

Original Research Article

Detection and clinical characteristics of *Entamoeba histolytica* infection among children in Kirkuk-Iraq

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Abstract: Amoebiasis is one of important health problems in developing countries. It is commonly reported among human beings with different frequency of distribution in different parts of the world. The aim of this study was to detect clinical signs and symptoms and to compare sensitivity and specificity of conventional stool examination and qualitative enzyme immunoassay (EIA) panel kit to detect *E. histolytica*/*E. dispar* among children in Kirkuk hospitals. The method in a study was carried on 800 stool samples from children attended Kirkuk hospitals for period from February 2007 to end of January 2009, as well as another 100 children samples of the near ages of the patients as a control group. The ages of children were ranging from below one month to 12 years old. The stool samples were examined by direct stool examination and qualitative enzyme immunoassay (EIA) panel kit. In results the morphology of *E. histolytica* cysts for which modified D'antoni's iodine and 1% lugol's iodine (weak iodine solution) was clearer than that strong iodine solution. It was found that the sensitivity, specificity, and efficiency of Enzyme immunoassay (EIA) test for *E. histolytica*/*E. dispar* were 91.07%, 98% and 93.2% respectively. The clinical signs and symptoms were increased when the bacterial infections associated with *E. histolytica*/*E. dispar* infections.

Keywords: Detection *E. histolytica* and *E. dispar*, clinical characteristics.

INTRODUCTION

It is now known that amoebiasis is caused by two very similar species *E. histolytica* and *E. dispar*. *E. histolytica* and *E. dispar* are genetically distinct but closely related protozoan species [1]. The former is the cause of all invasive diseases, with an estimated 100,000 fatalities each year [2]. Persistent diarrhea should not be confused with chronic diarrhea which is recurrent or long lasting-diarrhea due to noninfectious causes, such as sensitivity to gluten or inherited metabolic disorders [3].

Microscopic diagnosis of *E. histolytica*/*E. dispar* complex on stool samples requires technical expertise because of the existence of similar amoebas or artifacts that can be misdiagnosed as *E. histolytica*/*E. dispar*.

Entamoeba histolytica is a pathogen or invasive parasite, whereas *E. dispar* and *E. moshkovskii* are non-pathogenic and non-invasive parasites that are identical morphologically to *E. histolytica* [4]. There are at least eight amoebas (*E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. hartmanni*, *E. polecki*, *Iodamoeba butschlii* and *Endolimax nana*) which live in

the human intestinal lumen, however, these are generally accepted as commensal organisms except *E. histolytica* [5].

Diagnosis via microscopic examination of single stool specimen has a low sensitivity and may be missed. Therefore, up to 50% of *Entamoeba* infections are because of the intermitted shedding of the parasites which take the microscopic examination of 3 consecutive stool-specimens to reach sensitivity over 90% [6]. It has been accepted that the positive predictive value (PPV) of microscopic diagnosis of *E. histolytica* is low and that alternative causes for the complaints with which the patient presented should always be taken seriously [7].

In order to find simple, inexpensive and reliable diagnostic techniques for detecting intestinal infections with *E. histolytica*, Triage Parasite panel Enzyme Immunoassay has been developed and tested in various studies [8,9]. For evaluating the performance of commercially available Triage parasite panel Enzyme Immunoassay kit for detecting *E. histolytica*/*E. dispar*, *G. lamblia*, *C. parvum*. It is rapid, easy to use and can

be used as a screen for immediate testing of stool specimens [10]. In Kenya [11] evaluated the Triage Micro Parasite Panel in detecting *E. histolytica*/*E. dispar*, *G. lamblia* and *C. parvum* compared to O&P examination in 266 stool samples, they found that the sensitivity and specificity results for Triage Micro Parasite Panel were for *E. histolytica*/*E. dispar* and *Giardia lamblia* 100%, 100% for each and for *C. parvum* 73%, 100%. There was no evidence of cross reactivity using the kit with other parasites identified in the stool samples.

The aim of this study was to detect *E. histolytica* and *E. dispar* in children and clinical symptoms associated with this infection in Kirkuk City.

MATERIALS AND METHODS

The study was carried out on patients attended Kirkuk Pediatric hospital and pediatric wards in Al-Hawija and Kirkuk General Hospitals. The period of study was from beginning of February 2007 to the end of January 2009. A total of 800 children with invasive diarrhea who requested medical advice were included in this study, their age were ranging from below 2 years up to 12 years

In addition, one hundred children matched by age, sex, socio-economic strata were chosen as a control group amongst children brought to Al – Salam primary Health care center (PHCC) near kirkuk pediatric hospital for routine children medical care .

A full history was taken from the parents of each child regarding the clinical features like fever abdominal pain, tenesmus, vomiting and rectal prolaps. These patients that admitted to hospital not received antibiotics and patients who received antibiotics excluded from the study.

Fresh stool specimen were collected from the subjects into sterile containers and transported in to a cooled box (temperature approximately 10 °C). Stool samples were divided into three portions within two hours of collection on arrival at Kirkuk pediatric hospital laboratory. One portion was for the direct examination of parasites; the second portion was cultured for bacteriological examination while the third portion was stored immediately at -20°C and tested later by a new qualitative enzyme immunoassay (EIA) panel kit.

Stools were examined macroscopically for parasites and microscopically for ova and cysts using wet mount technique, Stools were examined macroscopically for parasites and microscopically for ova and cysts of parasites by direct microscopy. Stool specimens were examined unstained or stained with Lugol's or D'Antonis Iodine iodine for Chlamydia.

Iodine for Chlamydia. The pHs of stool specimens was determined with pH paper.

Stool specimens were cultured within the same day of collection on MacConkey agar (MA), Salmonella-Shigella agar (SSA), Sorbitol MacConkey agar (SMA), Thiosulphate citrate bile sucrose agar (TCBS). The specimens were also enriched in both tetrathionate and alkaline peptone water. The first is subcultured onto SSA and the second onto TCBS.

MA and SSA were used for isolation of *Escherichia coli* (EPEC, EIEC), *Salmonella* spp. and *Shigella* spp. TCBS is used for isolation of *Vibrio* species, SMA was used for isolation of *Escherichia coli* O157:H7 and alkali treatment method for *Y. enterocolitica*. Plates were incubated at 37°C for 24-48 hours except those used to identify *Y. enterocolitica* by incubation at 28°C [12].

Enzyme immunoassay (EIA) Test

Assay procedure Triage parasite panel is a qualitative enzyme immunoassay (EIA) which is a single immunochromatographic strip coated with monoclonal antibodies specific for *E. histolytica*/*E. dispar* antigen (29 KDA) and for antigens of *G. lamblia* and *C. parvum* (Biosite Diagnostics, San Diego, Calif).

A qualitative enzyme immunoassay (EIA) is used according to the following manufacturer's directions. The assay procedure involves the addition of 4.5 ml of specimen diluents to the specimen tube sample (0.5 ml) and the mixture is vortexed for at least 10 seconds. The mixed sample is centrifuged at 1,500 Xg for at least 5 minutes. The supernatant is poured into the sample filter device and is filtered into filtrate tube. The filtered sample (0.5 ml) is then added to the center of test device with a transfer pipette. Enzyme conjugate (140 µl) is added to the center of the membrane. Six drops of washing solution is added to the membrane. This step is repeated twice then four drops of the substrate is added to the membrane followed by 5 min incubation at 25 °C. The device is then read and the results are interpreted. Positive results are visualized as purple black lines in the appropriate positions in the result window. The EIA test was carried on 343 samples (100 *E. histolytica* positive test, 100 *E. histolytica*, negative by Conventional microscopic examination, 124 *E. histolytica*/*E. dispar* associated with bacteria and 19 dual and triple infections).

The following term and equation were used for detecting the efficiency of laboratory methods in detecting *Entamoeba histolytica*/*E. dispar*; TP=True positive, TN=True negative, FP=False positive, FN=False negative. Sensitivity=TP/(TP+FN), Specificity=TN/(TN+FP).

To calculate sensitivity, specificity and efficiency of Triage parasite enzyme immunoassay kit, the following formulas [13].

EIA [▼]	CME	
	Positive	Negative
Positive	a (True positives)	b (False positives)
Negative	c (False negatives)	d (True negatives)

▼ Triage parasite enzyme immunoassay.

$$\text{Sensitivity} = \frac{a}{a + c} \times 100$$

$$\text{Specificity} = \frac{d}{b + d} \times 100$$

$$\text{Efficiency} = \frac{a + d}{a + b + c + d} \times 100$$

The specimens which are positive with both Triage parasite panel enzyme immunoassay and CME are considered true positive (a). A number of specimens which were negative for *E. Histolytica* by both methods were considered to be true negative (d). Specimens that were Positive by CME and negative by triage parasite enzyme immunoassay were considered false Negative (c). Specimen that is negative by CME and positive by Triage Parasite Panel Enzyme Immunoassay are considered false positive (b).

For the purpose, 324 selected specimens are tested for EIA test following CME. They were 124 specimens *E. histolytica* associated with bacteria, 100 *E. histolytica* alone and 100 *E. histolytica* negative. The triple and dual infections are not included due to possibility of cross reaction of *G. lamblia* and *C. parvavum* with *E. histolytica/E. dispar*. Statistical analysis is done using the chi-square (χ^2) of independency and homogeneity test with Yates

correction. Probabilities of ($P < 0.05$) were considered statistically significant [14].

RESULTS

The prevalence of parasitic infection among patients was as the following: *E. histolytica/E. dispar* 42.5%, and *E. vermicular* is 0.62% while in control groups the rate of *E. histolytica* was 2%. From examination of 20 stool samples using 7 types of iodine solution, it was found that the best morphology of *E. histolytica* cyst was observed, using both modified D'antoni's iodine and routinely used 1% Lugols' iodine. While the morphology of parasite was not clearly visible using other types of iodine as Lugols iodine 5%, gram iodine, Alberts iodine, iodine for staining chlamydia. (Figures 1,2 &3), indicates that the structure of the parasite was best seen when using modified D'antoni's iodine and Lugols' iodine than using iodine for staining Chlamydia and their morphology of parasites was not significantly varied when using other types of iodine stains.

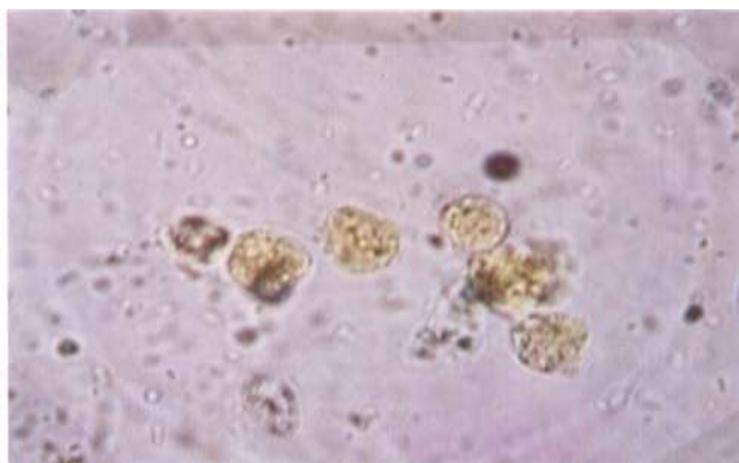


Fig-1: Photograph of *E. histolytica* Cyst Stained with Modified Da'ntonis' Iodine (X1000).

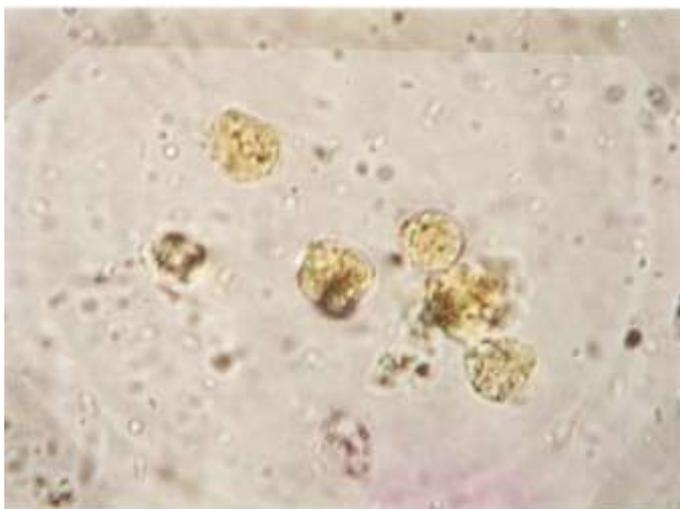


Fig-2: Photograph of *E. histolytica* Cyst Stained with Lugols' Photograph of *E. histolytica* Cyst Stained with Iodine for Chlamydia (X1000).

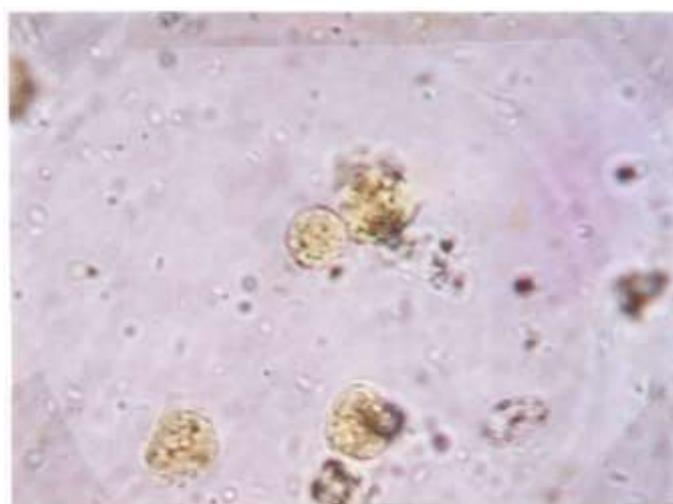


Fig-3: Photograph of *E. histolytica* Cyst Stained with with Iodine for Chlamydia (X1000).

Sensitivity and Specificity of EIA Test

The results of 124 samples which were positive for *E. histolytica* by CME and bacteria with culture methods, were compared with EIA test for *E. histolytica*/*E. dispar*.

dispar indicates that out of 124 samples only 116 were positive for *E. histolytica*/*E. dispar* with EIA test and 8 were negative as shown in Table 1.

Table 1: Comparison between the Accuracy of CME[▼] and EIA[■] Test for Enteropathogens

Type of pathogen	Bacteria associated with <i>E. histolytica</i> (CME)	Traige parasite panel (EIA) test for <i>E. histolytica</i> / <i>E. dispar</i>	
		Positive	Negative
<i>P. aeruginosa</i>	42	40	2
EIEC	25	24	1
EPECI	25	24	1
<i>S. flexneri</i>	12	12	0
<i>S. typhi</i>	12	10	2
Non O157:H7 <i>E. coli</i>	3	3	-
<i>P. shigellodies</i>	5	3	2
Total	124	116	8

▼CME: Conventional microscopic examination. ■ EIA: Enzyme immunoassay.

To calculate sensitivity, specificity and efficiency of Triage parasite enzyme immunoassay kit, 324 specimens were tested for EIA test following CME. They were 124 specimens *E. histolytica* associated with

bacteria, 100 *E. histolytica* alone and 100 *E. histolytica* negative. It was found the sensitivity of EIA was 91.07%, specificity 98% and efficiency 93.2% as follows:.

		CME	
	+	-	Total
+ <i>Triage</i>	204	2	206
<i>E. histolytica/</i>	(a)	(b)	
- <i>E. dispar</i>	20	98	118
	(c)	(d)	
(EIA)	224	100	324

The sensitivity of EIA test was: $\frac{204}{204 + 20} \times 100 = 91.07\%$

The specificity of the test was: $\frac{98}{2 + 98} \times 100 = 98\%$

The efficiency of the test was: $\frac{204 + 98}{204 + 2 + 20 + 98} \times 100 = 93.2\%$

For testing the sensitivity, specificity and efficiency of EIA for *E. histolytica* positive and *E. histolytica* negative specimens were tested. It was found

that the sensitivity of the test was 88%, specificity 98% and efficiency was 93% as illustrated in the following formula.

		CME	
	+	-	Total
+	88	2	90
	(a)	(b)	
-	12	98	110
	(c)	(d)	
	100	100	200

Sensitivity = $\frac{88}{88 + 12} = 88\%$

Specificity = $\frac{98}{2 + 98} = 98\%$

Efficiency = $\frac{88 + 98}{88 + 2 + 12 + 98} \times 100 = 93\%$

Multiple infection are mostly encountered in those cases with *E. histolytica/E. dispar* that are 125 patients: 40 with *P. aeruginosa*, 24 with EPEC I, 24 with EIEC, 12 with *S. flexner*, 10 with *S. typhi*, 3 with Non O157:H7 *E. coli* and 3 with *P. shigelloides* (Table

4-11a), 6 with *G. lamblia* and (3) with *C. parvum*. The rate of bacterial infections associated with *E. histolytica/ E. dispar* was higher than parasitic infections (Table 2).

Table 2: Distribution of *E. histolytica/ E. dispar* with enteropathogena using EIA test among 125 patients

Enteropathogens	Number	Percentage
<i>Pseudomonas Aeruginosa</i>	40	32.0%
EPECI	24	19.2%
EIEC	24	19.2%
<i>S. flexneri</i>	12	9.6%
<i>S. typhi</i>	10	8.0%
Non O157:H7 <i>E.coli</i>	3	2.4%
<i>P. shigelloides</i>	3	2.4%
Total	116	92.8%
<i>G. lamblia</i>	6	4.8%
<i>C. parvum</i>	3	2.4%

Clinical Signs and Symptoms

The clinical signs and symptoms associated with *E. histolytica* are shown in Table 3. It was found that tenesmus (82.35% was predominantly seen in amoebiasis followed by mucus in stool (40.88%); fever (38.82%); vomiting (32.94%) and rectal prolaps (31.76%).

In general the clinical signs and symptoms were increased when the bacterial infections associated with *E. histolytica*/*E. dispar* infections. The detail of clinical signs and symptoms for each organisms associated with *E. histolytica* mixed infection are illustrated in Table 3. Statistically there was significant difference in clinical signs and symptoms between two main groups of infections *E. histolytica*, and *E. histolytica*/*E. dispar* associated with bacteria ($P < 0.05$).

Table 3: Clinical characteristics for *E. histolytica* and associated bacteria

Pathogens	No	Mucus in stools No. (%)	Fever No. (%)	Vomiting No. (%)	Tenesmus No. (%)	Rectal Prolaps No. (%)
<i>E. histolytica</i> / <i>dispar</i>	340	139 (40.88)	132 (38.82)	112 (32.94)	280 (82.35)	108 (31.76)
<i>E. histolytica</i> / <i>dispar</i> associated with bacteria						
<i>P. aeruginosa</i>	40	12 (30)	30 (75)	21 (52.5)	19 (47.5)	6 (15)
EPEC 1	24	12 (50)	21 (87.5)	9 (37.5)	23 (95.8)	16 (66.6)
EIEC	24	23 (95.8)	24 (100)	15 (62.5)	24 (100)	12 (50)
<i>Shigella flexneri</i>	12	12 (100)	12 (100)	6 (50)	10 (83.3)	10 (83.3)
<i>Salmonella typhi</i>	10	8 (80)	10 (100)	4 (40)	10 (100)	7 (70)
Non O157:H7 <i>E. coli</i>	3	1 (33.3)	2 (66.6)	2 (66.6) 3 (100)		2 (66.6)
<i>Plesiomonas shigelloides</i>	3	3 (100)	3 (100)	1 (33.3)	3 (100)	1 (33.3)
Total	116	71 (61.20)	102 (87.93)	58 (50)	92 (79.31)	54 (46.55)
P<0.05						

DISCUSSION

In this study, different types of iodine solution are used in wet mount of stool samples examination. The structure of the parasite was most clearly seen using modified D’Antoni’s iodine as shown in Figure 1 and 1% lugol’s iodine as shown in Figure 2 which is routinely used in our diagnostic laboratory. The morphology of cysts in the use of this weak iodine solution is clearer than that of the use of Chlamydia and stock solution of lugol’s iodine as shown in Figure 3. This reflects that the strong iodine is not as efficient as weak iodine solution. It has been reported that the strong iodine tend to coagulate the faecal particle and destroy the refractile nature of the organism [15]. It is also recommended by WHO [16] that the stock iodine solution 5% to be diluted to 1% and freshly prepared every two week for wet mounts technique. The other iodine solution were inferior than 1% iodine solution and modified D’Anton’s iodine, therefore, these two iodine solutions were routinely used in general stool examination in this study. Shetting and Prabhu [17] found that D’Anton’s iodine was much better than saline or buffered methylene blue for detection of *E. histolytica* cysts while saline and buffered methylene blue were equally good for detection of *E. histolytica* trophozoites.

In order to find simple, inexpensive and reliable diagnostic techniques for detecting intestinal infections with *E. histolytica*, Triage Parasite panel Enzyme Immunoassay has been developed recently and tested in various studied [8,9]. For evaluating the performance of commercially available Triage parasite panel Enzyme Immunoassay kit for detecting *E. histolytica*/*E. dispar*. *G. lamblia*, *C. parvum*, 324 specimens examined by conventional microscopy were compared with the results of Triage *E. histolytica*/*E. dispar* kit. Comparing the sensitivity and specificity of EIA test when applied on the whole 324 samples which include *E. histolytica* associated with bacteria, *E. histolytica* alone and negative samples, the sensitivity of EIA on the whole samples was 91.07% which is slightly higher than that of which tested on *E. histolytica* specimens alone that is 88% while the specificity of the test is 98% in both whole samples and *E. histolytica* alone and efficacy being 93%. The sensitivity and specificity of EIA test applied in this study is almost identical to that referred by the Biosite diagnostics San Diego Calf Company which referred to sensitivity 87% and specificity 99%. The finding of this study was in agreement with that reported by other studies who reported the sensitivity of the test ranging from 68.3% - 95% and specificity ranging from 97% - 99% [18, 19].

The high sensitivity and specificity of the EIA test and its simplicity to be used in our diagnostic laboratory in the future, because it is costly in the present time. As it is mentioned earlier, microscopic examinations of one single stool specimen has low sensitivity [20, 21]. This reflects that stool antigen assays are more sensitive and specific than microscopy for diagnosis of *E. histolytica* [22]. Diagnostic problems arise when only cysts are identified in stools of healthy or diarrhoeic individuals. A commercially available laboratory test based on the identification of specific *E. histolytica* antigens in stool is able to discriminate *E. dispar* cysts [18]. However, the high cost and lack of knowledge of this test have hindered its use in clinical laboratories, especially in countries where amoebiasis is endemic. Until these new diagnostic tests are widely available to clinical laboratories, these samples should be reported as containing *E. histolytica/E. dispar* [23].

Stool antigen assay has been shown to be as sensitive and specific as culture with isoenzyme analysis and to outperform microscopy for detection of *E. histolytica* in areas of endemicity [18].

Furthermore, detection of positive zones of *E. histolytica/E. dispar*, *G. lamblia* and *C. Yparvum* by EIA test could be considered as an alternative method for performing simultaneous discrete detection of *Giardia*, *Cryptosporidium* and *E. histolytica/E. dispar* specific antigen in patient faecal specimens. *G. lamblia* or *E. histolytica* can be detected by EIA test even in the absence of intact organisms (cysts or trophozoites). This reflects to greater sensitivity of EIA tests compared with microscopy.

Another parasitic agent detected was *G. lamblia* with *E. histolytica/ E. dispar* in 6 patients (0.75%) and *C. parvum* with *E. histolytica/E. dispar* in 3 patients (0.37%). *Cryptosporidium*, and *Giardia lamblia* enteric pathogen, waterborne, which has been looked for in Iraq [6, 24, 25] of which reports are rare in the area which may be because of a specific diagnostic method is not being used routinely during stool examination in our country.

Clinical Signs and Symptoms

In this study several parameters are added to aid through them in trying to help the clinician to make a rapid judgment as to the probably causative agent in the presenting case of gastroenteritis. These parameters are: presence of blood, mucus in stool, consistency of the stool, presence or absence of fever, tenesmus and prolaps of the rectum.

The clinical signs and symptoms of amoebiasis are varied from one child to another. In general the main clinical signs and symptoms in case of amoebiasis were as the following: mucus in stool 40.88%, fever

38.8%, vomiting 32.9%, and tenesmus 82.35%. These findings are in accordance with that reported by Peter *et al.* [26]. Thus the researchers see that the wide spectrum in the rate of clinical signs and symptoms among the studied groups of children might be due to *E. dispar* infection misdiagnosed by *E. histolytica* [27]. Zaki *et al.*; [28] have been reported that the presence of both types of parasite (*E. histolytica/E. dispar*) and / or different strains of either parasite in the same patient could be one of the reasons for the differences in signs and symptoms in infected persons.

Detection of rectal prolaps in cases of children infected with amoebiasis may be due to complications such as intestinal stricture formation amoeboma, which lead to abdominal pain or difficulty with defecation. In addition to that the disease can progress to severe involvement of the colon with dilatation and paralysis resembling a toxic megacolon or ulcerative colitis [27]. The increase in clinical signs and symptoms in case of *E. histolytica/E. dispar* associated with bacterial infection reflects the synergistic effect of bacterial and *Entamoeba* infection. This finding is also observed by Wittner and Rosenbam [29] and Mirelmam [30] who found that the virulence of *E. histolytica* increased when the culture seeded with *E. coli* and *S. typhi* infections. Finding of mucus in all cases of diarrhoea in children suffering from shigellosis reflects the invasiveness of *Shigella spp*

It is concluded that the prevalence of *E. histolytica/E. dispar* was high in Kirkuk province. The morphology of *E. histolytica/E. dispar* cysts using modified D'antoni's iodine and 1% lugol's iodine is more evident than strong Iodine solution.

The sensitivity, specificity and efficiency of EIA test was high for detection of *E. histolytica / E. dispar*. *E. histolytica/E. dispar*s were mostly associated with *P. aeruginosa* followed by EPEC, EIEC, *S. flexneri*, *S. typhi*, Non O157:H7 *E. coli* and *P. shigellodies* respectively.

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