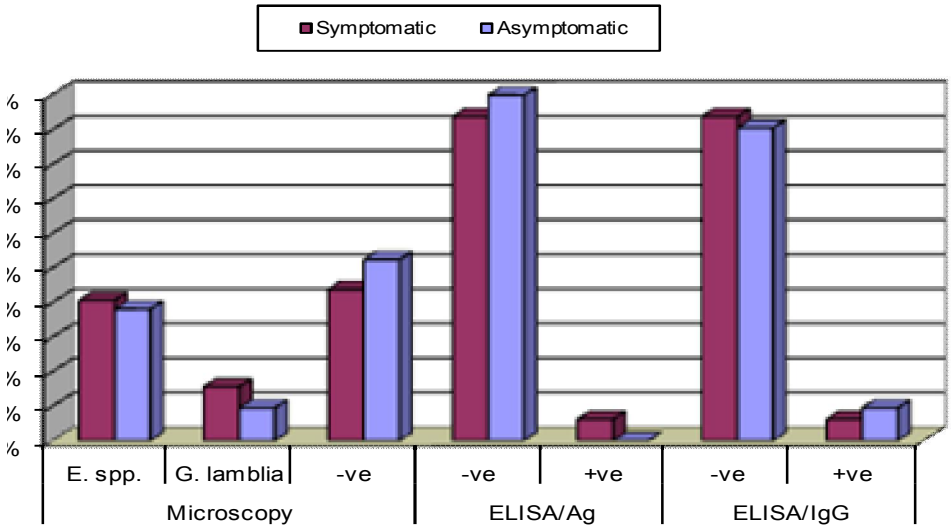

Fig. 1: Comparison between symptomatic and asymptomatic groups regarding; microscopy (X^2=0.578; P-value=0.749), ELISA /Ag in stool (X^2=1.364; P-value=0.243) (and ELISA /IgG in serum (X^2=0.195; P-value=0.659).

4. Discussion

The high percentage (39.6%) of individuals infected with amoeba was found as expected. It is known that prevalence of amoebiasis in Gaza Strip communities is high as the diagnosis performed by a single microscope stool examination.

Microscopic examination remains the gold standard method for diagnosing intestinal *Entamoeba* infection; however, it cannot differentiate between *E. histolytica, E. dispar* and *E. moshkovskii*. Morphologically, *E. polecki, E. coli and* E. *hartmanni* can be differentiated from *E. histolytica* but some of their diagnostic morphologic features overlap depending on the quality of the smears, creating issues for the differential diagnostic identification. Additionally, accurate diagnosis of intestinal parasites is mostly dependent on the level of expertise of the microscopist (Ravdin, 1994 and Leo*et al.,* 2006).

The sensitivity of microscopy ranged from 10-60% in the best conditions and presence of leukocytes or non-pathogen species in feces can lead to false positive results (Garcia and Bruckner,1997).Other researchers suggest that microscopic examination of the stool is sufficient to diagnose *E. histolytica* in the presence of characteristic microscopic findings (Stanley, 2003).

The present work represents, for the first time in Gaza, the use of commercially antigen detection kit to estimate the proportions between *E. histolytica/E. dispar* in addition to routine microscopy. Also the IgG kit was used to detect specifically the proportions of *E. histolytica* prevalence. Using microscopy, 21(39.6%) subjects were suspected to be positive for *E. sp.*, of which 13(40.6%) were symptomatic patients and 8(38.1%) were asymptomatic. Theseresults agree with the general microscopic prevalence in Gaza which is ranged from 24%-53% in different localities (Yassin *et al.,* 1999 and Al-Hindi, 2009).

Using *Entamoeba* ELISA antigen (ELISA/Ag) kit, it was found that only 2 (3.7%) symptomatic patients were positive for *E. histolytica/E. dipar* complex and the same subjects were also positive by microscopy. None of asymptomatic subjects were found to be positive for the same parasites. The results of ELISA/Ag revealed that all the positive samples by microscopy, except two samples, were negative by ELISA test. Also, none of the negative samples by microscopy were found to be positive by ELISA/Antigen.

The difference between microscopy and ELISA/Ag test may be attributed to the quantity of the pathogen in stools with a low number of cysts; according to the manufacturer of the ELISA kit some specimens may give weak reactions that are inconclusive (Gonin and Trudel 2003). Also, the quantity of small cysts of *E. coli* and large cysts of *E. hartmanni* was probably misdiagnosed as *E. histolytica/E. dispar* in the direct microscopy (Ravdin, 1994).

There haven't been yet studies reported in Gaza Strip where ELISA antigen detection method had been used to diagnose amoebiasis to compare with the data

in this study. One study using molecular techniques was done by (Adnan *et al.,* 2005); 92 positive stool specimens by microscopy, *E. histolytica* was 64 (69.6%) and of *E. dispar* was 21 (22.8%) when examined by PCR. Mixed infection with *E. histolytica* and *E. dispar* was evident in 7 specimens (7.6%). These percentages are considered very high compared to the data presented in this study.

In a similar to this study, the prevalence of infection of *E. histolytica* using antigen detection tests was reported to be 4.7% in a survey conducted with 680 asymptomatic children aged 2-5 years in Bangladesh (Haque *et al.,* 1999). Another similar studyin Ismailia (Egypt) showed that out of 50 stool samples of patients supposed to be *E. histolytica* by microscopy, *E. histolytica* alone was detected in only 5 (10%) samples and in association with *E. dispar* in 8 (16%) samples by using PCR. On the other hand, 20 samples (40%) were *E. dispar*. The other 17 samples were negative. *E. coli* and *E. hartmanni* were commonly misdiagnosed as *E. histolytica* (Rayan, 2005).

A study was conducted in Northeastern Brazil, 4.1% prevalence rate was obtained with microscopy for *E. histolytica/E. dispar* but with both PCR and ELISA, the prevalence of *E. histolytica* was 0% (Sandra, 2004). In Ghana, (Jaco*et al.,* 2003) found a high prevalence 98 (39.8%) of the *E. histolytica/E. dispar* complex with microscopy, but they identified by PCR only one *E. histolytica* case. On the other hand, (Gonin and Trudel, 2003) found that ELISA/Ag was less sensitive than microscopy and PCR in differentiating *E. histolytica and E. dispar* in stool samples.

In the present work, ELISA/Ag test revealed a sensitivity of 100% and the specificity of 62.7%. The PPV was 9.5% and a NPV was 100%. This result agrees with (Barwari and Ismael 2011) that have found by ELISA/Ag test a sensitivity of 98.7% and specificity of 58.3% compared with microscopy. But, this test has no ability to differentiate between *E. histolytica* and *E. dispar*. This technique can be used as an additional standard for diagnosis, epidemiology, and quality control for amoebic infections.

In the present study, microscopy has 9.5% sensitivity and 100% specificity in comparison with ELISA/Ag. Delialioglu *et al.*(2004) found that microscopy had low sensitivity and high specificity in comparison with ELISA, which is similar to this study.

The overall rate of seropositivity among all groups for IgG antibodies was 4(7.5%), only 2 (6.2%) of the symptomatic individuals that were positive by microscopy and ELISA/Ag were seropositive and 2(9.5%) of the asymptomatic individuals were seropositive but they were negative by microscopy also by ELISA/Ag. This suggests that individuals may have been infected before and recovered from infection (Haque *et al*., 2000).

The seropositivity rate was higher than the rate of ELISA antigen infection because antibodies were demonstrated to persist for at least one year (Abd-Alla *et al.,* 2002). Although the ELISA/Ag result can't differentiate between *E. histolytica* and *E. dispar*, serologic result ELISA/IgG reflects the incidence of *E. histolytica* infection because *E. dispar* does not show a positive serologic test result (Petri, 1996 and Abd-Alla *et al.*, 1998).

The presence of G. *lamblia*, that was found in 7 specimens negative to *E. histolytica/E. dispar* by both microscopy and *Entamoeba* ELISA, indicated that *G. lamblia* did not cross-react with *E. histolytica* in the ELISA/Ag and ELISA/IgG methods. Other studies also reported an absence of cross reaction with non- *E. histolytica* intestinal parasites (Grundy, 1982; Randall *et al.,* 1984 and Haghighi and Rezaeian, 2005).

In the present study the *E. histolytica* ELISA/ IgG test revealed a sensitivity of 50%, the specificity. of 61.2%, the PPV was 6.2% and a NPV was 93%. Anti-amoebic antibodies develop at the end of a week following exposure in symptomatic patients and persist for years (Petri and Singh, 1999 and Ravdin, 2000).

The results of this study highlight the importance of accurate identification of *Entamoeba species*. In the diagnosis of amoebiasis, a simple detection by microscopy is not sufficient cause for clinical treatment. However, techniques such as ELISA detection, described here, should be used for complete identification at the species level of parasites. It is extremely important for exact diagnosis and for providing correct epidemiological data to have a precise identification of *Entamoeba species*. Accurate diagnosis of amoebiasis is crucial in order for physicians to prescribe proper treatment. These results present important information that will help clinicians decide whether to apply an antiamoebic treatment or search for a different etiology for the presented symptoms. Only the specific detection of *E. histolytica* confirms a diagnosis of amoebiasis, while the identification of other nonpathogenic amoebas can lead clinical investigators to search for different pathologies with similar symptoms that would not be considered without this information. Quantitative serological data constitute valuable information on the endemicity of an area, and the results will gain reliability from use of two complementary tests instead of one.

In conclusion, the present study has demonstrated that *E. histolytica and E. dispar* infections are very low in Gaza population samples. Prospective serious studies are needed to clearly

define the epidemiology of *E. histolytica* infection. ELISA tests are inexpensive, yield objective results and do not require experienced microscopists and can therefore be recommended for screening of stools worldwide. Thus, determination of the true prevalence of *E. histolytica*is possible and the use of antiparasitic drugs is unnecessary.

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