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Original article

Evaluation of Infection of Entamoeba Histolytica and Entamoeba dispar by using ELISA among Patients in Kirkuk City

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Abstract:

- ***Background*:** The differentiation between *Entamoeba histolytic*a (pathogenic) and *Entamoeba dispar* (nonpathogenic), two morphologically indistinguishable species of *Amoeba*, is crucial for making treatment decisions and advancing public health understanding.
- ***Methods:*** In the present investigation, spanning from January 1, 2023, to May 20, 2023, fecal samples were procured from a cohort of 220 pediatric patients, aged below 15 years, who presented with symptoms of diarrhea and/or abdominal discomfort. These patients sought medical attention at the Pediatric Hospital, Azadi Teaching Hospital, and Kirkuk Teaching Hospital, all located in Kirkuk City. The samples that exhibited positive results under microscopy were subjected to additional analysis using ELISA technique.
- ***Result:*** The current data revealed that out of 220 stool samples examined with microscopy and iodin preparation, 93(42.27%) specimens were positive for *E. histolytica/E. dispar* trophozoites and cysts, while the remaining 127(57.73%) were negative for any amoebic stages. the male more infected than women and highest among children 1-5 years. The percentage of positive specimens that were tested with *E. histolytica / dispar* ELISA, these samples were microscopy positive and further confirmed with DRG ELISA. Out of 93 stool specimens, 59(63.44%) were positive, while the remaining specimens 34(36.56%) were negative despite been tested positive by microscopy. The DRG stool ELISA revealed sensitivity and specificity (69.28 % and 97.91 %) respectively and predictive value of (97 %).
- ***Conclusions:*** The standard diagnostic tool in the health sector is microscopic inspection, which cannot distinguish between pathogenic and nonpathogenic amoeba ELISA is an alternate method for microscopy screening confirmation. males infected more than women and 1-5-year-olds more than others.

- **Keywords:** *E. histolytica*, *Entamoeba dispar*, ELISA.

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INTRODUCTION

Amebiasis is a parasite infection that can result in a range of clinical presentations, spanning from asymptomatic colonization by the protozoan *Entamoeba histolytica* in humans, to the development of severe fulminant colitis and non-intestinal amebiasis [1]. Nevertheless, it is challenging to differentiate between E. dispar and E. histolytica based on their morphology alone. The prevalence of the disease is more pronounced in locations characterized by tropical and subtropical climates, with a larger incidence of reported cases observed in nations classified as developing countries [2,3]. roughly 10% of the global population is parasitized by *Entamoeba histolytica* and *Entamoeba dispar*, with roughly 90% of these infections being asymptomatic. However, it is estimated that amebiasis is responsible for causing up to 110,000 fatalities annually [4,5]. Amebiasis has the potential to be spread via the consumption of fresh food and beverages that are contaminated with cysts of E. histolytica, each having four nuclei [5]. Infection with Entamoeba histolytica possesses the capacity to induce dysentery and extraintestinal ailments, but E. dispar is widely regarded as a benign commensal [6]. As per the World Health Organization (WHO), amebiasis ranks as the third most fatal ailment, preceded only by malaria and schistosomiasis [7]. The diagnosis of intestinal amebiasis is established by the detection of cysts or trophozoites of *E. histolytica/E. dispar* in fecal samples. Additionally, assays that analyze amoeba antigens in feces have been found to be valuable [8]. Microscopy continues to serve as the primary diagnostic technique for amebiasis and is widely employed in the majority of poor nations. Nevertheless, it is important to note that the method in question lacks the ability to distinguish between *E. dispar* and *E. histolytica*. Furthermore, the effectiveness of this method in detecting E. histolytica is highly reliant on the proficiency of the technician. In comparison to alternative techniques like immunofluorescence (IFA), antigen detection, and polymerase chain reaction (PCR), this method has been demonstrated to exhibit lower levels of sensitivity and specificity [9].

PATIENT and METHOD

Study Population and Design: From 1/1/2023 to 20/5/2023, general stool examination was done to patients for detection of *E. histolytica / dispar* infection. A total 220 patients of less than 15 years, whom attended the parasitology section. The chosen patients were suffered from abdominal pain and diarrhea. A questionnaire form was given to each one include name, age, address and symptoms.

Samples collection: Fresh stool samples were collected in a clean sterile screw disposable plastic container, a part of the specimen was processed directly to wet mount examination. A small part (0.5 ml - 3 ml) of stool specimens were put in sterile screw cap containers and kept at -20℃ until being examined by ELISA. Serum samples were collected from patients of microscopically positive results for *E. histolytica / dispar*.

Stool examination: Macro and Microscopic examination were done for each stool specimen, the microscopic examine was performed by direct wet mount method using normal saline and lugol's iodine solutions. Enzyme-linked immunosorbent assay (ELISA) for detection of galactose/N-acetyl Dgalactosamine lectin for *E. histolytica* in stools;(*E histolytica* II Test, Tech Lab, Blacksburg, VA, USA,

Sensitivity 96.9–100, Specificity 94.7– 100) was also done according to manufacturer's instructions. In a 96-microtiter ELISA well plate pre coated with polyclonal antibodies binding adhesion assays were done. 0.1 ml of diluted specimen (stool specimen diluted 1:1 in diluent provided with the kit) were added. One drop of conjugate clonal antibodies specific for adhesion from *E. histolytica*; coupled to horseradish peroxidase were added to a well too. A positive and negative controls were included in each test. The wells were incubated and washed by ELISA washer, substrate and stop solution were added, the absorbent was read by ELISA reader at 450 nm.

E. histolytica/E. dispar stool antigen ELISA (DRG Instruments GmbH,Germany)

The DRG ELISA stool antigen assay was performed on 90 microscopy positive stool specimens according to manufacturing company.

E. histolytica II (monoclonal ELISA for detecting *E. histolytica* adhesin in fecal specimen) (TECHLAB Inc., Blacksburg, Virginia, USA)

The ELISA assay was performed on 79 stool specimens that were positive by DRG ELISA *E. histolytica / E. dispar* stool antigen, according to manufacturing company.

Statistical Analysis:

The statistical analysis was performed with Graph Pad Prism analytical software and comparison were made where required via $\chi 2$ test. Data were considered non-significant if P value >0.05, while significant data difference is considered if P value <0.05.

Ethical approval

This research was conducted at Azadi Teaching Hospital in accordance with the ethical principles outlined in the Helsinki Declaration. Prior to collecting any samples, verbal and written consent were obtained from all participating patients. The study protocol, participant information, and consent forms were thoroughly reviewed and approved by a local Ethics Committee, as documented under reference number 6563, dated January 1, 2023, to ensure compliance with ethical standards.

RESULTS

The prevalence of *E. histolytica/E. dispar* in stool specimens

Table (1): The current data revealed that out of 220 stool samples examined with microscopy and iodin preparation, 93(42.27%) specimens were positive for *E. histolytica/E. dispar* trophozoites and cysts, while the remaining 127(57.73%) were negative for any amoebic stages.

Table (1): The ratio of positive and negative specimens for *E. histolytica / E. dispar*.

E. histolytica/dispar	No.	%
Positive	93	42.27
Negative	127	57.73
Total	220	100.00

Gender Distribution of patients infected with *E. histolytica/E. dispar*

Table (2): Exhibited that the rate of *E. histolytica / E. dispar* infection was higher in males 64 (68.82%) than females 29 (31.18%). Statistical analysis revealed significant difference between genders with P=0.013.

Table (2): Gender distribution of *E. histolytica / E. dispar*

Gender distribution	No.	No. positive	Positive %	*P* *
Male	126	64	68.82	**0.013**
Female	94	29	31.18	
Total	220	93	100.00	

** Chi-square test*

Age distribution of patients group

Table (3): The rate of *E. histolytica / E. dispar* infection was highest among children 1-5 years 44 (47.31%); followed by <1 years 22 (23.66%); 6-10 years 19 (20.43%) and 11-15 years 8 (8.60 %); Statistic analysis revealed significant difference (P=0.048).

Table (3): Age distribution of *E. histolytica / E. dispar* infected individuals.

Age /years	No. of positive	%	No. of patients	%	*P* *
<1	22	23.66	34	15.45	0.048
1-5	44	47.31	92	41.82	
6-10	19	20.43	70	31.82	
11-15	8	8.60	24	10.91	
Total	93	100.00	220	100.00	

* *Mann Whitney test*

Distribution amoebiasis according to residency

Table(4): The most patients infected with amoebiasis were residing the city (66.67%) while the remaining (33.33%) were from rural areas. Significant difference found with P=0.047.

Table (4): Residency distribution of *E. histolytica / E. dispar*.

Distribution of infection according to residency	No. examined	No. Positive	Positive %	*P* *
Urban	141	62	66.67	0.047
Rural	79	31	33.33	
Total	220	93	100.00	

* *Chi-square test*

Duration of diarrhea in patient group

Table (5): The current study revealed that most of the patients 132(60%) complaining of diarrhea were having runny bowel motion for less than three days, while the only 16(7.27%) had diarrhea for a period over 10 days as depicted in Table 5.

Table (5): Duration of diarrhea in tested individuals.

Duration of diarrhea/days	No.	%
<3	132	60.00
4-7	72	32.73
>8	16	7.27
Total	220	100.00

ELISA for *E. histolytica / E. dispar*

Detection of *E. histolytica / E. dispar* in stool specimens

Table (6): The percentage of positive specimens that were tested with *E. histolytica / dispar* ELISA, these samples were microscopy positive and further confirmed with DRG ELISA. Out of 93 stool specimens, 59(63.44%) were positive, while the remaining specimens 34(36.56%) were negative despite been tested positive by microscopy. The DRG stool ELISA revealed sensitivity and specificity (73.17% and 96.42%) respectively and predictive value of (98.94%).

Table (6): Detection of *E. histolytica / dispar* antigen in stool specimens by DRG ELISA

DRG ELISA *E. histolytica/dispar*	No.	%
Positive	59	63.44
Negative	34	36.56
Total	93	100.00
Sensitivity	73.17%	
Specificity	96.42%	
Predictive value	98.94%	

Detection of *E. histolytica* antigen in fecal specimens

Table(7): Our data demonstrated that the sample that produce positive result with DRG *E. histolytica /E. dispar* ELISA were discriminated via TechLab E. histolytica ELISA that detect the presence of only

E. histolytica alone in fecal samples. Out of 93 examined specimens, only 24(25.81%) were positive while the remaining 69(74.19%) were negative, as depicted in Table 7.

Table (7): Detection of *E. histolytica* antigen in fecal specimens via TechLab ELISA.

TechLab ELISA	No.	%
Positive	24	25.81
Negative	69	74.19
Total	93	100.00
Sensitivity	69.28%	
Specificity	97.91%	
Predictive value	98.97%	

DISCUSSION

The current study involved microscopy detection *of E. histolytica/dispar* in stool specimens from children below 15 years, these specimens were tested with ELISA that capture both *E. histolytica/dispar*, the positive specimens were submitted to ELISA that capture only *E. histolytica* in stool samples. In addition, it included the confirmation of *E. histolytica* specific DNA in those microscopies' positive specimen.

As it is obvious, out of 220 stool specimen the rate of microscopy positive samples was 42.27%, this result is in line with a study conducted by Uslu H, *et.al*., in Malaysia who reported the presence of *E. histolytica/dispar* at a rate of 31.1% of in stool specimen examined with trichrome staining [(10)]. However, our result is in conflict with Das S, *et.al* research who reported the presence of *E. histolytica/dispar* in stool samples diagnosed with variable techniques and found that microscopy positive samples represented only 3.17% of tested patients [(11)]. It also differs from a study conducted in Malaysia by Ngui *et.al*, that found microscopy positive amoebic form in 17.6%, as well as, Previous research has also revealed a high frequency of Entamoeba infection, in rural communities ranging from 9.4% to 21.0% [(12)].

The discrepancies in the data shown above could be attributed to the fact that microscopy detection of amebiasis is mainly rely on personal skills and experience thus false positive or even false negative reports of amoebic form could be obtained and no accurate data could be generated through microscopic examination. In addition, most of the preparation techniques of microscopic detection could not cover

the whole specimen as only a small portion of fecal specimen many be utilized for the test and larger quantities of stool may cover more forms of the parasite. However, microscopy despite being accessible to many health setting still it lacks the required sensitivity and sometime specificity because it is not possible to distinguish between amoebic species according to morphological features [13]. Thus, microscopy despite being available and most of health facilities it does require enough expertise to facilitate detection of amoebic form and further confirmation of positive result is mandatory to allow accurate incidence of the parasite and reflect the true picture of the disease prevalence.

Regarding to the sensitivity of microscopy, our data revealed this technique has been improved with innovations such sample concentration by sedimentation and distinguish between hematophagous trophozoites of *E. histolytica* in fecal samples, allowing the differentiation to be determined more readily [11]. Also, staining technique of trophozoite could help identifying aspect of the parasite and differentiating them from identical *E. dispar*.

This is agreed with Al-Damerchi, *et.al.*, who demonstrated that the sensitivity and specificity of microscopic examination were 91%, 44%, and the accuracy of wet mount (60%) respectively. This is also consistent with a research conducted on microscopic examination and antigen detection which demonstrated that antigen detection tests using ELISA and PCR techniques were more sensitive and specific for identifying Entamoeba species than microscopy [14]. Hence, diagnosis of the parasite most not rely on microscopic detection and a superior technique to a microscopy must be provided in reference laboratories [15,16].

Regarding gender distribution, our data revealed that male patients infected with the parasite were higher than females with 68% and 31% respectively. These data are in consistent with Hamza, *et.al* who recorded infection of *E. histolytica* was higher in males (55.2%) than in females (44.8%), and there was no correlation between genders (P> 0.05) [17]. Our results are contrast to Al-Damerchi *et al*., who reported elevated infection rate of amoebiasis in females rather than males [14,15]. In addition, Ngui R, *et.al* demonstrated that infected people with amoebiasis is higher in females (19.1%) than males (15.9%) [12]. Add to this, our data are not in line with Flaih *et al.*, who reported that the proportion of females were 50.4% just above the number of males 94.6% infected with amoebic dysentery [16].

The difference between our result and other findings may be explained by the fact that males often have weaker immune systems, and many diseases are often more prominent in females and this could be due to physiological and ecological factors, which are typically hormonal in nature, are usually the cause of these variances due to sex-specific behaviour or morphology and disease exposure can change depending on ecological conditions [17]. Additionally, the difference between our data and the data of other studies could be attributed to the variation in sample size or the bias selection of patients included in the study as well as to the male behavior pattern to spend more time with their friends out in the streets which significantly increasing the risk of ingestion of contaminated water or food.

In terms of age distribution, the result generated in this research found that the highest infection rate was in children below 5 years, these data is in agreement with Hamza, et.al data that recorded similar

infection rate in patients below 5 years [15]. While it conflicts with Flaih et al., study who reported elevated rate of infection among age group 5-14 years [16]. Added to this, Ngui *e al*., study illustrated that infection rates by age categories, it was found that adults (23.9%) had higher rates than children (15.3%) [12].

The data confliction indicated the effect imposed by this parasite on children below five as they are more vulnerable to infections due to the fact that their habits of manipulating things around them may increase the risk of acquiring the infective form more readily. In addition, children and toddler at this age group are more often experience to discover the surrounded environment and are carefree to ingest anything they encounter. The variation also in age group may indicate difference in study design in which children are the only assist group while other group may include opened age group which reflect significant variation between researches with regard to age group.

As far as patient residency is concerns, our data reported that most of the patients infected with amoebiasis 66.67% were residing in urban areas. These data are close with Hamza *et al*. who found that most of his patient group were residing in urban areas[17]. These data are dissimilar with Flaih *et al*. who mentioned that 69% of the participants were residing in rural areas [16]. The difference in our data and other studies indicates that the inclusion and exclusion criterion in each study as well as the geographical distribution of patients and the area each researcher conducted the study in. Our data illustrated that ELISA for *E. histolytica /E. dispar* has detected 63.44% positive amoebic form out of 93 specimens. Our results are in disagreement with a Bayoumy et al., study that reported 31.6% of amoebiasis detected via ELISA method [18]. The result displayed here is differ from another report which indicated that an ELISA bases detection of amoebic form was positive in 31.6% [19]. The variation in the results found in our data and other reports could be due to the fact that each research has study population that cover certain criterion as well as the basic tool used in each research for the initial diagnosis of stool amoebiasis which mainly rely on microscopy which is greatly influence by the personal skills and experience to detect the parasite stages. In addition to the basic principle of ELISA used in the assessment which could have variable sensitivity and specificity [19]. The basic principle of stool antigen ELISA is proposed to capote either *E. histolytica/dispar* or *E. histolytica* alone, in each circumstances specificity must be high in order to detect the presence of any amoebic forms. However, compared to microscopy, the microscopy is capable of detection of the three common species of Entamoeba, i.e. *hsitolytica, dispar* and *moshkoviskii* [20]. The above-mentioned ELISA are only capable of capturing two of the three species and hence lowering the specificity rate of ELISA dedicated for capturing *E. hsitolytica/ dispar*. Also, it does require the parasite to be at certain load in order to allow the kit to detect the presence of amoebic antigen in the tested specimens and in the case of in appropriate or in adequate sample reconstitution false negative result may obtained. Over that, false positive or false negative results could be encountered because ELISA technique required trained laboratory staff to proceed with ELSA protocol, and inexperience technician could significantly increase invalid data generated from ELISA [20, 21]. Moreover, each ELISA kit has specific target to which the antibody coat will react, thus if the amount of antigen in sample was bellow detectable level, then false negative result will produced, therefore despite an ELISA test has as credible specificity and reliability, it has some drawbacks for instance,

contamination of the wells with samples from other wells due to poor techniques, inaccurate addition of the reagents, sample dilution may increase the possibility of negative result, in appropriate washing process of the well may result in contamination and false positive data will generated [22].

Our data revealed that the sensitivity of each ELISA applied in the research were 73% and 69% respectively. Our data are in line with Uslu *et.al* research who conducted a comparison of different methods for detection of amoebiasis and reported a sensitivity for ELISA at 64% [10]. On the contrary, these data are in conflict with another research that indicated a sensitivity of ELISA test of 96% compared to microscopy [23]. Hooshyar *et al*., study reported a sensitivity of 96% for ELISA compared to microscopy [24,25]. Moreover, Cafaro *et al*., refereed to ELISA test's sensitivity and specificity at (93% and 75%, respectively) [26] It has been demonstrated that ELISA is useful for ordinary tasks. According to a study by Al Damerchi et al., this test has shown to have good sensitivity and specificity for detecting *E. histolytica* antigen in stool specimens from individuals with amebic colitis and asymptomatic intestinal infection [14].

The variation of the sensitivity in the ELISA dedicated for amoebic detection in each research could be due to the fact that each ELISA has sensitivity point at which it can detect target antigen as well as the parasite load in each sample which can greatly affect the sensitivity of an ELISA test. In addition, other factor that may influence the sensitivity of an ELISA is the inadequate mixing of fecal specimen during sample preparation and processing which can reduce the recovery of the parasite in samples.

CONCLUSION

The common diagnostic tool in heath sector relies on microscopic examination and this method is not satisfactory as it cannot discriminates between pathogenic and nonpathogenic amoebas, Whereas ELISA technique is an alternative method for confirmation of microscopy screening. the male more infected than women and the age between 1-5 more infected than others.

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Conflicts of interest:

There are no conflicts of interest**.**

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