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Multiplex-PCR for diagnosis of *Entamoeba histolytica* and *Escherichia coli* O157 in the patients of Wasit Province, Iraq

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Abstract--This study was carried out at Wasit Province / Iraq in cooperation with Fairouz Hospital/ unit of microbiology. Samples were collected from many places at Wasit province. It included 100 stool samples from Iraqi patients with diarrhea. The study was conducted during the period from October to December, 2021 to investigate the prevalence of intestinal parasites and *Escherichia coli* O157 bacteria. Only 65 stool sample (65%) were positive for *Entamoeba histolytica* and *E.coli* O157. Rate of infection in current study with intestinal parasites was 65% among diarrheal cases were that from those samples *E.histolytica*. The rate of *E.histolytica* infection was 65 (65%) samples, while only 14 *E.coli* O157 isolates (21.5%) were positive using PCR technique. The higher rate of parasitic infection 15 (23.1%) was recorded in the age group (1-10) years old, while for *E.coli* O157 infection the rate was 4 (6.1%) in the age group (1-10) years old. Regarding gender males for *E.histolytica* rate was 60% male and 40% females, for *E.coli* O157 rate was 50% to 50% in this study without significant differences.

Keywords--*E. histolytica*, *E.coli* O157, PCR, feces, human.

Introduction

In developing countries, enteric infections and diarrheal diseases represent some of the main causes of hospitalization and a serious health problem (1). However, for reasons not quite understood, *E. histolytica* transition to an invasive form and following invasion gives rise to amebic colitis with symptoms ranging from diarrhea, ameboma, and life-threatening extraintestinal invasion to the liver (2, 3). This suggests that changes in the gut environment may contribute to the pathogenesis of *E. histolytica*, leading to invasive amebiasis (4, 5). Occurrence of diarrhea is

closely associated with deficiencies in the water supply and sanitation systems and poor personal hygiene conditions, which are common in developing countries. These diarrheal diseases can be caused by bacteria like *Escherichia coli* O157 and parasites such as *E. histolytica* (6). The exposure to these pathogens is favored by the climate and is mainly related to the lack of adequate hygiene and sanitation conditions and the consumption of contaminated ready-to-eat (RTE) foods and vegetables (7).

The bacterial species *E. coli* represents one of the main hazards in food and water sources (8). However, not all *E. coli* cause diarrheal diseases (9). Its association with the occurrence of diarrhea is based on the presence of genes that encode different virulence factors such as Shiga toxin-producing *E. coli* or enterohemorrhagic *E. coli* (STEC/EHEC) (10). The presence and/or association of these virulence factors determine the *E. coli* pathogenic types that cause high morbidity and mortality worldwide and are collectively known as diarrheagenic *E. coli* (DEC) (11).

In developing countries, the growing use of antibiotics in veterinary medicine, mainly by small poultry farmers, has been associated with increased levels of antimicrobial resistance in *E. coli* strains, in fact, meat and meat products are frequently linked to the prevalence of antimicrobial resistance genes (12). Since bacteria are highly prone to exchanging genetic material, these resistance genes can be transferred to other bacteria, particularly Enterobacteriaceae (13). The sale of RTE street food, despite representing an important source of income for many unemployed families, has often been associated with outbreaks of diarrheal diseases (14). This increases the risk of diarrheal diseases, which are responsible for 35% of the total mortality, the incidence of diarrheal diseases has led to the overuse of antibiotics. Often, these antibiotics can be purchased at informal markets without a prescription, this non-medically assisted antibiotic intake is also frequently associated with incomplete antibiotic therapy, contributing to the increase in antimicrobial resistance (15, 16).

Co-infection with pathogenic organisms, especially enteropathogenic bacteria, may be an important factor that contributes to alteration of normal enteric microbiota and immune regulation, enhancing the virulence of *E. histolytica* in disease pathogenesis (17, 18). Epidemiological studies have reported frequent presence of enteropathogenic bacteria in coinfection with symptomatic intestinal amebic infection (19). Under *in vitro* culture conditions, *E. histolytica* interaction with enteropathogenic bacteria for as little as 1 h enhanced parasite adherence and cysteine protease activity, with increased cytopathic activity (20). Another study showed that short-term culture (12 h) of a pathogenic *Escherichia coli* serotype with an *E. histolytica* strain that had lost its capacity to produce amebic liver abscess (ALA) in hamsters restored parasite virulence by producing ALA (21).

Materials and Methods

Ethical Consideration

The proposal of the study was approved by the Ethical Committee of Wasit University, the patients were interviewed and the ones who were willing to participate and sign an informed consent were recruited.

Samples collection

This study was carried out in Fairouz Hospital at Wasit province. This study included the collection of 100 feces samples, which were obtained from various areas in Wasit province during the period from the beginning of October to the end of December, 2021. The stool samples were collected from diarrheal patients who attended Fairouz Hospital and some private laboratories in clean, airtight plastic bottles and examined directly. The following information was recorded on each sample, such as: name, age, gender, as well as the residence of patients.

Isolation and identification of *E.histolytica*

All stool specimens were examined under microscope after taking small amount on glass slide with normal saline and covered with cover slip to examined under 40 x for trophozoite and cyst forms (22).

Isolation and identification of *E.coli*

Culture

The stool samples were cultured on MacConkey sorbitol agar, MacConkey agar and blood agar (23).

Triple Sugar Iron (TSI) medium

The TSI medium contains phenol-red reagent, ferrous sulfate, sodium thiosulfate (to detect the production of hydrogen sulfide gas) and three sugars glucose, lactose and sucrose, the concentration of glucose in the medium is 0.1 concentration of the other two sugars (24).

PCR identification

Stool DNA Extraction

DNA from stool samples were extracted by using Presto™ Stool DNA Extraction Kit and done according to company instructions (25).

Primers

The PCR primers for detection *Entamoeba histolytica* based on small subunit ribosomal rRNA gene and detection of Shiga toxin 1 subunit A (stx1A) producing *Escherichia coli* were designed in this study using NCBI-Genbank (AB608092.1-MK635343.1) and primer 3 plus design. These primers was provided from ScientificResercher.Co.Ltd, Iraq as following table:

Primers	Sequence 5'-3'		Product size
<i>Entamoeba histolytica</i> ssrRNA gene	F	ATTGGAGGGCAAGTCTGGTG	616 bp
	R	GCCTTGTGACCATACTCCCC	
<i>Escherichia coli</i>		GGATGATCTCAGTGGGCGTT	423 bp

Shiga toxin 1 subunit A (stx1A) gene	GTTACACAATCAGGCGTCGC
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Multiplex PCR

The mPCR technique was performed for detection *Entamoeba histolytica* based on small subunit ribosomal rRNA gene and detection of Shiga toxin 1 subunit A (stx1A) producing *Escherichia coli* from stool samples. This method was carried out according to (26).

Results and Discussion

The total number of samples were 100 feces samples collected from people with diarrhea who attended Fairouz Hospital in Wasit province. *E.coli* O157 appearance on MacConkey agar were fermented lactose, while on Sorbitol- MacConkey agar was diagnosed based on morphological, culture and biochemical tests, where it appeared in the form of short gram-negative bacilli and n standard lactose-containing MacConkey Agar, so this strain is indistinguishable from other lactose-fermenting *E. coli*. Unlike most *E. coli* strains, *E. coli* O157:H7 ferments sorbitol slowly or not at all and it lactose fermented on the MacConkey agar as shown in figure (1A) and (1B) (27), while on blood agar it appeared a typical enterohemolytic phenotype on blood agar plates after 18 to 24 h of incubation, figure (1C) (28), which is a distinctive feature of *Escherichia coli* from the rest of the genera of the intestinal family.

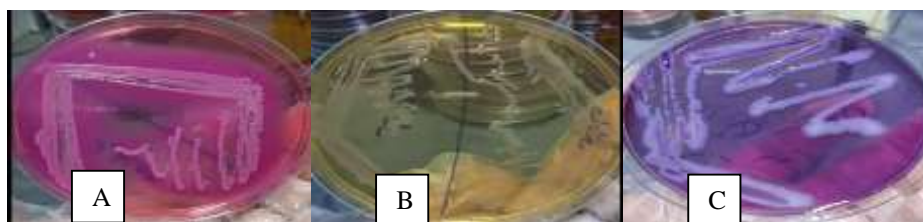


Figure 1. Growth of *E.coli* O157 bacteria on different media.

A- MacConkey Agar B- Sorbitol- MacConkey agar C- Blood agar.

On TSI culture medium *E.coli* O157 was able to ferment glucose, lactose and sucrose, with CO₂ production, bubbles and cracks and even displacement of the environment are observed, the whole tube becomes acidic and the culture medium turns yellow (24) figure (2).



Figure 2. Growth of *E.coli* O157 bacteria on TSI medium

The rate of infection in current study with *E.histolytica* was 65% among diarrheal cases, while only 14 *E.coli* O157 isolates (21.5%) were positive using PCR technique, table (1), table (2) and figure (3), for the period from the beginning of October until the end of December 2021.

Table 1. Percentage of infection with *E.histolytica* and *E.coli* O157

Infectious agent	Number of Examined Samples	Positive Samples (%)
Diarrheal Group	100	100 (100%)
<i>E.histolytica</i>	65	65 (65%)
<i>E.coli</i> O157	14	14 (21.4%)

Table 2. Percentage of infection with *E.histolytica* and *E.coli* O157

Infectious agent	Number of Examined Samples	Negative Samples (%)
Diarrheal Group	100	100 (100%)
<i>E.histolytica</i>	35	35 (35%)
<i>E.coli</i> O157	51	51 (78.8%)

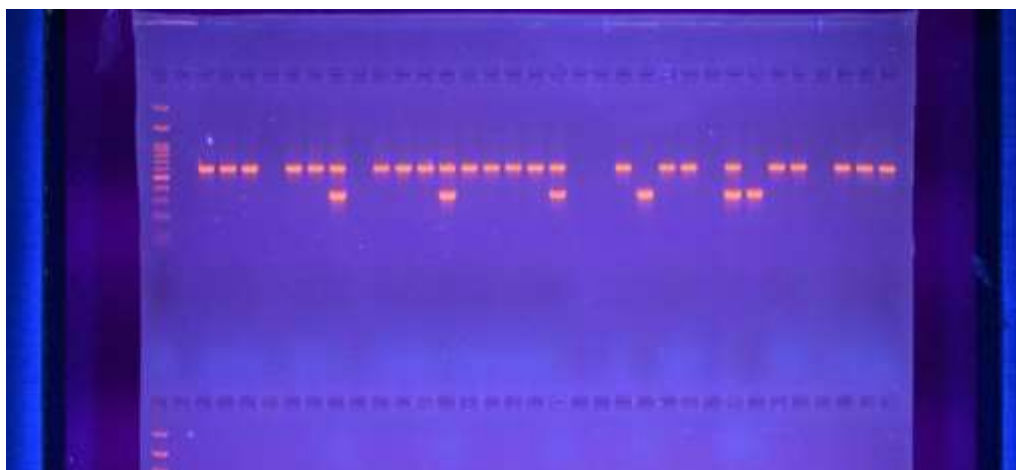


Figure 3. Detection of *E.coli* O157 using PCR technique.

The Current study outcomes were agreed with previous studies (29-31) who showed that reports have shown prevalence of *Entamoeba* more in the stool of diarrheal and other GI discomfort patients as compared to the stool of healthy control subjects. But our data were non-compatible with (32) who showed a difference in the infection rate, as the percentage of tissue-type amoeba infection was recorded at 25% (33) and (34) as it was among GI discomfort patients, the prevalence of *E. histolytica* was more (5%) in their study. The reason for the difference may be attributed to many reasons, the most important of which are the regional differences in the habits and living conditions of the participants.

Regarding *E.coli* O157 and method of detection by PCR as molecular method is more reliable mode of detection as compared to microscopy as it can differentiate between the morphologically similar species. These findings were in agreement with other studies which recommend PCR to be a better detection tool for bacterial infections (35, 36). Also in one study, a real-time PCR detection method based on a combined concentration method was proposed for the rapid screening of cabbage contaminated with low *E. coli* O157:H7 count (37)

The examined stool samples were collected from different age groups, starting from one year to 70 years, and the results showed correlation between infection rates *E.histolytica* parasite and *E.coli* O157 bacteria. As the percentage of *E.coli* O157 was 27.8% for the age group (1-10) years old, which recorded the highest rate of infection, and the lowest percentage of infection in the age group (60-70) years, as it was 3.7% as shown in table (3).

Table 3. Distribution of *E.histolytica* and *E.coli* O157 infection according to age groups

Age groups / Years	<i>E.histolytica</i>	<i>E.coli</i> O157
1-10	15 (23.1%)	4 (6.1%)

11-20	10 (15.4%)	2 (3.1%)
21-30	7 (10.8 %)	3 (4.6%)
31-40	8 (12.3%)	1 (1.5%)
41-50	7 (10.8 %)	4 (6.1%)
51-60	8 (12.3%)	0 (0.0%)
61-70	10 (15.4%)	0 (0.0%)
Total	65 (100%)	14 (21.4%)

Table (3) shows the total number of people examined and the infection rates for parasite according to the age groups under study. The total infection rate with *E.histolytica* was 65 %. It was found that the highest rate of infection with *E.histolytica* was within the age group (1-10) years, it reached 23.1%, and the lowest infection rate was within the age group (21-30) years with a rate of 10.8 %, and for *E.coli* O157 6.1% in both (1-10) and (41-50) age groups.

These results were in agreement with previous studies in Iraq who mentioned that the highest rate of infection with *E. coli* O157 bacteria was recorded for the category (1-10) years it reached 8.82% (38). And the study by (24) as they mentioned the same outcomes. Also (39) who concluded that amebiasis was a substantial burden on the overall health of the cohort children. But this study differed with study by (43) who found the high prevalence of *E. histolytica* and other unidentified enteropathogens as major potential causes of pediatric AGE in hospitalized Lebanese children, and (40) as they revealed that prevalence was 10% for *E. coli* O157. Also The highest isolation rate (3.19%) was in children aged 1–4 years old (41).

Table 4. Distribution of *E.histolytica* and *E.coli* O157 infection according to the gender

Gender	<i>E.histolytica</i> infection %	<i>E.coli</i> O157 infection %
Male	39 (60.0%)	7 (50%)
Female	26 (40.0%)	7 (50%)
Total	65 (100%)	14 (100 %)

Regarding *E.histolytica* infection related to gender results showed male are more prevalent than female, the present study outcomes were in agreement with (42) who showed that the infection rate was higher in males as it is in females. And with (43), where the rate of infection with *Entamoeba histolytica* among males was 62

%, and in females it was 38%. But these results were not agreed with (45), (44) and (45), as each of them recorded a higher incidence of intestinal parasites in females than males. *E.coli* O157 infection rate was similarly in both gender, these results were compatible with (46) study included average of 21% of the sampled animals in the positive *E.coli* O157.

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