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# Detection and differentiation of Entamoeba histolytica and Entamoeba dispar by enzyme linked immuno sorbent assay

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## ABSTRACT

***Amoebiasis is an important parasitic disease in human. The two species Entamoeba histolytica and Entamoeba dispar are morphologically identical but E. dispar is none pathogenic and is not associated with symptomatic amoebiasis. In this study, from July to December 2013, 397 (212 male, 185 female) stool samples from in and out patients in Kirkuk Azady Teaching Hospital were examined microscopically. 97 samples of them were positive for Entamoeba histolytica / dispar. Blood samples were collected from E. histolytica / dispar positive patients, and the sera were examined by ELISA for differentiating the two species and evaluating the IgG levels in their serum. The overall rate of E. histolytica / dispar detected microscopically was 24.4%, while when the positive samples examined by ELISA technique 89.7% of them were E. histolytica and 10.3% were considered to be E. disbar. The serum samples of 27.58% of the patients whom had E. histolytica were positive for IgG antibody. The most age group which was infected with E. histolytica / dispar in both sexes were 41-50 years with rates of 39.13, 34.6 % for each of males and females respectively. A significantly high frequency (62.9, 94.8 %) of E. histolytica / dispar positive samples were contained RBC and pus cells respectively for each cell type, and the highest rate (28.8, 39.1%) were for those samples contained three pluses respectively for each of RBC and pus cells. The conclusion is that there is a big necessity of a serology confirmatory test after microscopic detection of E. histolytica to avoid un necessary treatment.***

***Keywords:*** *E. hitolytica / dispar , Differentiation, ELISA.*

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# الكشف والتمييز بين الاميبا النسيجية و الاميبا المتغيرة بواسطة الاليزا

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## الملخص

**داء الاميبات هي احدى الامراض الطفيلية المهمة في الانسان، النوعين النسيجي والدسبار متماثلة شكلا، لكن نوع الدسبار غير ممرض وغير ذو علاقة بظهور الاعراض المرضية. في هذه الدراسة، ابتدأ من شهر تموز والى شهر كانون الأول لسنة ٢٠١٣، فحص مجهريا ٣٩٧ ( ٢١٢ ذكر، ١٨٥ انثى ) نموذج غائط للمرضى المراجعين والراقدين في مستشفى آزادي التعليمي في كركوك. ٩٧ عينة منهم كان موجبا للاميبا النسيجية\ الدسبار. جمعت عينات دم من المرضى ذوي نتائج الغائط الموجبة لأجل فحص المصل بطريقة الاليزا للتفريق بين النوعين، وتقدير نسبة الاجسام المضادة نوع IgG في مصولهم. النسبة الكلية لاميبا النسيجية\ الدسبار بالفحص المجهري كان ٢٤,٤%، بينما عندما فحصت هذه العينات الموجبة بطريقة الاليزا كان نسبة ٨٩,٧% منها لاميبا النسيجية ونسبة ١٠,٣% اعتبر موجبا لاميبا الدسبار. مصول ٢٧,٥٨% من مرض اميبا النسيج كان موجبا لاجسام IgG. كانت المرحلة العمرية الاكثر إصابة باميبا النسيجية\ الدسبار في كلا الجنسين هي ٤١-٥٠ سنة بنسبة ٣٩,١٣% ، ٣٤,٦% لكل من الذكور والاناث على التوالي. نسبة تردد عالية معنويا ( ٦٢,٩%، ٩٤,٨%) من العينات الموجبة للأميبا النسيجية\الدسبار احتوت على خلايا دم حمراء مع خلايا التهابية على التوالي لكلا النوعين من الخلايا، والتردد الاعلى كانت للعينات الحاوية على ( +++ ) وبنسبة ٢٨,٨ ، ٣٩,١% لكل من خلايا الدم و الالتهابية على التوالي، الاستنتاج هو انه هناك حاجة كبيرة لإجراء فحص مصلي تأكيدي بعد اجراء الفحص المجهري لتجنب اخذ العلاج الغير الضروري.**

**الكلمات الدالة:** اميبا النسيج \الدسبار ، التميز بينها ، الاليزا .

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## 1. INTRODUCTION

Amoebiasis is one of important human parasitic disease. *Entamoeba histolytica/ dispar* parasitize approximately10% of the world population of which 90% of infections are asymptomatic. It has been estimated that 40 million to 50 million people develop clinical amoebiasis annually, resulting in up to 100,000 deaths [1].

*Entamoeba histolytica* is anaerobic protozoan intestinal parasite, pathogenic species of *E. histolytica* was first described by Fedor Lösch in 1875 that causes amebic dysentery. *E. histolytica* can invade other body sites by penetrating the intestinal mucosa, including liver, lung and cerebral and genitourinary [2]. *Entamoeba dispar* was described by Emile Brumpt in 1925 but dismissed as a synonym of *E. histolytica*. *E. dispar* is now accepted as a distinct species. It is none pathogenic and is not associated with symptomatic amebiasis in humans. Morphologically it resembles *E. histolytica* [3]. Amoebiasis is the third most common cause of death due to parasitic infection after malaria and schistosomiasis.

Laboratory diagnosis of *E. histolytica*/*dispar* is primary based on the finding of a trophozoite or cyst in the stool smears [4]. Now new approaches of identification of the two species are based on detection of specific antigen and DNA in stool and other clinical samples. Several molecular diagnostic tests, including traditional and real-time polymerase chain reaction (PCR), antibody detection tests like enzyme-linked immunosorbent assay (ELISA) and a variety of antibody assays are commercially available [5].

The two species are found throughout the world, but like many other intestinal protozoa, they are more common in tropical countries or other areas with poor sanitary conditions. High rates of amoebiasis occur in the Indian subcontinent, the far east, western and southern Africa, and parts of South and Central America [2]. In Iraq and other countries the prevalence and the differentiation of the two species was the subject of many studies, PCR technique were used for diagnosing and differentiating the two species in Tikrit and Baghdad [6, 7]. Also ELISA technique were used in each of Kirkuk, Basrha, Duhok, Egypt, Turkey, Canada, Mexico City [8, 9].

The aim of the current study was to detect and differentiate between *E. histolytica* and *E. dispar* in human isolates by ELISA technique , because it's very important to determine whether the patient has to require treatment or not, to avoid the side effects of the unnecessary medications.

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## 2. MATERIALS AND METHODS

**2-1-Population study:** From July to December 2013, general stool examination was done to patients for detection of *E. histolytica / dispar* infection in Azadi Teaching Hospital. A total of 212 male and 185 female were eligible, 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.

**2-2-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°C until being examined by ELISA. Serum samples were collected from patients of microscopically positive results for *E. histolytica / dispar.*

**2-3-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.

**2-4-Blood samples collection:** from 87 patients 2-3 ml of venous blood was drawn carefully and transferred into disposable plan tube, the specimen was left for (15-30) min, then centrifuged at 300rpm for 5 min to separate clear serum. The sera were kept at (-20$^{o}$C) till used for ELISA test.

**2-5-ELISA test:** The qualitative immunoenzymatic determination of antibodies against *E. histolytica* is based on the ELISA (Nova Tec Immunodiagnostica. GmbH, Germany) technique. Micro titer strip wells are pre coated with *E. histolytica* antigen to bind corresponding antibodies of the specimen. After washing a wells to remove all unbound

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sample material horseradish peroxidase(HRP) labelled protein a conjugate is added. This conjugate bind to the captured *E. histolytica* specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethyl benzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of *E. histolytica* specific antibodies in the specimen. Sulphoric acid is added to stop the reaction. This produces a yellow endpoint color. Absorbance at 450 nm is read using an ELISA micro well plate reader.

**2-6-Statistical analysis:** $\chi^2$ (chi-square) test in style of independent and in style of homogeneous, Duncans multiple - range test style of comparison between the levels of the factors were used. The significant level used was $P< 0.05, 0.1$.

## 3. RESULTS

This study had designed for detection and differentiation between *E. histolytica* and *E. dispar* in Kirkuk city. The result in Table (1) showed that the overall prevalence of *E. histolytica/dispar* was 24.4%, there were significant differences between the sexes for the infection. The infection rate was 27.4% in male comparing to the female rate ( 21%). Figures (1, 2) shows the cystic and trophozoite stages of *E. histolytica / dispar* as seen under compound light microscope by 40x lens.

**Table (1):** *Entamoeba histolytica / dispar* prevalence by direct microscopic examination according to sex.

| Sex | Examined No. | +ve No. | % | -ve No. | % |
|---|---|---|---|---|---|
| Female | 185 | 39 | 21 | 146 | 79 |
| Male | 212 | 58 | 27.4 | 154 | 72.6 |
| Total | 397 | 97 | 24.4 | 300 | 76.6 |
| $\chi^2$ value | Evaluated $\chi^2$ value =5.0, $\chi^2$ value of $P< 0.05$ =3.84 (significant**) | | | | |

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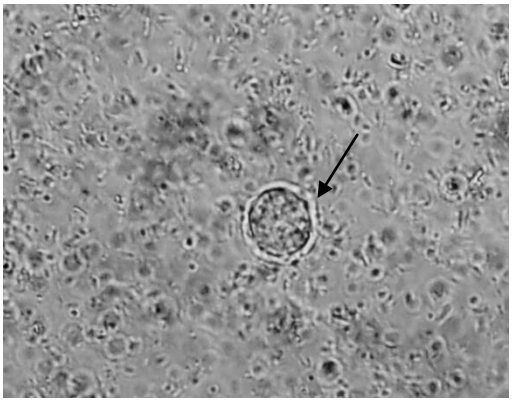

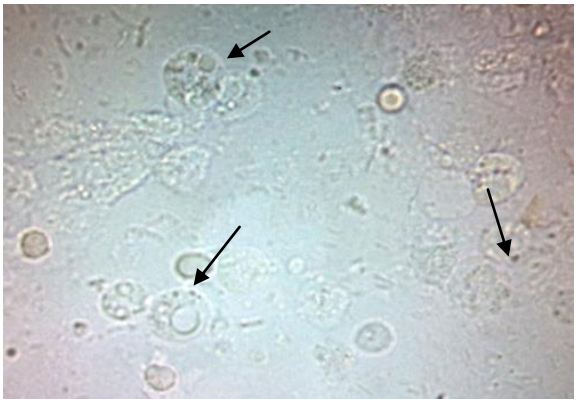

**Fig. (1):** *Entaoeba histolytica* cyst stage **Fig. (2) :** *Entaoeba histolytica* trophozoite stage (compound light microscope 40x)

Among 97 microscopically *Entamoeba histolytica / dispar* positive samples, 87 were positive with rate of 89.7 % when examined by ELISA kit specific to *E. histolytica*. The negative (10) samples were considered to be *Entamoeba dispar* with frequency rate of 10.3%, Table (2) .

**Table (2):** *Entamoeba histolytica* prevalence by ELISA stool antigen

| Sex | *Entamoeba* +ve No. microscopically % | *E. histolytic* +ve No. % | *E. dispar* +ve No. % |
|---|---|---|---|
| Male | 58<br>27.4 | 52<br>89.65 | 6<br>10.34 |
| Female | 39<br>21 | 35<br>89.74 | 4<br>10.25 |
| Total | 97<br>24.4 | 87<br>89.7 | 10<br>10.3 |
| $\chi^2$ value | Evaluated $\chi^2$ value =0.004, $\chi^2$ value of P< 0.05 =5.99 (none significant) | | |

IgG rate detected by ELISA kit in both male and female could be noticed in Table (3). The IgG frequency rate was 27.58%, with no significant differences between the sexes .

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**Table (3):** IgG rate detected by ELISA kit

| Sex | Total Entamoeba +ve No. % | E. hitolytica +ve No. by stool antigen % | Serum IgG +ve No. % |
|---|---|---|---|
| Male | 58<br>27.4 | 52<br>89.65 | 15<br>28.84 |
| Female | 39<br>21 | 35<br>89.74 | 9<br>25.7 |
| Total | 97<br>24.4 | 87<br>89.7 | 24<br>27.58 |
| $\chi^2$ value | Evaluated $\chi^2$ value =0.0001, $\chi^2$ value of P< 0.05 =5.99 (none significant) | | |

Table (4) shows that the significantly highest frequency rate in males were among <1, 21-30 and 41-50 age groups, with the rate of 34.61, 34.7, 39.13% respectively for each age group. While the lowest age group which were infected was 11-20 years with the rate of 15 %.

**Table (4):** *Entamoeba histolytica / dispar* prevalence by direct microscopic examination according to age group in male.

| Age group | Total N0. examined | +ve No. | % | -ve No. | % |
|---|---|---|---|---|---|
| <1 | 52 | 18 | 34.61 | 34 | 65.39 |
| 1-10 | 54 | 10 | 18.51 | 44 | 81.49 |
| 11-20 | 20 | 3 | 15 | 17 | 85 |
| 21-30 | 23 | 8 | 34.7 | 15 | 65.3 |
| 31-40 | 13 | 2 | 15.38 | 11 | 84.62 |
| 41-50 | 23 | 9 | 39.13 | 14 | 60.87 |
| >50 | 27 | 8 | 29.62 | 19 | 70.38 |
| Total | 212 | 58 | 27 | 154 | 72.6% |
| $\chi^2$ value | Evaluated $\chi^2$ value =17.8, $\chi^2$ value of P< 0.05 =12.6 (significant**) | | | | |

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The result in Table (5) revealed that the most female age group which were infected with *E. histolytica/dispar* was 41-50 and >50 years , with the rate of 28.57 and 34.61 % respectively for each age group. The lowest age group which were infected was <1 year with the rate of 11.9 %. With no significant differences between them.

**Table (5):** *Entamoeba histolytica / dispar* prevalence by direct microscopic examination according to age group in female

| Age group | Total No. examined | +ve No. | % | -ve No. | % |
|---|---|---|---|---|---|
| <1 | 42 | 5 | 11.9 | 37 | 88.1 |
| 1-10 | 58 | 13 | 22.41 | 45 | 87.59 |
| 11-20 | 17 | 4 | 23.5 | 13 | 76.5 |
| 21-30 | 12 | 2 | 16.6 | 10 | 83.4 |
| 31-40 | 16 | 2 | 12.5 | 14 | 87.5 |
| 41-50 | 14 | 4 | 28.57 | 10 | 71.47 |
| >50 | 26 | 9 | 34.61 | 17 | 65.39 |
| Total | 185 | 39 | 21 | 146 | 21 |
| $\chi^2$ value | Evaluated $\chi^2$ value =12,2, $\chi^2$ value of P< 0.05 =12.6 (none significant) | | | | |

Tables (6) and (7) summaries the results of microscopic finding in relation to *E. histolytica/dispar* positive samples. A significantly high frequency (62.9, 94.8 %) of *E. histolytica /dispar* positive samples were contained RBC and pus cells respectively, and the highest rate (28.8, 39.1%) were for those samples contained three pluses respectively for each type of the cells. Figures (3), (4) shows the RBC and pus cells.

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**Table (6):** frequency of pus cells appearance in *Entamoeba histolytica / dispar* positive samples

| Sex | Total *Entamoeba* +ve No. | | Samples with pus | | | | | Without pus |
|---|---|---|---|---|---|---|---|---|
| | | | Few | + | ++ | +++ | Total | |
| Male | No. | 58 | 5 | 14 | 13 | 23 | 55 | 3 |
| | % | | 8.62 | 24.13 | 22.41 | 39.65 | 94.8 | 5.17 |
| Female | No. | 39 | 8 | 9 | 5 | 15 | 37 | 2 |
| | % | | 20.5 | 23.07 | 12.8 | 38.46 | 94.9 | 5.12 |
| Total | No. | 97 | 13 | 23 | 18 | 38 | 92 | 5 |
| | % | 100 | 13.4 | 23.71 | 18.55 | 39.1 | 94.8 | 5.1 |
| $\chi^2$ value | Evaluated $\chi^2$ value =44.5 $\chi^2$ value of P< 0.05 =9.49 (significant**) | | | | | | | |

**Table (7):** frequency of RBC cells appearance in *Entamoeba histolytica / dispar* positive samples

| Sex | Total Entamoeba +ve No. | | Samples with RBC | | | | | Without RBC |
|---|---|---|---|---|---|---|---|---|
| | | | Few | + | ++ | +++ | Total | |
| Male | No. | 58 | 3 | 9 | 8 | 17 | 37 | 21 |
| | % | | 5.17 | 15.51 | 13.79 | 29.31 | 63.7 | 36.2 |
| Female | No. | 39 | 4 | 7 | 2 | 11 | 24 | 15 |
| | % | | 10.25 | 17.94 | 5.12 | 28.2 | 61.5 | 38.46 |
| Total | No. | 97 | 7 | 16 | 10 | 28 | 61 | 36 |
| | % | 100 | 7.21 | 16.4 | 10.3 | 28.8 | 62.9 | 37.1 |
| $\chi^2$ value | Evaluated $\chi^2$ value =2.9 $\chi^2$ value of P< 0.1 = 2.7 (significant*) | | | | | | | |

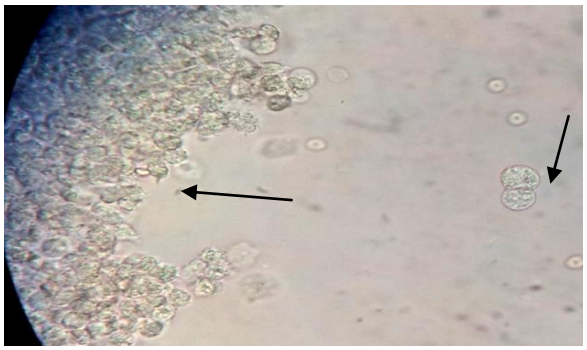

**Fig. (3) :** Pus cells aggregation

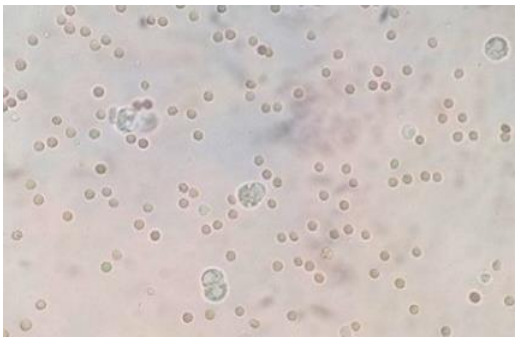

**Fig. (4) :** RBC and pus cells

(compound light microscope 40x)

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The Table (8) revealed that the most significantly common symptoms related with *E. histolytica / dispar* infection was abdominal pain with rate of 44.56% followed by diarrhea with rate of 38% from 94.84 % of all symptomatic infection.

**Table (8):** *Entamoeba histolytica / dispar* related symptoms

| Symptoms / Sex | | Fever | Vomiting | Diarrhea | Cough | Abdominal pain | Total symptomatic cases | Asymptomatic cases |
|---|---|---|---|---|---|---|---|---|
| Male | No | 12 | 8 | 19 | 4 | 22 | 54 | 4 |
| | % | 12.37 | 8.8 | 19.58 | 4.32 | 22.6 | 92.31 | 6.89 |
| Female | No | 13 | 9 | 16 | 4 | 19 | 38 | 1 |
| | % | 26.74 | 23.5 | 38.23 | 11.71 | 32.35 | 97.51 | 2.56 |
| Total | No | 25 | 17 | 35 | 8 | 41 | 92 | 5 |
| | % | 27.17 | 18.47 | 38 | 8.69 | 44.56 | 94.84 | 5.14 |
| $\chi^2$ value | Evaluated $\chi^2$ value =5.1 $\chi^2$ value of P< 0.1 =4.61 (significant) | | | | | | | |

The most residential area Table (9) which were infected was Qadsya with frequency rate of 24.7 % followed by Hay AL-Askary with frequency rate of 17.5 %, while shoraw had lowest frequency rate 4.1 %.

**Table (9):** *Entamoeba histolytica/dispar* prevalence according to residential area

| Residential area | Positive samples | % |
|---|---|---|
| Hay AL-wasty | 13 | 13.4 |
| Qadsya | 24 | 24.7 |
| Escan - Azadi | 10 | 10.3 |
| Hay AL-Askary | 17 | 17.5 |
| Hay AL-baath | 9 | 9.3 |
| Shorja | 9 | 9.3 |
| Penja ali | 11 | 11.3 |
| Shoraw | 4 | 4.1 |
| Total | 97 | 100 |

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## 4. DISCUSSION

In the current study the prevalence of *E. histolytica/dispar* in male and female in Kirkuk city were detected by microscopic examination and ELISA test. When 397 stool samples were examined microscopically, 97samples of them were positive for *E. histolytica/dispar* with rate of 24.4 %, identical rates were recorded by others, 25.66, 25.4, 20.4, 27 % [8,10,11,7]. Higher or lower prevalence (17.03, 53.6, 40.5, 15, 57.1%) were found by [6, 9, 12-14], and they were further added that the variation in the prevalence over time may be related to size of the population under study, the number of stool specimens examined per patient, and the duration of the study. The prevalence of amoebiasis in this study in male was 27.4% . while in female was 21% with significant differences between them. This espouse that amoebiasis infect male more than female, as indicated by [13, 15,16] that males were more infected than females, which may be due to that males are more susceptible or they spend more time than females outdoor. But this result was not in agreement with those reported by [2,17] that both sexes have an equal rate of infection, or that the infection rate in women is higher than man [18] .

Microscopic identification remains the most common routine means of diagnosis used in areas where amoebiasis is endemic. However, it is both insensitive and nonspecific (*E. histolytica* cannot be distinguished from *E. dispar* morphologically) [5]. This consented in the current study the microscopy positive samples were 97, these specimen when further examined by ELISA 87sample were *E. hitolytica* and 10 were *E.* dispar. This result indicate that in communities, the presence of either four-nuclei amoeba or trophozoites in the stool of a patient with diarrhea is not equal to amebiasis (the presence of the pathogenic *E. histolytica*), and other confirmatory test is required. The *E. histolytica*-specific ELISA was shown to be a sensitive and specific method for the rapid differentiation of the two species [8, 9 13, 14, 19]. In a study the result of the *E. histolytica*-specific ELISA was shown to be comparable to those obtained with the PCR [19]. It is important when one considers the currently available treatments, some of which have undesirable side effects, metronidazole for example is listed by the US National Toxicology Program (NTP) as reasonably anticipated to be a human carcinogen[20]. A rate of 27.58% of the ELISA positive specimen in present study had IgG antibody in their serum. Identical result revealed that among 82 samples only 10 were positive for serum IgG antibody against *E. histolytica* [13].The serum IgG antibody against *E. histolytica* was detected in 36.17 % using DRG *E. histolytica* serum IgG ELISA [8]. And were 32.7 % using TechLab ELISA [20], higher rate (97%) was recorded by [14].

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The high rate of serum IgG antibody against *E. histolytica* may be due the persistence of IgG antibodies for one year or more after the initial infection and the IgM antibody will disappear after short period of time, or due to the incomplete drug treatment, or the antibody was due to an extraintestinal infection [21, 22].

This study revealed that the most age group infected by *Entamoeba* was elderly peoples (39.13%) and children (34.6%) this may be due to fact that children begin to taste different foods especially after one years old, in addition they become in contact with more contaminated things during playing. In high prevalence in elderly may be because this age group often spends more of their leisure time outdoors which expose them to more contaminants ,or because of their behavior like eating out side and un washing their hands. This is close to the results recorded by [23] in Diwania, which demonstrated the occurrence of amoebiasis at a rate of 66% among 1-5 years of age. Also this agrees with the result of [12], whom revealed that the rate of infection was the highest (60%) in the age-group 1-10 years. And with those found by [13, 15].The R.B.C and pus cells were more seen in *E. histolytica* positive samples, similar results were indicated by others, high rate of *E. histolytica* stages contained samples had high pus and RBC cells [12]. A rate of 100% (5/5) of *E. histolytica* microscopy positive samples had blood in their stool, and similar rate 100% (2/2) of *E. histolytica* positive samples by ELISA had pus cells in their stool [9]. 34.5, 28.6 % of *E. histolytica* positive samples had bloody diarrhea[13, 24], this may due to that high number (89.7%) of examined samples were positive for *E. histolytica* the pathogenic amoeba that had the ability of invading the mucosa and engulfing the RBCs and causing inflammation at site of infection.The appearance of symptoms were more related with *E. histolytica* positive samples than those of negative one. The highest symptoms were for those patients suffered from abdominal pain (44.56%) and diarrhea (38%). This result is somehow in agree with that detected by [13] whom revealed 34.5 % of patients to have bloody diarrhea and 65.5% of them to have vomiting, and with that found by [14]in which 84, 64% of *E. histolytica* positive samples had acute and chronic diarrhea respectively. 13/14 of *E. histolytica* positive samples had symptoms versus 1/14 of a symptomatic one[25, 26]. A rate of 71.4 , 28.6% of *E. histolytica* positive samples had diarrhea and bloody diarrhea respectively[24]. This may due to same season mentioned above that most of positive cases were *E. histolytica* the pathogen that can cause symptoms. The highest frequency of *E. histolytica / disbar* was in Qadsya region (24.7%) followed by Hay-Alaskary (17.5). The prevalence of the parasite is

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differ from area to anther and from region to another inside the same city [6, 9, 13,14] the variation in the prevalence may be related to the differences in the environmental condition or due to poor hygiene and pooresty of populations, and the time of the study. The conclusion of the present study is that there is a necessity of a serology confirmatory test after microscopic detection of *E. histolytica* to avoid un necessary treatment, therefore we recommend that ELISA procedures based on reliable antigens or antibodies be used in this region. Because unfortunately, PCR methods are still too sophisticated and expensive for the public health systems of these communities.

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