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Diagnosis and Differentiation of *Entamoeba* Infection in Makkah Al Mukarramah Using Microscopy and Stool Antigen Detection Kits

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Abstract: *Entamoeba histolytica* infection is one of the significantly common pathogenic protozoa encountered in Saudi Arabia. Previous reports did not distinguish between *E. histolytica* and *E. dispar* as differential diagnosis is essential both for treatment decision and public health knowledge. Studies suggested that stool antigen assays are more sensitive and specific than microscopy for the diagnosis of *E. histolytica* infection. This study intended to determine the prevalence of *E. histolytica* and *E. dispar* by microscopy and two stool antigen detection kits: Triage parasite panel and TechLab *E. histolytica*. Stool specimens were collected from diarrhoeic patients in Makkah Al Mukarramah city. Parasite detection was performed by microscopy, Triage parasite panel and TechLab *E. histolytica* II. Differentiation between *E. histolytica* and *E. dispar* was performed using Tech Lab. Out of the 156 samples collected, 76.9% were infected. Microscopic examination revealed that, 64.8% were positive for *E. histolytica*/*E. dispar*, *Giardia lamblia* 1.9%, *Cryptosporidium* spp. 1.9% and other parasites 8.3%. 112 samples were found to be infected using Triage. 59.6% were infected with *E. histolytica/ E. dispar*, 1.9% was infected with *Giardia lamblia* and 1.9% with *Cryptosporidium* spp. Differentiation between *E. histolytica* and *E. dispar* performed by ELISA showed that 2.6%, samples were positive for *E. histolytica* antigen. The infection rate of *E. histolytica/E. dispar* was high and the Triage parasite panel kit was less sensitive than microscopy. The high incidence of *Entamoeba* infections are mainly of *E. dispar*. Also, *E. histolytica*-specific ELISA showed to be a sensitive, specific and fast method for the rapid differentiation of the two species.

Key words: Amoebiasis · Antigen · differentiation · ELISA · *Entamoeba dispar* · *E. histolytica* · Microscopy · Makkah · Saudi Arabia

INTRODUCTION

Amebiasis is an infection of human intestinal and extraintestinal organs by the protozoan parasite *Entamoeba histolytica.* The previously reported asymptomatic infections due to the non-pathogenic strains of *E. histolytica* currently are identified to be due to *E. dispar* [1]. Approximately 10% of the world's population is infected by either *E. histolytica* or *E. dispar* of which 50 million people have invasive disease due to *E. histolytica*. Infection is predominantly seen in the tropical and subtropical regions [2]. The annual death of 40,000-100,000 is due to *E. histolytica*, place amoebiasis as the second leading cause of death from parasitic disease worldwide [3]. The prevalence of amebiasis varies with the population of individuals affected, differing between countries and between areas with different socioeconomic conditions. Sometimes up to 50% of the population is affected in regions with poor sanitary conditions [4]. Humans are the primary known reservoir. Infection occurs by ingestion of cysts from faecal contaminated material such as water and food.

Previous studies have confirmed the existence of two genetically distinct but morphologically indistinguishable species of *Entamoeba: E. histolytica* and *E. dispar* [3]. Of these two organisms, *E. histolytica* is the pathogenic and the etiologic agent of amoebic colitis and liver abscess; while the other, *E. dispar* is a non-pathogenic species and has never been associated with disease. Differential diagnosis between the two species is essential both for treatment decision and public health knowledge [5, 6]. WHO have suggested that *E. histolytica* should be specifically identified and, if present, treatment is crucial; on the contrary if only *E. dispar* is identified, treatment is unnecessary [7].

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Traditionally, the diagnosis of *E. histolytica* infection has relied upon microscopic examination of cysts or trophozoites in fresh or fixed stool specimens. The trophozoites of *E. histolytica* are more likely than *E. dispar* to contain ingested erythrocytes. However, the two organisms are morphologically identical in appearance, which makes identification and differentiation by microscopy is imprecise. Besides standard microscopy is time consuming, requires expertise and at best only 60% sensitive [8, 9]. Isoenzyme analysis is considered the reference standard for discriminating *E. histolytica* from *E. dispar* [10]. A number of assays have been developed during recent years, such as serological methods and DNA detection systems, which are able to distinguish *E. histolytica* from *E. dispar* [9, 11, 12].

Efforts to improve the diagnostic testing have been developed in recent years. Antigen detection assays have proved to be very useful in the diagnosis of some parasitic infections, including *E. histolytica* and *E. dispar* [9, 13]. A number of researchers have reported the detection of amoebic antigen in stool samples to be sensitive and specific [5, 11-16]. Antigen-based ELISA has many significant advantages for the diagnosis of amoebiasis. Some of the assays are able to differentiate *E. histolytica* from *E. dispar*; and have excellent sensitivity and specificity. They can be preformed by none expert laboratory technicians and outperform microscopy in their potential as large-scale screening tools in epidemiological studies, such as waterborne outbreak situations [4]. A number of products are commercially available and have been evaluated. These assays range in sensitivity from 66.3 to 100% and in specificity from 92.6 to 100% [5, 9, 13, 14, 17]. Two of the commonly used kits are Triage parasite panel enzyme immunoassay and TechLab *Entamoeba histolytica* II [13, 18].

The Triage parasite panel enzyme immunoassay (Triage) (BIOSITE Diagnostics) is a qualitative enzyme immunoassay panel for the detection of *Entamoeba histolytica*/*E. dispar*, *Giardia lamblia* and *Cryptosporidium parvum* in fresh or frozen. The single immunochromatographic strip is coated with monoclonal antibodies specific for *E. histolytica/E. dispar* antigen 29 kDa and for antigens of *Giardia lamblia* and *Cryptosporidium parvum*. Antigens from clinical samples that are specific for these three parasites are isolated and immobilized on a membrane using specific antibodies. An antibody-enzyme conjugate then binds to specific sites on these antigens. The antigens are detected after the addition of substrate by the formation of colour bars in different areas depending on the parasite present and show on the test device as blue-black lines [9].

The TechLab (Blacksburg, Virginia) *Entamoeba histolytica* II kit is specific and sensitive for the detection of *E. histolytica* in faeces. This antigen detection assay captures and detects the parasite's Gal/GalNAc lectin from stool samples [17].

Various investigations have been carried out to determine the prevalence of entro-parasitic infections in Saudi Arabia. The studies revealed that *E. histolytica* infection is one of the significantly common pathogenic protozoa encountered. The reported prevalence rates were diverse according to the population considered and geographical location. The highest recorded prevalence was found during a household survey in Riyadh (30.3%) [19]. While, a former study conducted in Riyadh reported lower prevalence (16.83%) among patients' attending hospitals or health clinics [20]. The prevalence rate among school children was (5.2%) in Al-Asiah Qasim [21]; (4.1%) in Abha (Asir) [22]; (2.9) in Makkah [23]; finally, (6.8%) was detected among asymptomatic school children; while (14%) was reported among diarrhoeic school children in Jeddah [24]. The diagnostic methodology applied in these studies was primarily based on standard microscopy. None of the previous studies distinguished between *E. histolytica* and *E. dispar*, therefore do not address the true incidence and prevalence of *E. histolytica* and *E. dispar*. The relative prevalence of these *E. histolytica* and *E. dispar* is not yet fully known in Saudi Arabia. The aim of the present study is differentiate between the pathogenic *E. histolytica* and the non-pathogenic *E. dispar* and to determine the rate of their occurrence among specific group using commercial antigen detection kits, Triage Micro Parasite Panel and The TechLab, together with standard microscopy.

MATERIALS AND METHODS

Stool specimens were collected from symptomatic patients attending primary health clinics in Makkah Al-Mukarramah city and complaining from colitis during March to December, 2005. A portion of each stool specimen was fixed in 10% formal saline to be further examined by concentration technique. For ELISA a one-gram portion of fresh stools was stored quickly in a freezer at -20°C prior to analysis.

The parasite detection was performed by conventional microscopy on direct saline and iodine

wet mount preparations or using the formol-ether concentration technique and subsequent staining with Lugol's iodine solution. All smears were examined by experienced technician for the presence of *E. histolytica* /*E. dispar* complex cysts and trophozoites.

The ELISA kits were used on the frozen stool specimens. For Triage parasite panel (Triage), the assay procedure was completed according to the manufacturer's directions. The tubes, pipettes, devices, and all reagents are provided with the kit. The specimen preparation preformed by dispensing 4.5 ml of specimen diluent into tubes provided by the manufacturer. Stool samples were then added to these tubes and vortexed for 10 s. A filtration device was inserted into each specimen tube and then centrifuged for 5 minutes at 1,500 to 1,800 × g. The filtered sample (500 µl) is then added to the centre of the Detection Zone of the Test Device. Enzyme conjugate (140 µl) is added to the centre of the membrane and incubated for 3 min. The Detection Zone was washed twice by six drops of wash solution. Four drops of the substrate is then added to the membrane, followed by 5 minutes incubation. The device is then immediately read (Test Zones, three POS CTRL, and one NEG CTRL Zones) and the results are interpreted. Positive results are visualized as purple-black lines in the appropriate position in the results window. Positive and negative controls are included in the device, and the total time is approximately 15 min.

For the TechLab *E. histolytica* II test, the detection was carried out as suggested by the manufacturer. In brief, all stool specimens were diluted 1:1 in diluents provided with the kit. The assay microtiter wells (provided with the kit) were incubated with 200 µl of diluted specimen and one drop of MAb-enzyme conjugate for r 2 hours at (15-25°C). The contents of the well strips were then shaken out. Wells washed vigorously five times in wash solution. Following washing, the residual liquid was removed by striking the strip once against a paper towel, substrate solutions were added, and the strip was incubated at room temperature for 10 min. Two drops of intensifier was then added, and after an additional 10 min of incubation the well strips were read in a microtiter plate reader at 450 nm. A positive result was defined as an optical density reading of .0.05 after subtraction of the negative control optical density. Sensitivity was calculated as the number of true positives (number of true positives 1 number of false negatives); specificity was calculated as the number of true negatives (number of true negatives 1 number of false positives). (Sensitivity: 100%; specificity: 94.7%; correlation with zymodeme analysis = 96.8%).

Table 1: Parasite distribution among studied sample using microscopy

	E. histolytica/ E.dispar	*G. lamblia*	*Cryptosp.*	Others	Total Infected	Total
Females	52	0	1	3	56	76
Males	49	3	2	10	64	80
Total	101	3	3	13	120	156
percentage	64.7%	1.9%	1.9%	8.3%	76.9%	100.0%

Table 2: Parasite detected using Triage parasite panel

	E. histolytica/ E.dispar	*G. lamblia*	*Cryptosporidium*	Total Infected	Total
Females	48	0	1	49	76
Males	45	3	2	50	80
Total	93	3	3	99	156
percentage	59.6%	1.9%	1.9%	63.5%	100.0%

Table 3: Comparison of samples detected *E. histolytica/E. dispar* using microscopy and Triage parasite panel

	Trig +ve	Trig -ve	Total
Microscopy +ve	93	8	101
Microscopy -ve	0	55	55
Total	93	63	156

Table 4: *E. histolytica* samples detected by Microscopy and TechLab

	ELISA +ve	ELISA -ve	Total
Microscopy +ve	4	97	101
Microscopy -ve	0	55	55
Total	4	152	156

RESULTS

A total of 156 stool specimen were collected, seventy six (48.7%) were females and eighty (51.3%) were males. On the basis of microscopic examination, 120 (76.9%) samples were positive for parasitic infection; 64.8% (101) were for *Entamoeba histolytica*/*E. dispar*, 1.9% [3] *Giardia lamblia*, 1.9% [3] *Cryptosporidium* spp. and other parasites 8.3% [13] (Table 1). No mixed infection was detected. The Triage test accurately detected 99 (63.5%) positive samples, 93 (59.6%) *E. histolytica/E. dispar* complex, 3 (1.9%) *Giardia lamblia* and 3 (1.9%) *Cryptosporidium* spp. (Table 2). Both techniques were positive in 93 (59.6%) samples for *E. histolytica/E. dispar*. Samples positive for *Giardia lamblia* by microscopy were also positive with the Triage parasite panel kit. Also the three samples positive for *Cryptosporidium* spp. using modified acid-fast staining was detected positive using Triage parasite panel kit. The comparison of *E. histolytica/E. dispar* samples detected by Microscopy and Trig is summarized in (Table 3).

Differentiation between *E. histolytica* and *E. dispar* was performed using TechLab ELISA. The assay showed that only 4 (three females and one male) 2.6%, samples were positive for *E. histolytica* antigen. Comparison of microscopy and ELISA techniques showed that *E. histolytica* was detected in 4, of which suggest that the remaining 89 (44 males, 45 females) were *E. dispar* (Table 4).

DISCUSSION

Most epidemiological studies that identified *Entamoeba histolytica* infection in Saudi Arabia were performed without the distinction of the separate species, *E. dispar* and *E. histolytica*. This is possibly due to the inability, in the past, to differentiate *E. histolytica* from the more common, but non-pathogenic, *Entamoeba dispar*. As *E. histolytica* and *E. dispar* cannot be differentiated by routine microscopy. Therefore, there is an apparent need to carry out studies which distinguish the two species of *Entamoeba*. This investigation represents the first time research to use commercially antigen detection kit to estimate the proportions between *E. histolytica* and *E. dispar* in Saudi Arabia in addition to routine microscopy.

In the present study, the positivity of *E. histolytica /E. dispar* was high by microscopy and Triage parasite panel kit, 64.8% and 59.6% respectively. Triage test showed a good overall sensitivity and specificity for *E. dispar/E. histolytica*; *Giardia* and *cryptosporidium*. However, among the 55 Triage-negative, 8 were identified positive for *E. dispar/E. histolytica* by microscopic examination. Similarly ELISA was less sensitive than microscopy by Gonin and Trudel [6], whereas higher sensitivity was reported [17], who found that Entamoeba ELISA more sensitive and specific than microscopy compared to culture as the gold standard. Undoubtedly, direct microscopic examination has the advantage of being inexpensive compared to antigen detection tests. However, accurate identification of amoebae parasites mostly depends on the proficiency of the microscopist performance and interpretation. Results of microscopic examination therefore can differ between studies according to staff experience; as well as techniques used for diagnosis owed to the difficulty in differentiating *E. histolytica* / *E. dispar* from other similar amebae or artifacts. Many laboratories also suffer from the decrease number of well expertise staff. Over diagnosis *E. histolytica* / *E. dispar* and microscopical false positives have been reported [25, 26].

This difference may be also attributed to the quantity of the pathogen in stools: stools with a low number of cysts, according to the manufacturer of the ELISA some specimens may give weak reactions that are inconclusive. This may be due to a number of factors such as the presence of binding substances or inactivating enzymes in the faeces. Under these conditions, the specimen should be retested or a fresh specimen should be tested [6].

The TechLab *E. histolytica* II test revealed that from 93 patients with initial diagnosis of *E. histolytica / E. dispar* by microscopy and Triage test only 4 (4.3%) were *E. histolytica* positive. This indicates that the remaining 89 (95.7%) patients were *E. dispar*. The present work findings are consistent with those previously reported [26-30]. They showed a high incidence of *Entamoeba* infections are *E. dispar* (non-pathogenic amoeba) in the population. The study also indicates that *E. histolytica*-specific ELISA showed to be a sensitive and specific method for the rapid differentiation of the two species. The ability to differentiate *E. dispar* from *E. histolytica* should be used to diagnose suspected cases to reduce the number of unnecessarily treated patients.

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