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College of Veterinary Medicine
Department of Microbiology



Molecular Identification of Co-Infection Between *Entamoeba histolytica* and *Escherichia coli* in Two Districts of Thi-qar Province

A Thesis

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(Microbiology).

Presented by

Zainab Raed Jawad Al-Hameedawi

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Veterinary Medicine College /Wasit University

Supervision

Dr. Assistant. Prof Abdulaziz Saleh Al-Shammery

Dr. Assistant. Prof. Nothailah Rasheed Al-Kasar

2025 A.D

1447 A.H

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(يَرْفَعُ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ

وَرَحَاتٍ)

صدق الله العلي العظيم

المجادلة (11)

Supervisor Certification

We certify that this thesis (**Molecular Identification of Co- Infection Between *Entamoeba histolytica* and *Escherichia coli* in Two Districts of Thi-Qar Province**) was prepared under our supervision at the Department of Microbiology, College of Veterinary Medicine, University of Shatrah, in partial requirement for **Master Degree** in Veterinary Sciences / Microbiology.

Supervisor

Assistant Professor Dr. Abdulaziz Saleh Al-Shammery

College of Veterinary Medicine

University of Shatrah, Iraq

Supervisor

Assistant Professor Dr. Nothailah Rasheed Al- Kasar

Department of Microbiology

College of Veterinary Medicine

University of Shatrah, Iraq

In view of the available recommendation, I forward this thesis for debate by the examining committee

Signature

Assistant Professor Dr. Nothailah Rasheed Al-Kasar

Head of Microbiology Department

College of Veterinary Medicine

University of Shatrah, Iraq

Date :- 16 / 11 /2025

Declaration

I hereby declare that the thesis entitled (**Molecular Identification of Co -Infection Between *Entamoeba histolytica* and *Escherichia coli* in Two Districts of Thi-Qar Province**) is my original work except for quotations and citations, which have been fully acknowledged. Also, I declare it is not previously and currently submitted for other degrees at the University of Shatrah or other institutions.

Name: Zainab Raed Jawaad

Date: 11/16/2025

Examining Committee Decision

We are members of the examining committee read this thesis (**Molecular Identification of Co- Infection between *Entamoeba histolytica* and *Escherichia coli* in Two Districts of Thi-Qar Province**) and after examining the student (**Zainab Raed Jawaad Al-Hameedawi**) in her content, we found it is adequate for the award the **Master Degree in the Veterinary Medicine / Microbiology**.

Signature

Professor Dr.Haider Khamis Shanan
College of Veterinary Medicine

University of Shatrah

(Chairman)

Signature

Professor Dr. Afaq Talaib Farrhood
College of Science
University of Thi-Qar

Member

Signature

Assistant Professor Dr. Abdulaziz
Sahleh Abdulaziz
College of Veterinary Medicine
University of Shatrah

Supervisor

Approval of the College Committee on graduate, studies

Signature

Assistant Professor Dr.Nabeel Mahdi
Abd
Dean of College of Veterinary
Medicine
University of Shatrah

Signature

Lecture Dr. Muntadhar Mahommed Faliyy
College of Veterinary Medicine
University of Shatrah

Member

Signature

Assistant Professor Dr. Nothaila
Rasheed Hameed
College of Veterinary Medicine
University of Shatrah

Supervisor

Signature

Assistant Professor Dr. Nothaila
Rasheed Hameed
College of Veterinary Medicine
University of Shatrah
Head of the Microbiology department

Date of examination 31/12/2025

Dedication

To my beloved parents, whose guidance, encouragement, and unwavering support have been the foundation of my personal and academic growth.

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Summery

Intestinal infections caused by parasitic and bacterial pathogens constitute a major public health concern, particularly in developing regions. *Entamoeba histolytica* is a pathogenic protozoan responsible for amoebic colitis, which may progress to severe intestinal or extraintestinal complications. Conversely, *Escherichia coli* O157 is an enteric bacterial pathogen that can induce acute gastrointestinal disease with serious clinical outcomes. This study aimed to examine the correlation between positive samples of *Entamoeba histolytica* and *Escherichia coli*.

From October 2024 to the end of February 2025, 130 stool samples were collected from patients with diarrhea from Rifai hospital and Al-hussein hospital, private laboratories in the two districts of Al-Rifai and Al-Nasiriyah, in Thi-Qar Province. The patients age ranged from 1 to > 20years, and from both sexes. *E. histolytica* was identified by direct wet smear and PCR was used to detect the presence of infection such as *SSUrRNA* and *E.H.ssRNA* for *E. histolytica*. While identification of *E. coli* was preformed using culture on MacConkey, EMB agar and MacConkey sorbitol agar was employed as a differential medium to identify *Escherichia coli* O157, which is unable to ferment sorbitol, allowing its distinction from other *E. coli* strains. Followed by biochemical tests. PCR was used detect the presence of infection such as *rfbE*.

This study investigated *E. histolytica*, which was found in 66 out of 130 samples (50.76%). Conversely, *E. coli* was detected in 54 out of 130 samples (41.53%) based on morphological identification. Infection rate of *E. histolytica* by sex showed a higher rate in males (52.63%) compared to females (48.15%). The differences were not statistically significant. The infection rate of *E. histolytica* was higher in Al-Nasiriyah district (67.50%) compared to Al-Refai district (43.33%) with statistically significant differences ($P < 0.05$). The highest infection rates were observed in children aged 1–3 years (40.90%) and adults over 20 years (42.42%) revealed a significant statistical difference. XI

In comparison, infection rate of *E. coli* was higher in females (57.41%) compared to males (30.26%). The statistical evaluation showed a significant difference ($P < 0.05$). This study examines the prevalence of *E. coli* in patients (50%) than that in Al-Refai

(37.78%). Although the differences were not statistically significant ($P > 0.05$). The highest prevalence was recorded in individuals older than 20 years, accounting for (40.74%), followed by children aged 1–3 years with (27.7%). Statistically, the distribution of infection among the age groups was highly significant ($p < 0.001$). 28 (21.53%) were found to be positive for the co-existence of both *E. coli* and *E. histolytica*. The highest prevalence of co-infection was observed in the age groups 1–3 years (39.28%) and >20 years (35.71%), Statistical analysis showed a significant association between age groups and co-infection ($p < 0.05$). The co-existence of the two pathogens was higher in Al-Refai district (71.06%) compared to Al-Nasiriyah (28.94%), this difference was statistically significant ($P < 0.05$).

12 stool samples, previously examined microscopically for *E. histolytica*, were further confirmed using conventional and real-time PCR techniques, showed that 6 out of 12 samples (50%) tested positive for *E. histolytica*. While *E. coli* o157 was not detected by conventional PCR but was identified in 1 out of 24 samples (4.16%) using real-time PCR, the findings of the study showed significant variations ($P \geq 0.05$) in two pathogenic.

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List of Abbreviations

Abbreviations	Meanings
SPSS	Statistical Package for the Social Sciences
<i>SPP</i>	Species
PCR	Polymerase Chain Reaction
µm	Micrometer
DNA	Deoxyribonucleic Acid
UTS	Urinary Tract System
°C	degrees Celsius
EMB	Eosin methyl blue
PH	Potential of Hydrogen
STEC	Shiga toxin-producing <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
EAEC	Enteraggregative <i>E. coli</i>

DAEC	Diffusely adherent <i>E. coli</i>
HUC	Hemolysin-producing uropathogenic <i>E. coli</i>
rRNA	Ribosomal RNA
Afa/Dr	Adhesive Fimbriae of Dr family
LPS	Lipopolysaccharide
OPS	O-specific polysaccharide
LT	Heat-labile toxin
ST	Heat-stable toxin
RAPD	Random Amplified Polymorphic DNA
lbs/in ²	pounds per square inch
Mg	Milligram
μL	Microliter
GD	Genomic DNA
SSURNA	Small Subunit Ribosomal RNA
Sec	Second
TBE	Tris-Borate-EDTA

Chapter One

Introduction

1.1. Introduction

Enteric infections and diarrheal illnesses are significant causes of hospitalization and present a critical health problem in under developed nations (Salamandane *et al.*, 2021). Diarrheal-related illnesses remain a significant public health concern, particularly harming elderly individuals and small children (Liang *et al.*, 2024).

The correlations between microbes and parasites may interact in multiple ways that could influence the rate of infection of the *Amoeba* within intestinal infections and liver abscesses (Burgess and Petri, 2016). *Entamoeba histolytica*, a protozoan parasite, can remain in the human intestine for months or years without causing symptoms, *Amoebae* can cause intestinal damage and invasion for unknown reasons (Gyem *et al.*, 2025). *Escherichia coli* represents a major pathogen responsible for bacteremia in developed nations. To establish and apply efficient preventive measures, a deeper insight into the epidemiological patterns of invasive *E. coli* infections is essential (Bonten *et al.*, 2021).

Infection with *E. histolytica* leads to noticeable alterations in the composition and density of intestinal microbiota (Hassen and Alsalim, 2023). *E. coli* generally exists in a balanced relationship with humans for extended periods. These commensal strains rarely cause disease unless the host's immune system is compromised or the normal gastrointestinal barriers are disrupted, as in peritonitis. Typically, commensal *E. coli* resides and multiplies within the mucosal layer of the mammalian colon (Kaper *et al.*, 2004). This species is an important part of the human bacterial flora, particularly in the gastrointestinal tract and mouth. In vaginally delivered infants, the initial microbial inoculum enters through the birth canal. Orally to infants fed mother's milk (Hassen and Alsalim, 2023). *E. histolytica* exposure can cause many varieties of symptoms, including diarrhea, dysentery (Burgess and Petri, 2016). Liver abscesses, typically caused by parasitic or bacterial infections, a leading cause of hospitalization in low-middle income countries (Khim *et al.*, 2019). This can lead to amoebic colitis, also known as extraintestinal amoebiasis, occurs when amoebae spread to other organs through blood vessels (Gyem *et al.*, 2025). Insufficient water and sanitation facilities, along with inadequate personal hygiene, significantly contribute to the widespread presence of these conditions in developing regions (Petri *et al.*, 2008).

Chapter One.....Introduction

Trophozoites live in the human colon and rely on bacteria and simple sugars like glucose for energy their surfaces (Nagode *et al.*, 2023).The virulence mechanisms responsible for its pathogenicity are complex. However, endocytic processes and motility are widely accepted virulence determinants (Ahmad *et al.*, 2024). This suggests that other environmental factors may influence the disease, and mounting evidence indicates that the human intestinal bacterial microbiome plays a significant role in the development and progression of amebiasis (Burgess and Petri, 2016). Enteropathogenic *E. coli* enhances the motility of *E. histolytica* while diminishing the parasite's adhesion strength in fibronectin-rich matrices (Guo *et al.*, 2024). *E. histolytica* can use bacterial oxaloacetate to increase resistance to oxidative stress and promote survival in mice with experimentally-induced amebiasis (Shaulov *et al.*, 2018).

1.2. Aim of Study

To detect the correlation between *E histolytica* and *E. coli* infection and to evaluate the influence of these bacteria on intestinal amebiasis by.

- 1-Diagnostic study of *E. histolytica* by microscopic examination and PCR technique
- 2- Detection of *E. coli* by culture on MacConkey agar, MacConkey sorbitol EMB IMVIC and PCR technique.
- 3- Study correlation between positive samples of parasite and bacteria
- 4- Detection of infection rate and some factor such as location, age and sex.

Chapter Two

Literature of Review

2.1. Taxonomy

After the parasite was initially identified in 1875 by the scientist Losch, who noticed it in the feces of a Russian patient who had chronic dysentery with blood and mucus, the scientist William Osler reported in 1890 that a young man had amoeba dysentery. The young man passed away from a liver abscess because he did not receive treatment (Paniker and Ghosh, 2018). Quinck and Ross found the encysted stage of this parasite in 1893, and Schaudinn named it *E. histolytica* in 1903 due to its capacity to examine infected tissues and differentiate it from *E. coli* (Pinilla *et al.*, 2008). Schaudin Fritz gave this parasite the name *E. histolytica* in 1903 (Fotedar *et al.*, 2007a).

Dysentery remains a significant cause of illness and death worldwide, with approximately 55,000 fatalities each year attributed to *E. histolytica* infections (Shirley *et al.*, 2018). Clifford Dobell, an English parasitologist, reported in 1919 that several species of Amoeba exist, each featuring a compressed stage characterized by four nuclei (Lebbad, 2010). Brumpt found *E. dispar*, a non-pathogenic species that is morphologically identical to *E. histolytica*, in the same year. However, the lack of techniques to differentiate between these two parasite species made these investigations weak. Sargeant and Collagene, however, showed in 1978 that the pathogenic and non-pathogenic species of the genus *Entamoeba* could be distinguished using the isoenzyme typing technique. These two researchers made it feasible to use an isoenzyme analysis technique to differentiate between these two phenotypically competing amoebas (Fotedar *et al.*, 2007).

2.2. Classification of *Entamoeba histolytica*

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Sarcodina

Superclass: Rhizopoda

Class: Lobosea

Order: Amoebida

Suborder: Tubulinea

Genus: Entamoeba (Lösch,1875)

2.3. Morphology of *Entamoeba histolytica*

Throughout its life cycle, *E. histolytica* goes through multiple stages, including the trophozoite, pre cyst, cyst, metacyst, and meta cystic (Dahariya *et al.*,2020).

2.3.1. Cysts Stage

Cysts are infectious, non-motile growths that undergo excystation as they develop in the gastrointestinal system. With four nuclei, mature cysts have an average containing chromatin that frequently aggregate to form elongated bars, glycogen accumulates in a vacuole, and there is a 20 µm diameter wall comprised of chitin that appears smooth and refractile under light microscopy. These cysts are expelled with the feces (Samie *et al.*, 2006). Due to their resistance to external environmental conditions, these mature cysts are the infectious stage of the histolytica amoeba. They can survive in the waste that is present on the soil for eight days at a temperature of 28–34 °C. It was found that the cyst stage was unaffected by the addition of chlorine to water (Paniker and Ghosh, 2018). Figure (2-1).

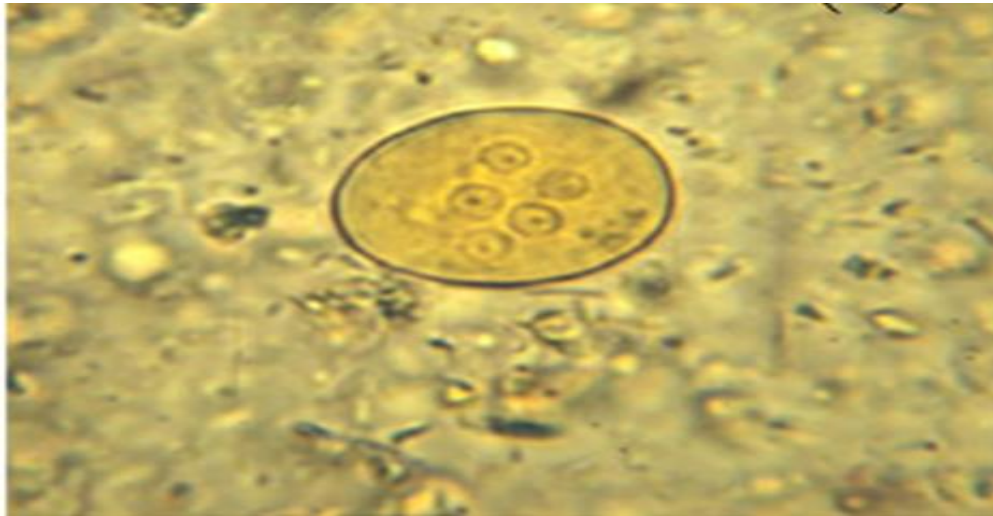


Figure (2-1): Cyst of *E. histolytica* (Ben Ayed and Sabbahi, 2017).

2.3.2. Pre Cyst Stage

Pre-encysted amoebas are round or oval, have colorless cells, are smaller than active amoebas, and lack food bodies. They move slowly and their pseudopodia lack distinct movements (Brown and Neva,1983). This stage, which does not result in illness in humans, is created when the active phase's activity slows down as a result of food particles being consumed it curls up and becomes smaller (Sastry and Bhat,2018).

2.3.3. Meta Cysts Stage

When the parasite exits the cyst and enters the small intestines lumen, the cytoplasm splits into multiple tiny amoebae based on the number of nuclei. These amoebae divide to produce eight single-nucleated *amoeba*, which have a diameter of roughly 8 micrometers and are smaller than the amoeba found in infected humans' intestines. These virions keep feeding and growing until they reach their typical size. Four tiny metacystic virions are formed as trophozoites, with each nucleus surrounded by a portion of the cytoplasm. (Sastry and Bhat ,2018). These amoebas move to the large intestine with food because they are unstable in the intestine (Samie *et al.*,2012).

2.3.4. Trophozoite Stage

Chapter Two.....Literature Review

This stage, also known as the feeding stage or the vegetative form, is a representation of the growing stage (Paniker and Ghosh, 2018). This stage is located in the host's large intestine, particularly in the rectum and cecum, and it spreads from there to infiltrate other organs like the liver, brain, and lungs (Uribe-querol and Rosales, 2020). Trophozoites exhibit an amorphous morphology with an average diameter of 15 to 30 μm , possess a rough endoplasmic reticulum or Golgi apparatus, feature helically orientated ribosomes, and lack traditional mitochondria (Lohia, 2003). As same the figure (2-2).

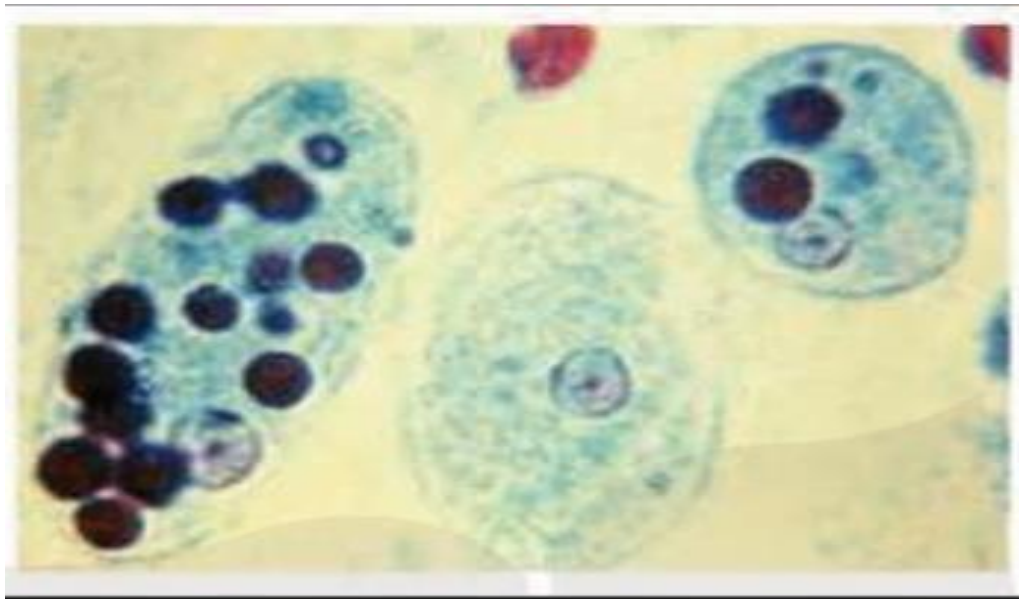


Figure (2-2): The trophozoite (the tissue *amoeba* parasite under a magnification of 100X. (Al Attar and Hamoo,2022).

2.4. Life Cycle

There are two active stages in the simple life cycle: the vegetative stage and the cyst stage (Satoskar *et al.*,2009). Consuming contaminated food or water can spread the infection, as can ingesting infectious cysts from contaminated hands or contaminated food. Without causing any harm, the cysts move through the stomach ((Ebert and Fields, 2020). The neutral and basic intestinal juices dissolve their outer wall when entering the bottom region of the small intestine, causing the release of four tiny feeding amoebas a process known as exocytosis. These amoeba split into eight tiny feeding amoeba (Clark *et al.*, 2007).The acidic environment does not cause them to exhibit any activity. The cysts are stimulated when they enter the small intestine's alkaline environment, which

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leads to exystation the process of escaping the cyst—and cytoplasmic and nuclear division, which results in the formation of eight tiny amoebae. The mature feeding stages and the post-cyst feeding stage are comparable (Ebert and Fields, 2020). The motile trophozoite form of *E. histolytica* inhabits the human colon where it multiplies and differentiates into cysts that are released into the environment. In turn, these cysts are responsible for transmitting the infection to another host via the fecal-oral route. The parasite invades the intestinal mucosa and causes many forms of invasive disease, including dysentery (Lin and Kao, 2013). Additionally, the trophozoites may reach the bloodstream through the intestinal mucosa barrier and spread to the resulting pathologic manifestations in the liver, lung, and other locations. These websites prevent them from leaving the host (Solaymani-Mohammadi and Petri, 2008). Sometimes the feeding stages stay in the intestinal lumen in a restricted way; this is referred to as an infection. Approximately 90% of infections are non-invasive in nature. They are referred to as an important source in the epidemiology of the parasite is thought to be the cysts that are expelled in the stool by asymptomatic carriers of the disease, known as asymptomatic carriers (Guillen and Nancy., 2021)

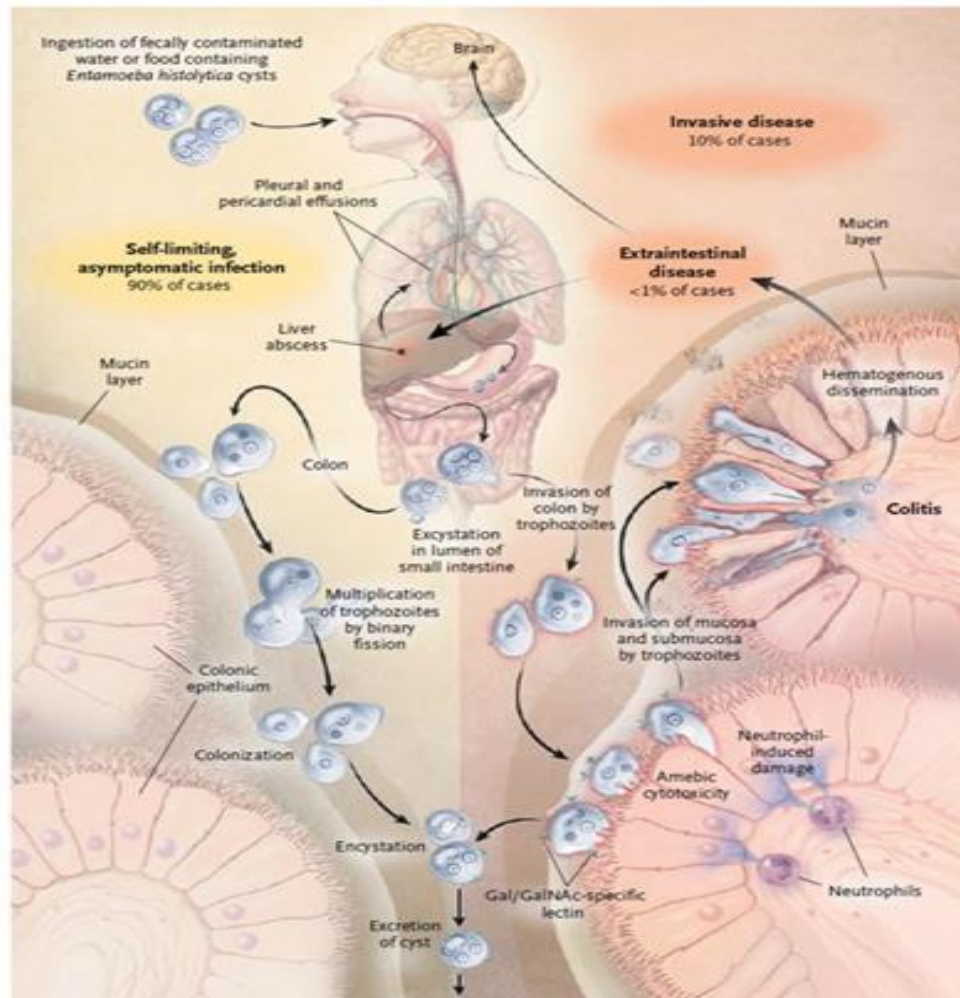


Figure (2-3): *E. histolytica* life cycle from (Haque *et al.*, 2003)

2.5. Genus: *Entamoeba*

Entamoeba species, are prevalent zoonotic protozoa of the intestine that can cause severe infections of the intestinal infection in humans and animals via fecal-oral transfer, resulting in considerable economic losses and public health issues (Wu *et al.*, 2025). The genus *Entamoeba* comprises a wide range of animals from various classes as well as numerous widely distributed species that have evolved to survive in human digestive (Zanetti *et al.*, 2021). Human and animal infections with *Entamoeba* spp endanger public health and result in large financial losses for the livestock sector. *Entamoeba* spp can potentially infect humans through animals (Wu *et al.*, 2025). Of the seven *Entamoeba* spp that are known to infect humans, *E. histolytica* is the most commonly recognized pathogen. According to reports, *Entamoeba* infections are uncommon in developed nations but widespread in developing ones.

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Finding antigens or DNA in the stool is the most accurate method of diagnosing these protozoan parasites(Servian *et al.*, 2022). *E. histolytica* is transmitted through contaminated food and water particularly in areas with poor sanitation (Guo *et al.*, 2024) . The primary *Entamoeba* spp that live in the human gastrointestinal tract are *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. polecki*, *E. bangladeshi*, and *E. hartmanni* (Cui *et al.*,2019). Cysts of *E. moshkovskii* share morphological similarities with those of the nonpathogenic *E. dispar* and the disease-causing *E. histolytica*. It is found in wild macaques and exhibits a close genetic affinity with *E. histolytica* and *E. dispar*, which asymptotically infects the human intestine, is another close relative. Only *E. histolytica* and *E. nuttalli* can pierce the intestinal epithelium of their host, despite the fact that all three species are closely related (Koing *et al.*,2021).

2.6. *Entamoeba histolytica*

The amoeba parasite *E. histolytica* infects approximately 45-50 million people each year, causing 40,000 to 100,000 deaths. It can cause life-threatening diseases such as hemorrhagic colitis or extra-intestinal abscess (Wesel *et al.*, 2021). *E. histolytica* is a unicellular parasite transmitted through contaminated food and water. It is located in the colon, and the majority of affected persons exhibit no symptoms. In certain individuals, the parasite may disseminate throughout both intestinal and extraintestinal tissues, resulting in amoebiasis. *E. histolytica* consumes bacteria present in the intestinal microflora (Shaulov *et al.*, 2018). The human protozoan parasite *E. histolytica* can reside in the human intestine for extended periods, ranging from months to years, without eliciting any symptoms in the host. *E. histolytica* can inexplicably devastate the gut mucosa and exhibit invasive behavior (König *et al.*, 2021). *E. histolytica*, the causative agent of amoebiasis, is a major contributor to pediatric diarrhea in South Asia and sub-Saharan Africa (Burgess and Petri, 2016) .

E. histolytica is an invasive, pathogenic protozoan responsible for amoebiasis and a major cause of diarrhea in underdeveloped countries.(Saurabh *et al.*, 2017). *E. histolytica* causing severe intestinal diseases and posing a serious threat to public health(Wang *et al.*, 2022).

2.7. Diarrhea Causes

Diarrhea is characterized by increased frequency, volume, and decreased stool consistency compared to usual. It is important to note that stool frequency varies with age, with infants experiencing the highest frequency. Dysentery is defined as the passage of blood and mucus in feces. Chronic diarrhea is defined as symptoms lasting more than 14 days, while persistent diarrhea lasts more than seven days(Vargas *et al.*, 2004). the most common causes of gastrointestinal infections in children, including bacteria, viruses, parasites, and protozoans (Sieloff *et al*,2013).

Infectious diarrhea refers to diarrhea that has an infectious cause and is frequently accompanied by cramping in the abdomen, nausea and vomiting (Guerrant *et al.*, 2001). Any form of diarrhea is a clinical sign that the intestines are secreting more water and electrolytes than they are absorbing (Masharipova,2023).

Diarrheal illness remains a significant public health issue, primarily impacting older adults and young children. The incidence of these diseases continues to be a global concern, despite ongoing efforts to prevent and control them(Liang *et al.*, 2024). Depending on the location, time, and population, acute diarrhea has different causes in every healthcare facility(Wulandari *et al.*, 2024). Developing successful public health strategies requires an understanding of the trends in diarrhea incidence and the factors influencing these trends(Liang *et al.*, 2024). Key environmental risk factors were identified. In order to prevent diseases in their children, mothers of children under five years old and members of endangered groups should be aware of these risk factors. Children's acute diarrhea can be prevented to prevent severe damage remarks to the health system(Vafae *et al.*, 2008).

2.8. Clinical Features

The incubation time generally ranges from 14 to 28 days, although it may vary from a few days to several months or years (Ben Ayed *et al.*,2017). Some people may have the parasite for years without experiencing any symptoms, but others may develop amoebic colitis or other symptoms all at once. The symptoms of amoebic colitis gradually worsen over a period of one to two weeks. Diarrhea is one of the most

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common that sets amoebic colitis apart from bacterial dysentery. While bacterial diarrhea is watery, amoebic colitis patients experience watery or bloody diarrhea, abdominal pain, cramps, and weight loss (Slater *et al.*, 2019). There are a number of reasons why amoebic dysentery symptoms can vary widely, including the amoeba strain that causes the illness, the host's innate and acquired resistance to this strain, the host's genetic susceptibility, interference with the normal flora, and other factors like poor nutrition symptoms, sex, and age (Loh *et al.*, 2013).

There are four intestinal conditions that are frequently acute: amoebic colitis, ascending colitis, appendicitis, and amoebic dysentery. 90% of infected individuals remain asymptomatic, with 10% of cases exhibiting symptoms (Gracia ,2007). In roughly 90% of cases, bloody diarrhea and diarrhea syndrome are present. Additionally, the patient has rectal tenesmus, which causes moderate colon pain, and diarrhea that happens three to five times a day. Mucus and blood are present in the liquid stool. Patients with intestinal bloody diarrhea do not experience fever or other systemic symptoms (Espinosa-Cantellano and Martínez-Palomo, 2000). The symptoms initiate as abdominal discomfort and tenderness, bloating, irregular bowel habits, dysentery with or without blood or mucus, tenesmus accompanied by bloody mucoid diarrhea devoid of fecal leukocytes, constitutional symptoms, toxic megacolon, and finally signs of peritonitis resulting from perforation (Haque *et al.*, 2006).

2.8.1. Extraintestinal Amoebiasis:

The *E. histolytica* parasite penetrates all intestinal layers and reaches the serosa layer, after which it spreads to other parts of the body. Other organs like the liver and lungs may receive it after it enters the lymphatic or mesenteric venules brain, spleen and reproductive organs (Fotedar *et al.*, 2007). In addition to coughing, the symptoms of amoebic pneumonia, which is caused by a trophozoite that extends from the diaphragm, are similar to those of a liver abscess. The most common symptoms include upper abdominal pain with fever, weakness, and weight loss. Sweating, nausea, and vomiting are possible side effects, and diarrhea and constipation do not alternate (Zeibig,2012).

2.9. Pathogenesis of *Entamoeba histolytica*

The parasite may be found in the colonic crypts, and successful colonization is contingent upon several things, including bowel movements, intestinal flora presence or absence, the host's diet, and the amoeba's ability to adhere to colonic mucosal cells. The virulence factors mentioned above then contribute to the pathogenesis of the parasite when its feeding stages are released in the intestine and begin to invade the tissue, causing ulceration in the intestine from the rectum and appendix or in the upper colon and possibly spreading along the colon (Steve and Chadee,2017).

The active penetrate the cells in the colon by their movement and the enzymes they possess histolysis, lytic which causes damage to the mucous membranes (Mahmud *et al.*,2017). The enzyme cysteine proteinase, produced by the tissue amoeba *E. histolytica*, is released into its environment. This enzyme targets intercellular proteins, including collagen and fibronectin, leading to the separation of epithelial cells from one another and causing them to adhere to the enzyme(Miller *et al.*, 2022). In order to facilitate tissue penetration and the analysis of intestinal mucosal cells, the parasite secretes lytic enzymes that break down the intestinal mucosa and the barriers created by the epithelial cells. These enzymes then reach the submucosal layer and the layers beneath it, where they analyze the host cells and form distinct ulcers that resemble pins, have raised edges, and are more common in the colon and mucosal area(Guillén and Nancy, 2023).

The parasite amoebic causes liver abscesses, which are among the most common side effects of the disease that affects extraintestinal sites in the human body. In rare instances, these abscesses can lead to clots in the inferior vena cava and hepatic vein (Martin *et al.*, 2017). The disease may progress to the liver, and when the active agents spread through the blood vessels, a liver abscess results. Liquefied liver tissue devoid of amoebas is called pus, and it is found in the center of the abscess. Liver abscesses typically occur in the right upper lobe of the liver and can be single or multiple. The number of white blood cells increases to 20,000 cells/ cm³and the rate of red blood cell sedimentation increases as it presses against the desert ducts (Mehlhorn ,2016). It can

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travel through the nose or mesenteric veins to the liver, where it can form abscesses in the hepatic-pyloric system. The lungs and other organs may also be affected, and abscesses may develop there (Samie *et al.*,2012).

2.10. Epidemiology of *Entamoeba histolytica*

2.10.1. Epidemiology in Iraq

The infection rate with *E. histolytica* is 3.8% in Karbala, Iraq (Al- Saqur *et al.*, 2017). Men are ten times more likely than women to develop an amoebic liver abscess, particularly those aged 18 to 50, who are at the highest risk. This information is based on a study conducted in Baghdad that reported an incidence of amoebic infection at 23.5% (Kantor *et al.*, 2018). A study on the prevalence of intestinal parasite infections was conducted in Baghdad. Stool specimens were collected from 1,071 youngsters aged 7 to 12. 27% of kids who attended the Children's Hospital in Kadhimiya were diagnosed with an *E. histolytica* infection(Salman *et al.*, 2019).

In 2014, the microscopic wet mount technique with iodine demonstrated that *E. histolytica* was detected in 12.9% of the cases examined in Sulaymaniyah (Abdullah *et al.*, 2020). In Najaf, a total of 372 samples were analyzed in a study investigating intestinal parasites, The incidence rate of *E. histolytica* was significantly high at 34.3% (Hussein *et al.*, 2021).

The study was conducted in Wasit Province, Iraq, in collaboration with the microbiology unit of Fairouz Hospital. Samples were collected from various locations within Wasit Province, the study included 100 stool samples from Iraqi patients experiencing diarrhea, previous studies indicated that 65% of diarrheal cases were associated with intestinal parasites, specifically *E. histolytica* (Ali and Flaeih, 2022). The frequency of the parasite *E. histolytica* has been investigated in several research carried out in different Iraqi provinces (Al-Sultany *et al.*, 2023).

2.10.2. Epidemiology in World

The prevalence of amoebic dysentery in Gaza City, Palestine, was 17 out of 309 stool samples (Agha Rodina and Teodorecsu,2002). An intestinal parasite study was carried out in Kuwait. Based on data gathered from 912 stool samples at one of the medical

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facilities, it was discovered that the incidence rate of dysentery had reached 5.9% (AL-Nakkas *et al.*, 2006). After analyzing 5,921 stool samples, the *E. histolytica* parasite infection rate in Australia was 2.9% (Fotedar *et al.*, 2007). High prevalence rates of intestinal infections are found all over the world according to, *E. histolytica* infection is among the top three parasitic infections worldwide, following schistosomiasis and malaria. One hundred thousand people die each year from *E. histolytica* (Yimer *et al.*, 2017). When 106 stool samples from Mexico were analyzed using the PCR technique, the prevalence of parasite infection was found to be 1.8% (Aguayo-Patrón *et al.*, 2017). The Brazilian population had a high prevalence of *Entamoeba spp.* (22%) and *E. histolytica* (3.1%), with the northern, northeastern, and central-western regions having the highest prevalences of up to 50% (Zanetti *et al.*, 2021).

2.11. Diagnosis

2.11.1. A microscopic Examination

The primary method for identifying and diagnosis the types of this genus is still the microscope because microscopic diagnosis relies on the number of nuclei in the cystic phase or the size and presence of chromatin granules surrounding the nuclear membrane of the nucleus in the active phase, both of which require high diagnostic accuracy and skill from laboratory personnel, particularly in cases of mixed infection (Tan *et al.*, 2010). Red blood cells are found in the active phase, while pyogenic cells, oocysts, and a small amount of blood are found in the feeding phase of stool samples. The nuclei of the feeding phases are not visible in the wet spots, although several red blood cells are visible (Paniker and Ghosh, 2018).

2.11.1.1. Direct Method

Two different techniques are included in the wet smear method for creating a wet smear, and they are First technique: applying dyes the nucleus and internal structures are made more visible during diagnosis by the use of dyes example Eosin methylen, Trichrome, Iodine, blue wrights, giemsa which are used to identify internal structures. Using a matchstick to combine a drop of feces with a drop of the solution, the forbidden

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dye used to make permanent smears is one of the most helpful dyes for diagnosis. The mixture is then viewed at 40x and 100x magnification (Guillen and Nacy 2021).

Second technique: physiological local solution is used to detect the active phase and the cyst.

2.11.2. PCR-Based Diagnosis of *E. histolytica*

PCR is a molecular technique that amplifies specific DNA sequences of the parasite, facilitating the direct identification of its genetic material in feces or tissue specimens. This method has enhanced sensitivity and specificity relative to conventional microscopic testing techniques. Real-Time PCR techniques provide exceptional sensitivity, enabling the detection of trace amounts of *E. histolytica* DNA, hence reducing false-negative results and enhancing diagnostic reliability (Roy *et al.*, 2005).

2.12. *Enterobacteriaceae*

Both humans and animals' intestines are home to a large number of Gram-negative bacteria that belong to the *enterobacteriaceae* family. There are numerous species of bacteria in this family, including *Escherichia*, *Salmonella*, *Shigella*, and *Proteus* (Riedel *et al.*, 2019). This includes genital infections, respiratory tract infections, urinary tract infections (UTIs), nosocomial infections, and wound infections. They are facultative or aerobic anaerobes, rod-shaped, and Gram-negative. The majority of the participants are lactose fermenters that are non-spore forming, flagellated, oxidase test negative, indole test positive, and grow best at 37 °C. Toxins, enzymes, capsules, flagella, and other virulence factors are among their many varieties (Oliveria *et al.*, 2017) .

2.12.1. Classification of *Escherichia coli*

The bacteria, *E. coli*, was formally named after its discoverer a few decades later. Ironically, the original strain's genome was sequenced. Later, it was discovered in Dr. Escherich's lab that he had isolated a benign member of the gut microbiota that lacked known virulence factors and pathogenicity islands such as the Shiga toxin (Meric *et al.* 2016). The incidence of *Shigella* and *Salmonella* was considered low when compared to other causes of gastroenteritis among children as enteropathogenic *E. coli* (Abbas *et*

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al., 2017). According to the Bergys classification system, *E. Coli* bacteria belong to the *Enterobacteria* phylum, which includes the following categories: (Garritty *et al.*,2005).

Domain: *Eubacteria*

Kigdom:Bacteria

Phylum:Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriace

Genus: *Escherichia*

Species: *coli*

2.12.2. *Escherichia coli*

E.coli species are members of the Enterobacteriaceae family, including *E. fergusonii*, *E. vulneris*, *E. hermannii*, *E. blattae*, *E. albertii*, and *E. coli*. The aforementioned species exhibit biochemical differences (Olowe *et al.*, 2017). Extra intestinal *E. coli* infections represent a growing public health threat (Clabots *et al.*, 2024). Normal intestinal microbiota. However, some *E. coli* strains can cause diseases in humans, other mammals and birds ranging from intestinal infections(Pokhareel *et al.*, 2023). *E. coli* is mostly Strains rarely cause illness in healthy people and survive in the intestines without causing any harm. However, a variety of pathogenic strains can result in extraintestinal illnesses or diarrhea in both healthy and immune compromised people (Gomes *et al.*, 2016). The human large intestine naturally contains *E. coli*. The majority of strains are benign, but some develop into pathogenic strains by acquiring bacteriophage or plasmid DNA that codes for invasion factors or enterotoxins: These pathogenic strains are the cause of urinary tract infections (UTIs), neonatal meningitis, septicemia, and diarrheal infections globally (Makvana and Krilov, 2015). Bloodstream infections are recognized as important nosocomial infections(Xiao *et al.*, 2022). The disease spreads through contaminated food and water(Guo *et al.*, 2024).

2.12.3. Characterization of *Escherichia coli*

It is a gram-negative, rod-shaped bacterium that propels itself using flagella instead of spores. Colonies of *E. coli* often have a smooth surface and a convex shape. On MacConkey agar, their colonies have a flat morphology, desiccated, and pink; yet, on Eosin Methylene Blue (EMB) agar, they exhibit a multi colored shine. O157:H7 cannot ferment sorbitol, distinguishing it from other *E. coli* serotypes; hence, it can be identified using sorbitol MacConkey agar. The *E. coli* bacteria in Triple Sugar Iron agar do not generate H₂S. The predominant strains produce the enzyme glucuronidase. The optimal pH range for growth is 4.4 to 9.0, with a suitable temperature of 36 to 37°C. The bacteria produce negative results in the oxidase, the urease and nitrite assays, but positive findings in the catalase assay, but most strains exhibit favorable results in the indole test (Kodaka *et al.*, 2004). Furthermore, it exhibits a positive result for the indole test, which serves as the most effective method to distinguish it from other members of the Enterobacteriaceae family, while yielding negative results for both the oxidase and urease tests. It additionally yields a positive result in the catalase test. Moreover, it does not use citrate as its only carbon source, and the methyl red test produces a positive result, whereas the Voges-Proskauer test generates a negative result (Hemraj *et al.*, 2013). Extra intestinal. *E. coli* infections represent a growing public health threat (Clabots *et al.*, 2024).

2.12.4. Pathogenic *Escherichia coli*

Two primary classifications of pathogenic *E. coli* have been delineated according to the spectrum of human diseases related to the bacteria: extraintestinal pathogenic *E. coli* and diarrheagenic *E. coli* (Opintan *et al.*, 2010). The six recognized pathogenic strains of *E. coli* that induce diarrhea.

Shiga toxin-producing *E. Coli* (STEC)

Enteropathogenic *E. coli* (EPEC)

Enterotoxigenic *E. coli* (ETEC)

Enteraggregative *E. coli* (EAEC)

Enteroinvasive *E. coli* (EIEC)

Diffusely adherent *E. coli* (DAEC)

are the different types of *E. Coli*. The one that hemorrhagic colitis and self-limiting diarrhea are among the gastrointestinal illnesses brought on by these *E. coli* strains (Mirsepasi-Lauridsen *et al.*, 2019).

2.12.4 .1. Shiga toxins- Producing *E. coli* (STEC) or Enteric Hemorrhagic *E. coli* (EAEC)

Over a span of 30 years of observation, enterohemorrhagic *E. coli* (EHEC) has historically been designated one of the most significant dangers of foodborne pathogens globally. EHEC mostly resides in the gastrointestinal tract of cattle, from whence it can disseminate to adjacent agricultural settings and persist for several months (Saberianfar *et al.*, 2019). A variety of foodborne bacteria is responsible for many diseases, ranging from mild diarrhea to severe dysentery, including hemorrhagic colitis (HUC). The most common serotypes of EHEC/STEC related to Human infections include O24, O45, O103, O111, O145, O121 and O157. EHEC isolates are distinguished by their ability to produce shiga-toxins, which consist of one active A subunit connected to one B5 subunit that promotes attachment to glycolipid receptors on the surface of target cells, resulting in systemic absorption. Shiga toxins inhibit protein synthesis by removing leftover adenine from 28S rRNA for the 60S ribosome (Melton-Celsa, 2014; Gould *et al.*, 2013; Moxley *et al.*, 2020).

2.12.4.2. Enteropathogenic *Escherichia coli*

This strain infects the intestine and causes diarrhea and damage to intestinal tissue called lesions, which leads to the destruction of microvilli, small hairs found in the normal intestinal lining located on the surface of body cells (Mare *et al.*, 2021).

2.12.4.3. Enteroaggregative *Escherichia coli* (EAEC)

Characterized by the AA pattern configuration on Epithelial cells in cultures of tissues. The AA pattern is characterized as a "stacked-brick" arrangement on the surface of cells in epithelial tissue cultures. Diarrhea induced by EAEC is characterized by watery consistency with mucus, potential presence of blood, Abdominal ache, mild pyrexia,

and vomiting. In 2011, a notable outbreak of a hybrid strain transpired in Germany, resulting in about 4,300 instances of diarrhea and 50 deaths (Ellis *et al.*, 2020).

2.12.4.4. Enterotoxigenic *Escherichia coli*

This strain is distinguished by its capacity to create colonization factors and enterotoxins. This strain induce diarrhea, which bind to specific receptors on epithelial cells (Zhang *et al.*,2022).

2.12.4.5. Enteroinvasive *Escherichia coli*

This strain is regarded as the etiological agent of dysentery. The disease manifests due to the invasion of *E. coli*, which penetrates and obliterates intestinal cells. The bacteria attach to the mucosal layer of the large intestine by endocytosis, potentially disseminating to adjacent cells (Michelacci *et al.*,2020).

2.12.4.6. Diffusely Adherent *Escherichia coli* (DAEC)

DAEC induces distinctive alterations in epithelial cells, resulting in diarrhea and epithelial damage that may precipitate further intestinal disorders. DAEC includes a collection of *E. coli* strains possessing genes that encode Afa/Dr adhesins, which can induce diarrhea in humans. It can infect youngsters and is regarded as the second leading cause of mortality in individuals under five years of age (Omolajaiye *et al.*, 2020).

2.12. 5. Virulence Factors

E. coli bacteria have multiple virulence factors that enhance their pathogenicity. The most significant of these is urinary tract infections. One element is the capacity to lyse red blood cells, as they include the hemolytic enzyme hemolysin, which exists in many forms of lysis: alpha, beta, and gamma. The initial type, alpha hemolysin, confers upon the bacteria the capacity to partially lyse human erythrocytes. The third type, gamma-hemolysin, enables the bacteria to lyse the red blood cells of various animals, except humans. The bacteria also include the cytotoxic necrosis factor, which lyses erythrocytes (Jawetz *et al.*,2016). Numerous virulence factors contribute to the pathogenicity of microorganisms; can identify those that are helpful in adhesion, biofilm formation, encapsulation, and toxin production (Karam *et al.*,2018).

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The ability of bacteria to produce a polysaccharide capsule containing the capsular antigen (capsular antigen K), as well as additional characteristics of the surface such as flagella, utilized for locomotion in motile organisms, and endotoxins, which comprise lipopolysaccharide (LPS), which are composed of endotoxins (somatic antigens), are additional virulence factors. These structures allow bacteria to colonize host cells, particularly the bladder lining, and also make it possible for them to evade the host's immune system, these antigens have the capacity to evade the host's immune system. They also contain curli fibers, which have chemical and physical characteristics that help bacteria produce biofilms. Additionally, bacteria have vesicles in their outer membrane that act as a facilitation system to help deliver bacterial toxins, enzymes, and adhesion factors to host cells. The presence of toxins, outer-membrane (OPS), and secreted outer-membrane proteins all contribute to the bacteria's increased capacity for infection (Terlizzi *et al.*,2017).

Adhesion is a critical virulence component in *E. coli*, and its attachment to uroepithelial cells is considered the preliminary phase of invasion (Kallas *et al.*,2020). Adhesion is contingent upon genetic factors that encode the adhesion factors that are required, such as pap, Sfa, eae, and fim. fimbriae. Fimbriae can be classified into three types: F-, S-, and P-fimbriae. These crucial virulence factors enable bacteria to attach themselves to host cells, promote colonization, and create a biofilm, all of which help the bacteria become more resistant to anti biotic (Spaulding *et al.*,2017).

2.12.6 Pathogenesis of *Escherichia coli*

Enteric *E. coli* is intrinsic flora of humans and serious pathogens responsible for considerable morbidity and mortality globally(Clements *et al.*, 2012) .

Disease Caused by *Escherichia coli*

2.12.6.1. Intestinal Diseases or Enterotoxins:

There are three categories of these toxins, two of which induce watery diarrhea: heat-labile toxin (LT) and heat-stable toxin (ST). Shiga toxins, analogous to those produced

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by Shigella bacteria, induce bloody diarrhea. The bacteria, rather than their toxins, can cause bloody diarrhea upon invasion of the intestinal lining, resulting in a condition known as dysentery (Levinsion,2016). EHEC, which induces severe illnesses in people, particularly EHEC. O157:H7 is transmitted via contaminated food (Wong *et al.*, 2000). The most prevalent serotype of enterohaemorrhagic *E. coli* (EHEC) is *E. coli* O157:H7, which has caused numerous outbreaks of waterborne and foodborne diseases in multiple countries. Recently, there has been a significant increase of non-O157 Shiga toxin–producing *E. coli* (STEC) strains, especially those linked to serotypes O26, O45, O103, O111, O121, and O145 (Farrokh *et al.*, 2013).

2.12.6.2. Urinary Tract Infections

This disease is prevalent and significant, arising from bacterial infection and its proliferation inside the urinary system, which includes the lower urinary tract (urethra and bladder) and the upper urinary tract (ureters and kidneys). The illness typically initiates in the lower urinary tract and is referred to as cystitis. Urethritis also arises following asymptomatic bacteriuria and presents with symptoms. It also manifests in catheter-associated urinary tract infections, which advance to the upper urinary tract, resulting in ureteritis, nephritis, and infections of the renal pelvis (pyelonephritis). Infection with *E. coli* bacteria, which causes urinary tract infections, is the main source of bacteremia (Foxman ,2014; Forsyth *et al.*,2018).

2.12.6.3. Meningitis

Bacterial meningitis is association with high mortality and morbidity rates, with mortality rates ranging from 10%to 15%particulary in the neonatal period. Extraintestinal pathogens such as *E. coli* is one of the most prevalent bacterial pathogens responsible for extraintestinal infections, including newborn meningitis. Early-onset meningitis is characterized by the onset of bacterial meningitis within three days of birth, whereas late-onset meningitis is defined as the occurrence of bacterial meningitis more than three days after birth (Liu *et al.*,2021).

2.12.6.4. Infections wound / Burns

A common worldwide issue, wound and burn infections result in about 150,000 fatalities each year. The ideal conditions for the colonization and spread of microorganisms are created by skin penetration and loss ((Khosravi *et al.*,2019). One of the most prevalent and destructive types of truma is burn injury, since the mechanical disruption of the skin's integrity causes burn patients to have a special vulnerability to a number of infections linked to weakened resistance. A protein-rich, vascular environment takes the place of the skin barrier, creating an ideal environment for microbial colonization and spread by one or more microorganisms anywhere and putting them in a better position to harm the host's sterile tissues (Fatema *et al.*,2021). Burn patients need to receive immediate specialized care to lower the risk of bacterial infections, which are a leading cause of death (Macedo and Santos, 2005; Akansel *et al.*, 2014). However, pathogenic microorganisms such as Gram-negative bacteria *Klebsiella*, *Escherichia coli*, *Pseodomans auroginosa*, *Acinobacteria baumannii*, and *pneumoniae* can colonize damaged skin due to wound exudate (Zhou *et al.*,2019; Hassan *al*,2021).

2.12.7. Epidemiology of *Escherichia coli*

E. coli, considered the most ubiquitous member of the Enterobacteriaceae family, can inhabit the intestines as part of the natural flora. Opportunistic strains of *E. coli* can become pathogenic when suitable circumstances occur (Gharajalar and Sofiani, 2017; Gurtler *et al.*, 2017). About half of the world's population suffers from urinary tract infections, which rank second only to respiratory infections in underdeveloped areas. Approximately 150 million cases of urinary tract infections occur annually worldwide. When there are fewer than 10 bacteria per milliliter of urine, it indicates an infection (Jaweez *et al* 2016).

The presence of *E. coli* bacteria in drinking water can cause major problems like diarrhea, enteritis, and in rare circumstances, death. *E. coli* bacteria are frequently used as a sign of fecal contamination of drinking water. *E. coli* bacteria are a concerning reservoir of antimicrobial resistance because of their prevalence in environments where

there is a high risk of environmental transmission to humans. Antimicrobial-resistant bacteria are widely distributed in environmental water (Mahmoud *et al.*,2020).

2.12.8. Diagnosis of *Escherichia coli*

2.12.8.1. Molecular Diagnosis

Genetic techniques depend on polymerase chain reaction or DNA sequencing analysis. Diagnosis relies on the amplification of the selected molecular target, understanding its sequence, and comparing it with the reference sequence available in the nucleotide database (Kosecka-strojek *et al.*,2019). 16S rRNA gene sequencing is a highly sensitive and extensively utilized diagnostic technique. The 16S rRNA gene exhibits excellent specificity for individual bacterial species, and the resultant nucleotide sequences are ascertained through comparison with those archived in databases (Clifford *et al.*,2012). Molecular analysis is the most useful method for reducing genetic diversity among bacterial isolates and strains. Various methodologies using molecular markers have been widely used to analyze variation within and between natural populations. Technologies used to study genetic diversity can be hybridization-based. One of the most important molecular technologies is RAPD, which is a DNA marker based on the polymerase chain reaction (PCR). RAPD is used to amplify genomic DNA using a single primer from the nucleotide sequence. It has been widely used to investigate genetic diversity and provides information that can help identify and differentiate species and phylogenetic markers. It does not require a small amount of genomic DNA and can produce high levels of polymorphism (Al-Judy and Majeed ,2013).

2.13. Co infection Between *Escherichia coli* and *Entamoeba histolytica*

Co-infections of the intestines with the parasite, and bacteria, accompanied by intensified clinical symptoms, are prevalent in areas where amoebiasis is widespread(Galván-Moroyoqui *et al.*, 2008). The high incidence rate may be attributed to nutrition and water contamination by parasites, as well as cleanliness and agricultural practices in tropical region (Mustafa *et al.*, 2022).

Chapter Two.....Literature Review

The parasite may disseminate throughout both intestinal and extra -intestinal tissues, resulting in amoebiasis. *E. histolytica* is a consumed bacterium found in the intestinal microflora; variations in the composition of colonic microflora are associated with the onset of symptomatic amebiasis in affected individuals, suggesting that this microflora may play a significant role in the disease(Shaulov *et al.*, 2018). Mainly caused by the toxicity's actions, that damage endothelium cells and stimulate immune system cell response (Khayoon *et al.*, 2025). This parasite, which feeds microorganisms in the intestinal system of its human host, can cause an effective inflammation reaction upon breaching the colonic mucous membrane (Shaulov *et al.*, 2018).

Chapter Three

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Chapter Three.....Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Equipments

Table (3-1): Equipments and instruments used in this study along with their company/country

No	Equipment and instruments	Company/ Origin
1	Autoclave	(HIRAYAMA / USR)
2	Centrifuge	GFL/Germany
3	Cotton Swabs	Estmed/ China
4	Cotton, Gloves	Broche/ Malaysia
5	Disposable syringe 5ml, 3ml	Sterile EO/China
6	Distillator	GFL/Germany
7	Electrophoresis	Cleaver/ England
8	Eppendorf tubes	BioBasic/ Canada
9	Hood	Labtech/Korea
10	hot plate and magnetic stirrer	Stuart (UK)
11	Incubator	Froilabo/ France
12	light microscope	Olympus/Japan
13	Oven	Sacora/ Japan
14	Petri dishes	TRUST/China
15	Power supply	Germany/ Agilent
16	Refrigerator	Dr DAIREI/ Denmark
17	Sensitive balance	Ohaus/ China
18	Slide and cover	China
19	Sterile cap	Superstar/ India
20	Thermocycler apparatus	Agilent/ Germany
21	Tubes, flask, beakers	Locav
22	Vortex mixer	Velp/ Europe

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3.1.2. Chemical Materials

The chemical substances utilized in the present study are included in table(3-2).

Table (3-2): Chemical and materials used in this study

No.	Material	Company/ Origin
1	Agaros	Bio basic/ Canada
2	Alcohol 70%	Al Joud /Iraq
3	GeneRuler 100 bp DNA (ladder)	Bioneer / Korea
4	Glycerol	BDH /England
5	Loading dye	Geneaid/ UK
6	Nuclease free water	Fisher/ Garantie
7	Safety dye	Bioneer/ Korea
8	Seder oil	BDH /England
9	Standard McFarland (0.5)	Bio Mérieux/ Franc
10	TBE	Bio basic/ Canada

3.1.3. Culturing Media

Table lists all of the culture medium utilized in this investigation, along with their manufacturers (3-3).

Table (3-3): Culture media used for this study

NO	Culture media	Company/ Origin
1	Brain hart infusion (BHI) broth	Bio lab/Hungary
2	Eosin methylene blue	Hi media /India
3	MacConkey agar	Hi media /India
4	MacConkey sorbitol	Hi media /India
5	Methyl Red-Voges –Proskauer	HIMEDIA/India
6	Peptone water	Liofilchem /Italy
7	Simon citrate	Liofilchem /Italy

Chapter Three.....Materials and Methods

3.1.4. Solutions, Stains and Reagents

Table (3-4): solutions, stains and reagents used in this study with their origins

No.	Solutions, stains ad reagent	Company / origin
1	Gram stain(crystal violet stain- iodine - ethanol-safranin)	Bio research/Jordan
2	Kovacs Reagent	HIMEDIA/India
3	Methyl red reagent	HIMEDIA
4	Normal Slain	
5	Voges -Proskauer VP1 -VP2	MothalathAl massy/Iraq

3.1.5. Kits

The kits made use of in the present study

No.	Kite	Company/ Origin
1	Master Mix	Geneaid/Taiwan
2	Presto™ Mini gDNA Bacteria Kit	Geneaid/Taiwan
3	Presto™ Stool DNA Extraction Kit	Geneaid/Taiwan

Chapter Three.....Materials and Methods

3.1.6. Primers

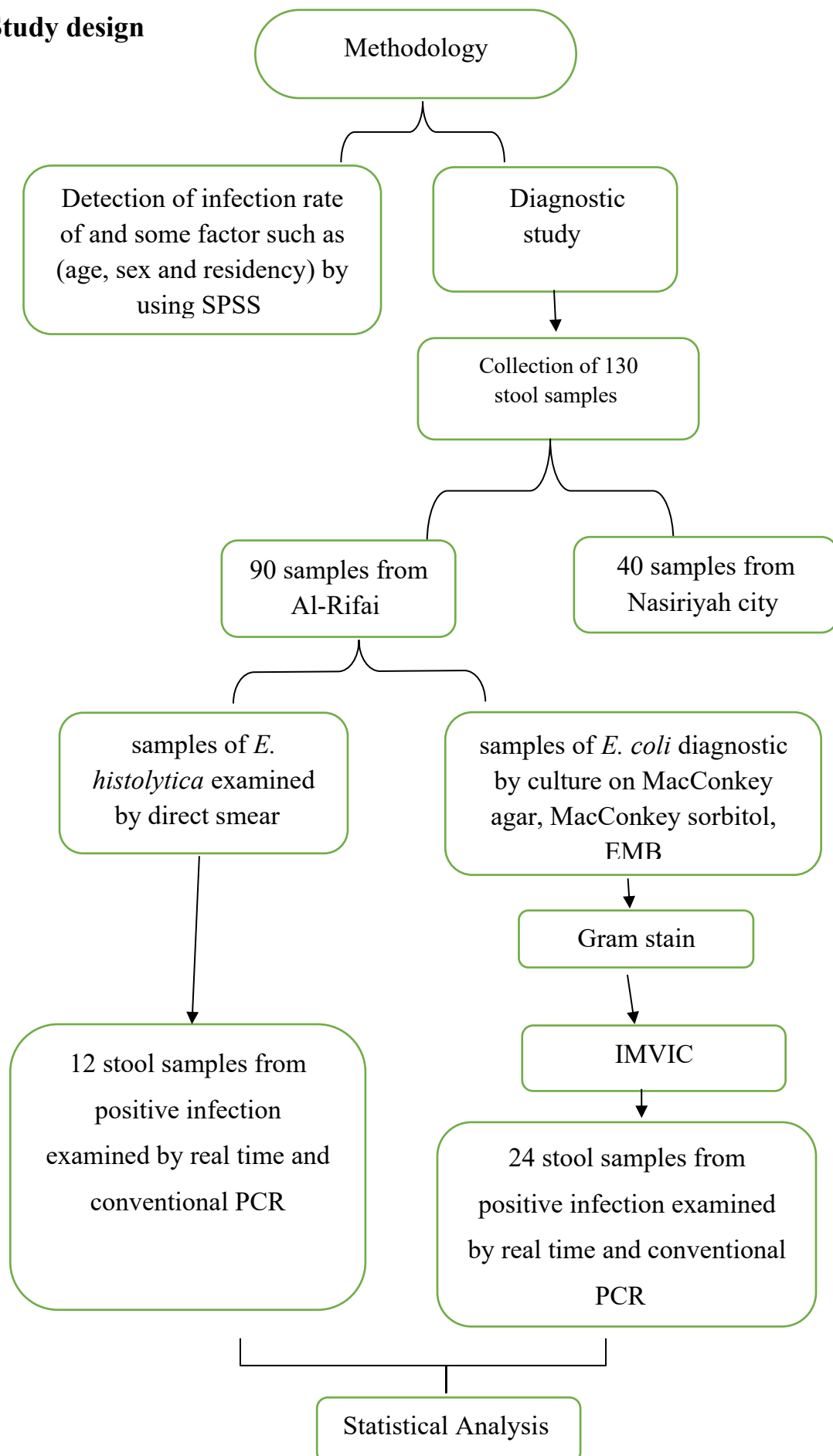
All primers utilized in this study are listed in table (3-5).

Table (3-5): sequences of primers used in gene amplification

Isolates	Gene	Primer Sequences (5' - 3')	Product size	Reference
<i>E. histolytica</i>	<i>E.H.ssR</i> <i>NA</i>	F*: GAATTGACGGAAGGGCACAC R*: AACTAAGAACGGCCATGCAC	149bp	(Al-Obaidi, 2009)
<i>E. histolytica</i>	<i>SSUrR</i> <i>NA</i>	F: TGGACTTCAGGGGGAGTATG R: TCAATCTCGGTACACCACTCA	545bp	(Al-Obaidi, 2009)
<i>E. coli</i>	<i>rfbE</i>	F: TCAAAAGGAACTATATTCAGAAGT TTGA R: CGATATACCTAACGCTAACAAAGCT AA	125bp	(Bahadorip and Jahandar, 2019)

3.2. Methods

3.2.1.Study design



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3.2.2. Collection of Samples

The study beginning from September 2024 to February 2025. Samples were collected from Rifai hospital and Al-hussein hospital in Thi-Qar province, in addition, samples were collected from several private laboratories, including Al-Bayan Private Laboratory (Al-Nasiriyah), Al-Noor Laboratory, and Al-Rifai Private Laboratory 130 stool samples were obtained from patients with severe to moderate diarrhea. The samples were analyzed by direct microscopic and stored in Frezer, within one hour of collection or stored at 4°C for a maximum of 18 hours before culturing. Patient information, including age, gender, and residency was documented.

3.2.3. Preparation of Culture Media

3.2.3.1. Culture Media

The culture media, which included MacConkey agar, Eosin Methylene Blue medium, MacConkey Sorbitol Agar, Simon's Citrate Agar, Peptone water, Methyl Red-Vogues, and Brain-Heart Infusion, were prepared in accordance with the manufacturer's instructions found on each medium's package. All culture media according to (Harrigan and McCance, 2014).

3.2.3.1.1. MacConkey Agar

The medium is prepared by mixing 50 g of dry powder with 1 L of distilled water, adjusting the pH to 7.1 ± 0.2 , and sterilizing it at 121°C for 15 minutes. Upon cooling to 45–50°C, the medium is transferred onto sterile Petri dishes. Prepared plates must be stored in a refrigerator at 4°C, shielded from light and moisture, and are viable for 2–3 weeks. Inoculated plates are incubated at 37°C for 18–24 hours, yielding pink colonies for lactose fermenters and colorless colonies for non-fermenters.

3.2.3.1.2. Sorbitol MacConkey Agar (SMAC)

It is made in a manner similar to MacConkey Agar, substituting lactose with 10 grams of D-sorbitol per liter. The pH is calibrated to 7.1 ± 0.2 and sterilized at 121°C for 15 minutes. Plates are poured, thereafter cooled, and stored at 4°C for a duration of up to three weeks. Inoculated plates are incubated at 37°C for a duration of 18 to 24 hours,

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non-sorbitol-fermenting *E. coli* O157:H7 appears colorless, whereas other bacteria that ferment sorbitol develop pink colonies.

3.2.3.1.3. Eosin Methylene Blue (EMB) Agar

For the purpose of making it, mix 37 g of powder with 1 L of distilled water, adjust the pH to 7.2 ± 0.2 , and then sterilize it at 121°C for 15 minutes. Upon cooling to around 50°C , the medium is transferred into sterile plates. Plates are maintained at 4°C , shielded from light, and remain viable for approximately one month. Inoculated plates are incubated at 37°C for 18–24 hours, resulting in *E. coli* producing a metallic green sheen, while non-lactose fermenters remain colorless.

3.3. Laboratory Diagnosis

3.3.1. Detection of *E. histolytica*

3.3.1.1. Wet Smear

The stool is dissolved in normal saline to facilitate the examination of the parasite's morphology and movement (Al-Kubaisy *et al.*, 2014). A compound light microscope with a magnification capacity of 100x was employed to identify the parasite. A drop of 0.9% saline solution was mixed with a swab collected from the stool of individuals with acute diarrhea on glass slides. The slides were examined under a microscope with a 40x magnification lens, subsequent to being affixed with coverslips. A parasite was identified, and its current stage was established (Flaih *et al.*, 2021)

3.4. Molecular Examination

3.4.1. Extraction DNA Parasite of *E. histolytica* from Stool Sample

1. Sample Lysis

Approximately 280 mg of stool transferred into a bead-beating tube with ceramic beads and added 800 microliters of ST1 buffer. Then we vortexed the sample for a brief time and let it sit at 70°C for five minutes. Thereafter, tape or an adapter was used to attach the bead-beating tubes horizontally to a regular vortex. The vortex was then turned on at its highest speed for 10 minutes at room temperature. Thereafter, the tubes were spun in a centrifuge at $8,000 \times g$ for 2 minutes to get rid of the foam that the

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detergents in the ST1 buffer generated. Thereafter, 500 μ l of the supernatant was put into a clean 1.5 ml microcentrifuge tube.

2. PCR Inhibitor Removal

Following the addition of 150 μ l of ST2 buffer to the sample and spun it for 5 seconds. The solution was then left at 4°C for five minutes. To eliminate rid of insoluble contaminants and possible inhibitors of PCR, the sample was spun at 16,000 $\times$ g for 3 minutes at room temperature. We carefully moved 500 μ l of the clarified supernatant to an inhibitor-removing column (the purple ring) that was put into a 2 ml centrifuge tube. The mixture was spun in a centrifuge at ambient temperatures for one minute at 16,000 $\times$ g. Thereafter, the column was thrown away, and the flow-through that was collected in the 2 ml tube was kept for the DNA binding phase.

3.DNA Binding

1. 800 microliters of ST3 buffer have been placed in the stream-through and dissolved well by shaking it strongly for a period of approximately five seconds.
2. A GD column (green ring) was placed in a 2 ml collecting container, and 700 μ l of the prepared specimen combination was added to the column.
3. The assembly was spun in a centrifuge at 16,000 $\times$ g for 1 minute at room temperature. Thereafter, the flow-through was removed.
4. The GD column was then put back into the original collecting container, together with the rest of the specimen mixture.
5. A second centrifugation was done in the same way, and then the stream-through section was thrown away, and the GD column was put back into the 2 ml collecting tube.

4.Wash

1. The GD column got 400 microliters of ST3 buffer.
2. Thereafter, the sample was spun at 16,000 $\times$ g for 30 seconds at room temperature.
3. The flow-through was taken out, and the GD column was put back into the 2 ml collection tube.

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4. Thereafter, 600 µl of wash buffer with 100% ethanol was added to the GD column and spun at $16,000 \times g$ for 30 seconds at room temperature.

5. After getting rid of the flow-through, the column was put back into the original collecting tube, and the washing process was done again with an extra 600 µl of wash buffer under the same circumstances.

6. The flow-through was thrown away again, and the column was put back into the collection tube. To fully dry the column matrix, centrifugation was done at $16,000 \times g$ for 3 minutes at room temperature.

5. Elution

The dried GD column was put into a sterile 1.5-ml microcentrifuge tube, and 70 microliters of Hot Elution Buffer 1 were carefully added to the center of the column matrix.

After that, the column was left to sit for at least two minutes to ensure that the elution buffer, TE, or water was completely absorbed. The pure DNA was extracted by centrifuging it at room temperature for 2 minutes at $16,000 \times g$.

3.4.2. PCR Detection of *SSUrRNA* gene of *E. histolytica*

Amplification of the *SSUrRNA* gene of *E. histolytica* DNA was carried out using the primers listed in Table (3.5). Each reaction tube had a final volume of 20 µl, containing 12.5 µl of Master Mix, 1 µl each of the forward and reverse primers specific to the target gene, 3 µl of DNA template, and nuclease-free water to complete the total volume. For electrophoresis, 5 µl of each DNA sample was combined with loading dye and carefully loaded into the designated wells. The samples were then subjected to electrophoresis under an electric field of 70 V for 45–60 minutes. The thermocycling conditions for amplification of the *SSU rRNA* gene are presented in Table (3-6).

Table (3-6): Program of *SSUrRNA* gene.

Step	Temperature, °C	Time	Cycle
Initial denaturation	94	5 min	1
Denaturation	94	45 sec	40
Annealing	57	45 sec	
Extension	72	60 sec	
Final extension	72	7 min	1

3.4.3. Agarose Gel Electrophoresis

1- Agarose gels with concentrations of 1% and 1.5% were made by dissolving 1 g and 1.5 g of agarose powder, respectively, in 100 ml of 1X TBE buffer. The amalgamation was sealed with aluminum foil to inhibit evaporation and cooked on a hot plate with magnetic stirring until the agarose was entirely dissolved. A 1% agarose gel was applied for electrophoresis after DNA extraction, while a 1.5% agarose gel was used for the examination of PCR-amplified gene products

2- The flask was removed from the hot plate and allowed to cool to a temperature range of approximately 45–55°C.

3- Once cooled, 8 µl of a safe DNA stain was added to the gel solution and mixed gently to ensure uniform distribution

3- A 16-tooth comb was positioned within the casting tray and immersed in the agarose solution to create sample wells.

4. A micropipette tip was used to remove any air bubbles after the molten agarose was gently poured into the casting tray.

5. After that, the gel was allowed to harden at room temperature for almost half an hour. Both the comb and the sealing tape were carefully removed after the gel had fully formed.

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6- After solidification, the gel tray was transferred into the electrophoresis tank, which was then filled with 1X TBE buffer until the gel was completely submerged.

7-DNA extracts or PCR amplification products were prepared by mixing 5 µl of each sample with 2 µl of loading dye (bromothymol blue) before being loaded into the wells. After that, electrophoresis was done at 70 V for 45 to 60 minutes.

8. Finally, as explained, the isolated DNA fragments or PCR results could be seen with ultraviolet light at a wavelength of 300 nm by (Podzorski *et al.* 2006).

3.4.4. The Isolation and Designation of *Escherichia coli*

3.4.4.1. Culture

After growing and purifying the bacterial isolates on different types of culture media, like MacConkey agar, eosin methylene blue, and MacConkey sorbitol, the morphological traits of the isolated colonies were looked at. The study looked at the isolated bacterial colonies' diameters, heights, edges, textures, colors, and shapes (Wanger *et al.*, 2017). To get pure, picked lactose-fermenting colonies and grew them again on brand-new MacConkey agar plates to achieve pure, well-isolated colonies. To verify for colonies at a green metallic sheen, separated colonies were spread in Eosin Methylene Blue (Lupindu, 2017). *E. coli* O157:H7 is clearly spreading on SMAC agar, where it observed essentially to be a pure culture with colorless colonies that non ferment sorbitol. This appearance differed from that of the common intestinal flora, which generally ferments sorbitol, resulting in the formation of pink colonies on the same medium. In contrast, its proliferation on MacConkey agar exhibited unique morphological and biochemical characteristics. The *E. coli* O157:H7 strain was indistinguishable from the fecal microbiota. SMAC medium facilitates the prompt identification of *E. coli* O157:H7 in stool cultures(March and Ratnam, 2012).

3.4.4.2. Microscopic Identification

A little quantity of the bacterial colonies was put on a microscope slide and combined with a drop of distilled water to create a smear. The smear underwent gram staining and

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was analyzed under a light microscope with an oil immersion objective at 100× magnification to assess bacterial coloration (McFadden2000).

3.4.4.3. Gram Stain and Procedure

The staining process distinguishes species within the domain Bacteria based on their cell wall construction, Gram-positive cells possess a robust peptidoglycan layer and exhibit a blue to purple coloration upon staining, Gram-negative cells possess a slender peptidoglycan layer and exhibit red to pink staining (Smith and Hussey, 2005).

3.4.4.3.1. Staining Procedure

1. The prepared smear was placed on a staining rack over the sink, and the principal stain, crystal violet, was applied to the smear for one minute, then rinse the smear under moderate tap water and apply Gram's iodine for one minute.
2. Apply Gram's iodine for one minute, after one minute, rinse the smear under moderate tap water.
3. The smear was then treated with a decolorizing agent, either acetone or 95% ethanol, for a maximum of 10 seconds.
4. Following this, the slide was gently rinsed with tap water, and a counterstain—either safranin or a 1:10 dilution of carbonyl fuchsin—was applied for under one minute, following one minute, wash the smear under gentle tap water and let it dry in the air.
5. Wipe the smear's back with tissue paper, add a drop of cedarwood oil, and look at it under a microscope with an oil immersion lens (100X) (Paray et al., 2023).

3.4.5. Biochemical Tests

3.4.5.1. Indole Test

It was put bacterial colonies in tubes filled with peptone water for this test. Thereafter, the tubes were kept at 37°C for an entire day. Then, one or two drops of Kovac's reagent were added to the medium. A red ring on the surface of the medium being tested shows that the result is positive (Forbes *et al.*, 2007).

3.4.5.2. Methyl Red Test

After putting colonies of bacteria into tubes with MR-VP broth, after incubation, 4–5 drops of methyl red reagent were added to the MR test tube. The tubes were then kept

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at 37 degrees Celsius for 24–48 hours. Development of a stable red color indicated a positive methyl red test (Hemraj *et al.*, 2013).

3.4.5.3. Voges-Proskauer's Test

It was put Methyl Red-Voges-Proskauer (MR-VP) media into tubes that had groups of bacteria in them. After that, 10 drops of VP1 (alpha-naphthol) were added, followed by five drops of VP2 (KOH), and the tubes were shaken hard. After 30 minutes, the tubes were checked to see if they had changed color. A change in color from yellow to red indicated that the bacteria could break down carbohydrates and make acetoin (Brown and Smith, 2017).

3.4.5.4. Citrate Utilization Test

After streaking and stabbing, bacterial colonies were put into tubes with Simmon's citrate medium. The tubes were then kept at 37°C for a whole day. The change in color from green to blue means that result will be positive. The objective of this experiment was to determine the bacteria's ability to use citrate as its only carbon source and ammonium salts as a nitrogen source (Brown and Smith, 2017).

3.4.6. Preservation

1-Short -Term Preservation

The isolated cultures were safely stored for a short time in closed tubes containing a broth made from brain and heart infusion with 15 per cent glycerol shortly after being incubated at 37°C for 18 hours (Rohman *et al.*, 2013).

2.Long-Term Preservation

A loop filled with pure bacterial culture was inserted to inoculate a broth made from brain and heart infusion in a screw-top tube. Following 18 hours at 37°C, the broth was ready to use. The addition of glycerol at last stored the inoculum at -20°C for six months. The inoculum was prepared using a 15% glycerol concentration and standard saline (0.9% sodium chloride) (Mahmoud, 2020).

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3.4.7. Extraction from Bacteria Sample

It utilized the Pres Mini gDNA bacteria kit to get genomic DNA from all of the *E. coli* isolates. The manufacturer recommends this procedure.

1. Sample Preparation

The overnight BHI broth containing the isolated bacteria (up to 1×10^9) was then transferred to a 1.5 ml microcentrifuge tube, centrifuged for 1 minute at 14-16,000 rpm, and the supernatant was discarded. Put in 180 μ l of GT buffer and then used a vortex machine or pipette for mixing the cell pellet again. Put in 20 μ l of proteinase K. For at least 10 minutes, the temperature was kept at 60°C. Turn the tube upside down every three minutes while it is incubating.

2.Lysis

200 Microliters of GB buffer were placed into the sample and mixed using vortexing for ten seconds, subsequently incubated at 70°C for a minimum of ten minutes to guarantee complete lysis and clarity of the sample.

The tube was flipped every three minutes to guarantee consistent mixing. Concurrently, the elution buffer necessary for the DNA elution phase was preheated to 70°C.

3. DNA Binding

1.200 microliters of pure ethanol were incorporated into the sample lysate and mixed promptly by vigorous shaking

2. If a precipitate forms, disaggregate it well using a pipette, then place a GD column in a 2 ml container.

3. Then, transport this mixture (with any solid precipitate) to the GD column and spin it at 14,000 to 16,000 x g for 2 minutes.

4. Remove the 2 ml collection tube containing the flow-through and place the GD column into a new 2 ml collection tube.

4. Wash

1. 400 microliters of W1 buffer were inserted in the GD column.

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2. Then, for 30 seconds, move it at 14,000 to 16,000 rpm and eliminate the flow-through.
3. Put the GD column back in the 2 ml collection tube and put 600 µl of a wash buffer on it. Ensure that ethanol has been applied.
4. For 30 seconds, spin the centrifuge at 14,000 to 16,000 rpm, then throw away the flow-through.
5. Thereafter, they put the GD column back in the 2 ml collection tube.
6. Finally, you centrifuged the column matrix for 3 minutes at 14,000 to 16,000 rpm to dry it.

5. Elution

It was put the dried GD column into a sterile 1.5 ml microcentrifuge tube and then added 100 µl of warmed Elution Buffer 1 straight into the middle of the column matrix. Let the buffer used for elution soak in for at least three minutes. Then, spin it in a centrifuge at 14,000–16,000 rpm for 30 seconds to remove the pure DNA out.

3.4.8. Realtime PCR Detection of *Ehr* and *rfbE* genes

The qPCR was performed using SYBR Green Master Mix according to the manufacturer's protocol. The primers sequences of the detection *Eher* for *E. histolytica* and *rfbE* genes for *E. coli* respectively, were listed in Table (3.8). The RT-PCR reaction mixture was 20µL, as listed in Table (3-7).

The thermocycling programs of *Ehr* and *rfbE* genes were listed in Table

Table (3-7): Reaction component of RT-PCR

Component	Volume
DNA	3 µl
Forward primer (10µM)	1 µl
Reverse primer (10µM)	1 µl
2x SYBR Green Master Mix	12.5 µl
ddH ₂ O	2.5 µl
Total volume	20 µl

Table (3-8): Program of *ehr* and *rfbE* genes

Step	Temperature, °C	Time	Cycle
Initial denaturation	94	3 min	1
Denaturation	94	20 sec	40
Annealing	60	60 sec	
Extension	72	20 sec	

3.5. Statistical Analysis

Descriptive statistics was employed, and the categorical variables (cities, age groups, and identification techniques) were expressed as percentages (%). Fisher's exact test conducted the comparison between categorical variables. Odd ratio was calculated based on Baptista-Pike, and relative risk was calculated based on Koopman's asymptotic score. The significance level was 0.05, and only two-sided significant value was chosen for all statistical tests. All statistical tests and graphical presentation by Graph-pad Prism

Chapter Four

Results

4.1. The Detection Rate of *Entamoeba histolytica* Based on Morphological Techniques

The results show that *E. histolytica* was found in 66 out of 130 samples (50.76%), while 64 samples (49.23%) were negative when checked under a microscope

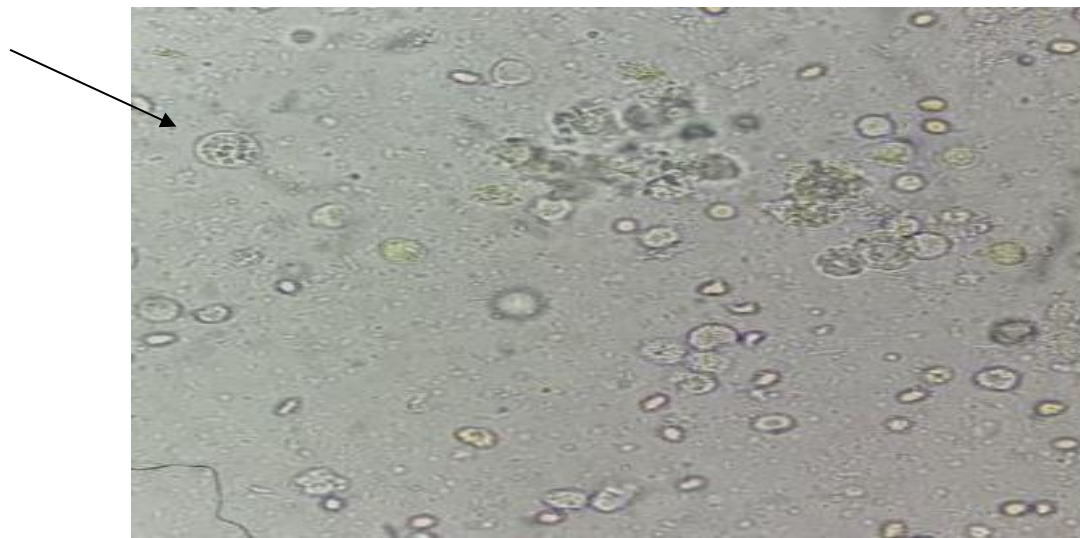


Figure (4-1): Demonstrates of *E. histolytica* under 40X light microscopic direct wet smear

Table (4-1): Detection rates of *E. histolytica* from stool samples based on morphological identification

Microorganisms	No of samples	Positive growth	Negative growth
<i>E. histolytica</i>	130	66(50.76)	64(49.23)

Chapter Four.....Results

4.1.1. The Distribution of *Entamoeba histolytica* Infection According to Sex

Table (4-2) represents the distribution of *E. histolytica* based on the sex of the patients. The infection rate was higher in males (40 out of 76, or 52.63%) compared to females (26 out of 54, or 48.15%). However, the differences were not statistically significant. Additionally, the odds ratio (OR) was 1.197 (confidence interval 0.5954 - 2.427), indicating no association between sex and the infection rate.

Table (4-2): The association of the patient sex and the *E. histolytica* Infection

<i>E. histolytica</i>	Male	Female	Total	P-value
Positive	40 (52.63%)	26 (48.15%)	66(50.76%)	0.7221
Negative	36 (47.37%)	28 (51.85%)	64 (49.23%)	
Total	76	54	130	

4.1.2. The Distribution of *Entamoeba histolytica* Infection Based on Residency

The results shown in Figure 4 illustrate the relationship between *E. histolytica* infection and residency (Nasiriyah vs. Al-Refai). The infection rate was higher in Al-Nasiriyah City compared to Al-Refai City 27(67.50%) vs. 39(43.33%)), with statistically significant variations ($P < 0.05$). Patients residing in Al-Nasiriyah City were nearly three times more likely to be infected than those in Al-Refai City (OR = 2.716, CI = 1.276 5.938).

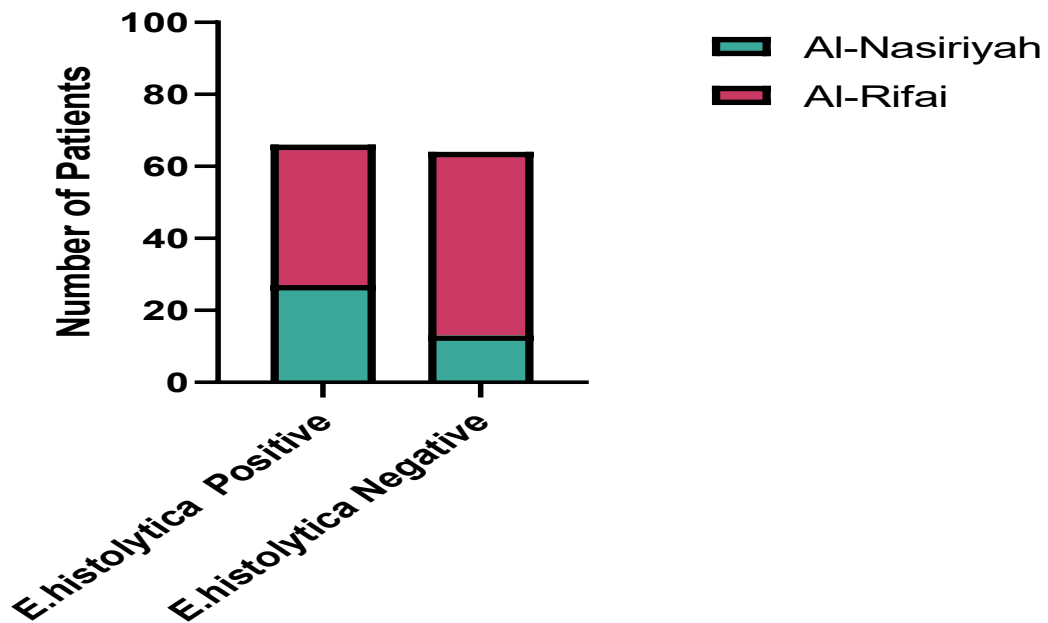


Figure (4-2): The association of the patient residency and the *E. histolytica* infection.

Chapter Four.....Results

4.1.3. The incidence of *Entamoeba histolytica* Infection Across Different Age Groups

The highest infection rates were observed in children aged 1–3 years (27/66, 40.90%) and adults over 20 years (28/66, 42.42%). Goodness-of-Fit analysis revealed a significant difference in infection distribution among the age groups ($X^2 = 51.902$ for children, $P = 0.000$; $P < 0.0001$ for adults), indicating that age is a significant factor influencing susceptibility to infection.

Table (4-3): The association of the patient age and *E. histolytica*

Age group	Age group	No. of positive (%)	P-value (X^2)
Young	1-3 years	27 (40.90)	0.000 (51,902)
	4-7 years	3(4.54)	
	8-12 years	2(3.03)	
Adult	13-16	2(3.03)	<0.0001 (42.71)
	17-20	4(6.06)	
	>20 years	28(42.42)	
	Total	66	

Chapter Four.....Results

4.2. The Detection Rate of *Escherchia coli* Based on Morphological Techniques

The results show that *E. coli* was found 54 out of 130 samples (41.53%) grew well on culturing media (MacConkey agar, MacConkey sorbitol and EMB) and biochemical test (IMVIC) as the same figures (4-3, 4-4, 4-5).

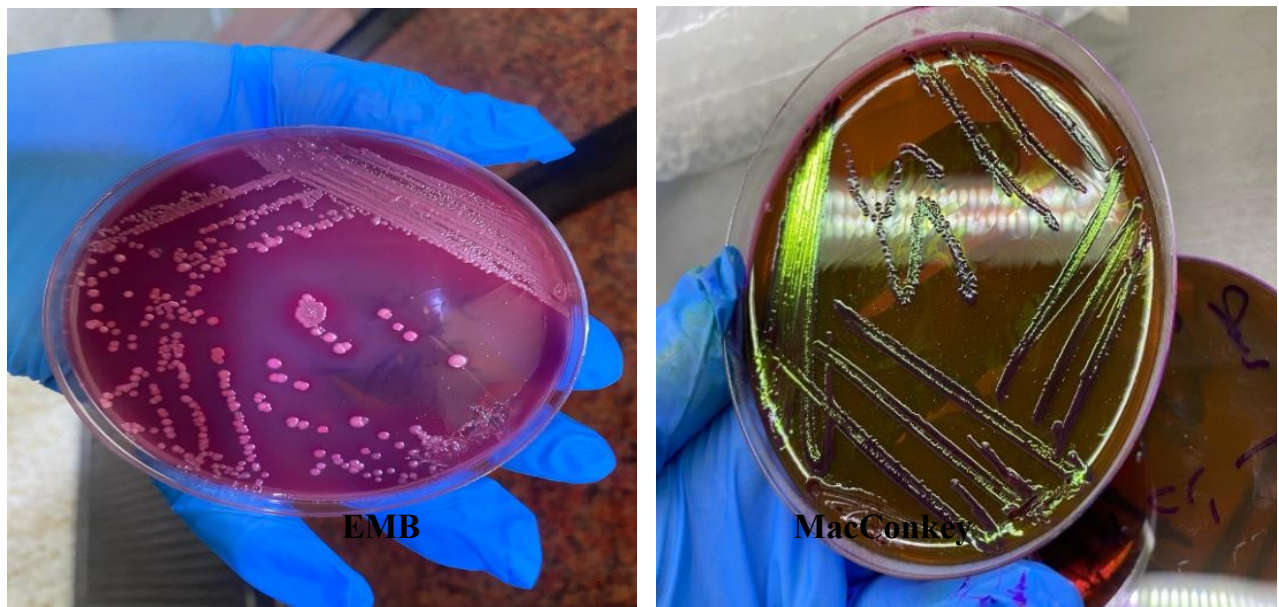


Figure (4-3): Demonstrates the result of *E. coli* on MacConkey, EMB

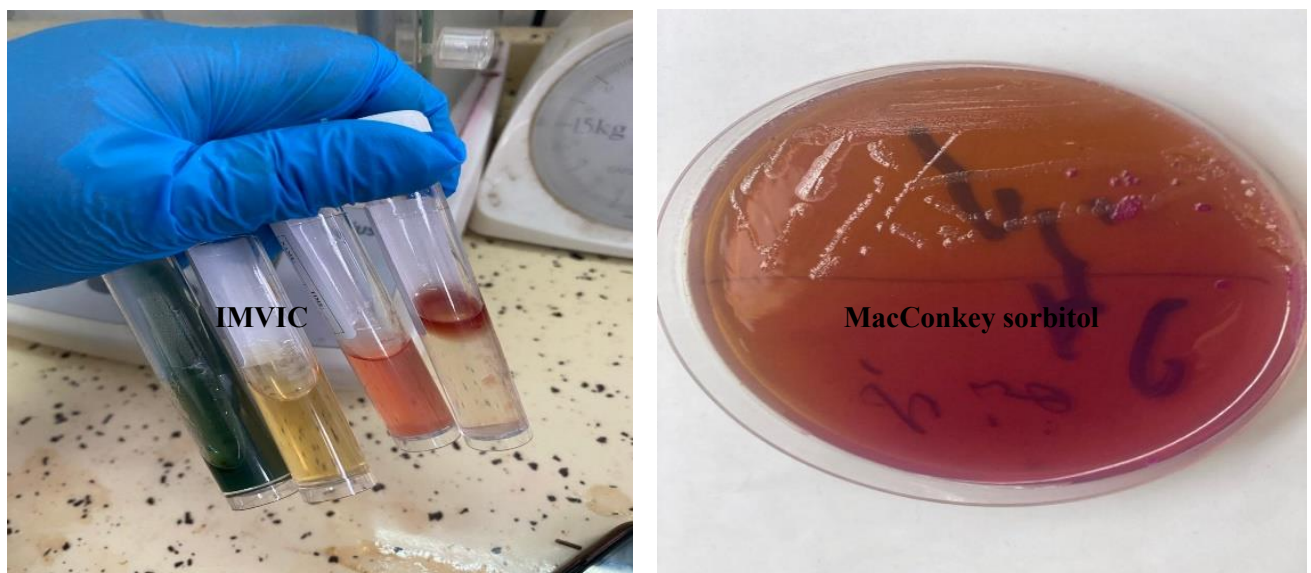


Figure (4-4): Demonstrates the colonies of *E. coli* with IMVIC, and growth *E. coli* O157 on MacConkey sorbitol agar.

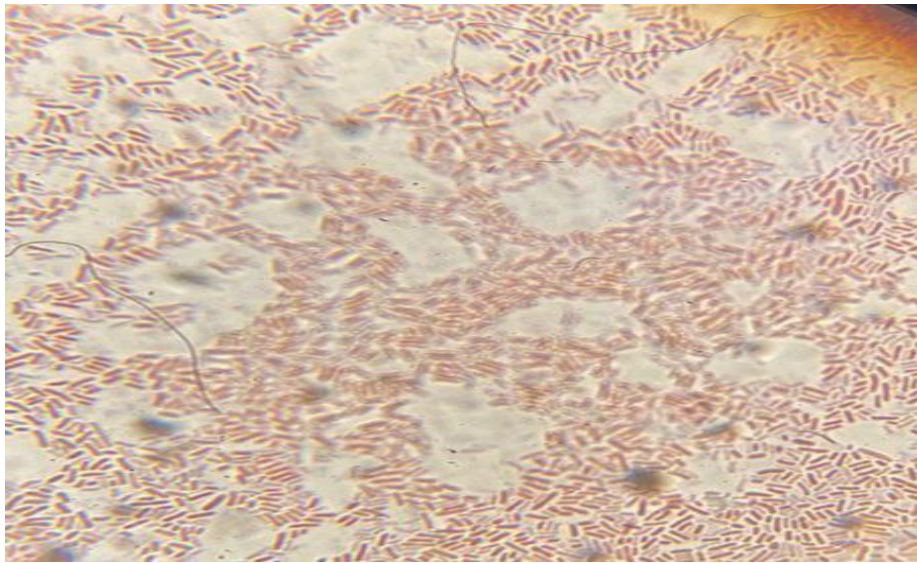


Figure (4-5) Demonstrates the result of bacteria Gram negative under 100X microscopic using Gram stain

Table (4-4): Detection rates of *E.coli* from stool samples based on morphological identification

Microorganisms	No. of samples	Positive growth	Negative growth
<i>E. coli</i>	130	54(41.53)	76(58.47)

4.2.1. Distribution of *Escherchia coli* Infections Based on Sex of Patients

Distribution of *E. coli* infections was analyzed based on the sex of the patients, as shown in Figure (4-2). The infection rate was higher in females 31(57.41%) compared to males 23(30.26%). The statistical evaluation demonstrated a difference of statistical significance ($P < 0.05$).

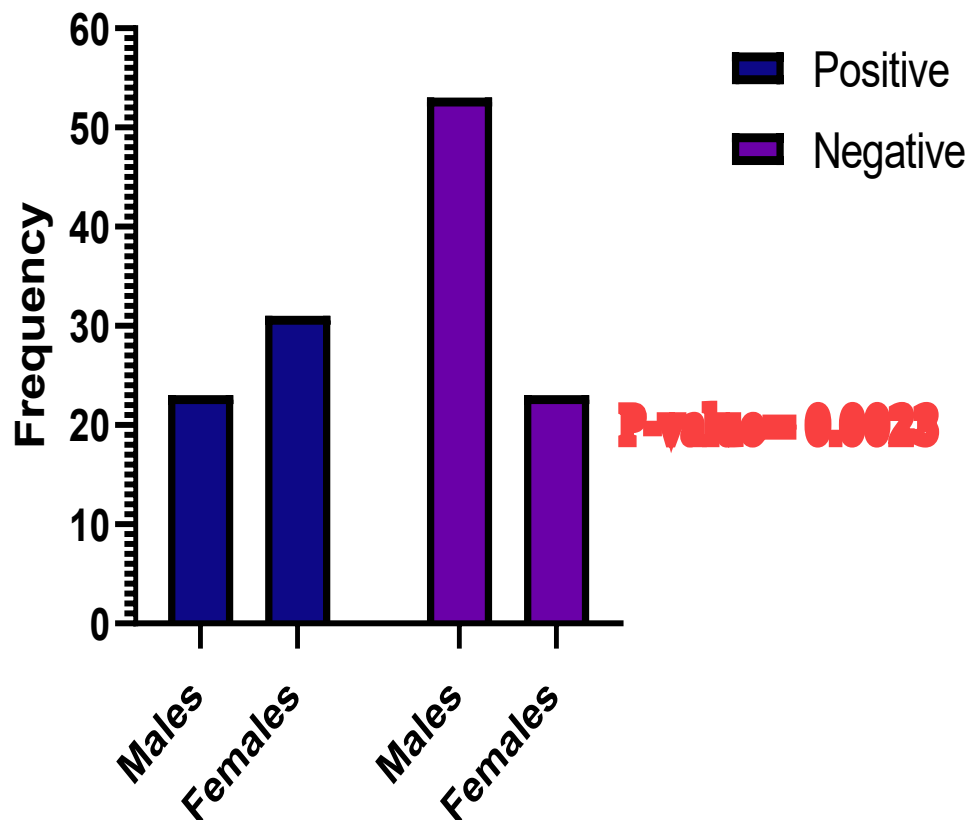


Figure (4-6): The distribution of *E. coli* infection based on the patient sex

4.2.2. Distribution of *Escherchia coli* According to Residency

Figure 7 shows the regional distribution of *E. coli* according to residency. The infection rate was higher in Al-Nasiriyah City 20(50%) compared to Al-Refai 34(37.78%). Although the differences were not statistically significant ($P > 0.05$), patients in Al-Nasiriyah City exhibited an infection rate nearly double that of patients in Al-Refai (OR = 1.6; CI = 0.78 – 3.48).

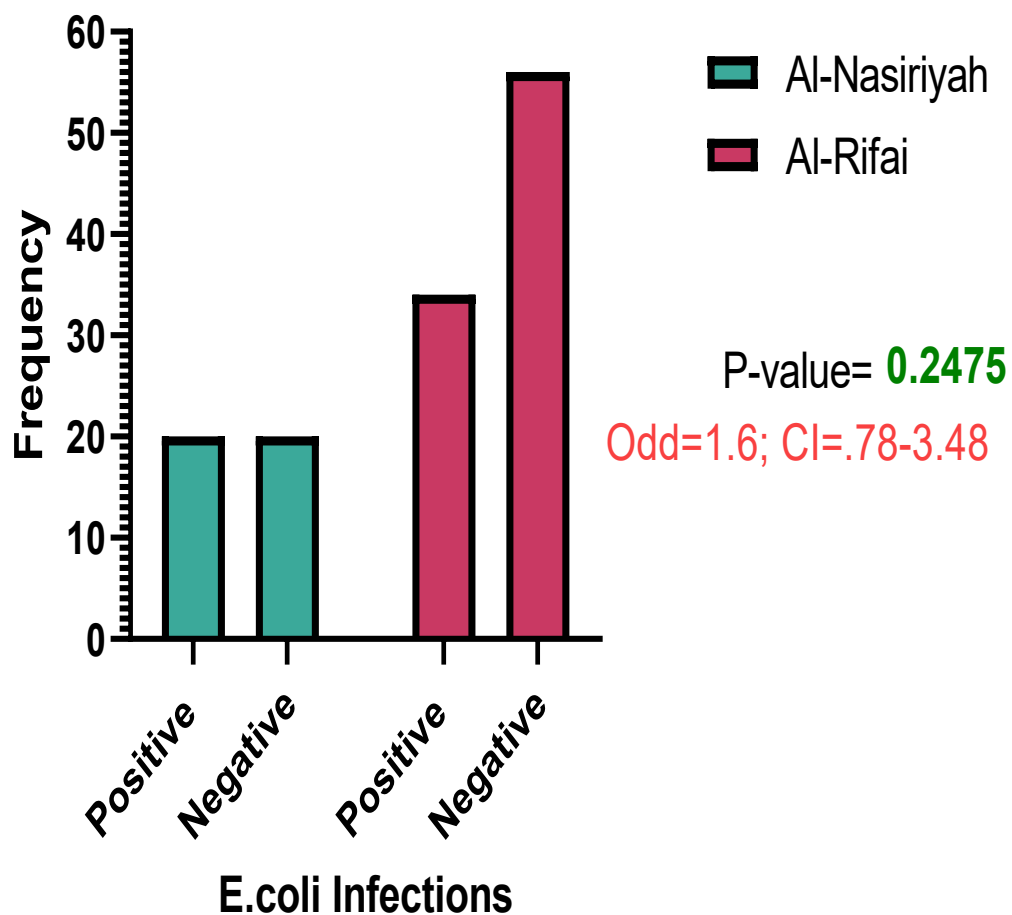


Figure (4-7): The prevalence of *E. coli* infection related with the patient's geographic location

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4.2.3. The Distribution of *Escherchia coli* Infections According to Age

The results of the current study regarding the distribution of infection according to age groups are presented in Table (4-5). Among the six age groups, the highest prevalence was recorded in individuals older than 20 years, accounting for 22/54 (40.74%), followed by children aged 1–3 years with 15/54 (27.7%). Lower prevalence rates were observed in the age groups 4–7 years (12.96%), 17–20 years (7.40%), and in both the 8–12 and 13–16 years age groups, each recording the lowest prevalence of 5.55%. Statistically, the distribution of infection among the age groups was highly significant ($p < 0.001$).

Table (4-5): The distribution of *E. coli* infection based on the patient's age groups

Age group	No. of positive (%)	P-value (X^2)
1-3	15(27.7)	0.000 (31.185)
4-7	7(12.96)	
8-12	3(5.55)	
13-16 years	3(5.55)	0.000 (32.82)
17-20 years	4(7.40)	
>20 years	22(40.74)	
Total	54	

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4.3. Coexistence of *Entamoeba histolytica* and *Escherchia coli* Among the Age Groups

Findings of this study revealed that out of 130 patients, 28 (21.53%) were found to be positive for the co-existence of both *E. coli* and *E. histolytica*. The highest prevalence of co-infection was observed in the age groups 1–3 years (11/28, 39.28%) and >20 years (10/28, 35.71%), whereas the lowest prevalence was recorded in the age groups 8–12 years and 13–16 years (1/28, 3.57% each). Statistical analysis showed a significant association between age groups and co-infection ($p < 0.05$).

Table (4-6): Coexistence of *E. histolytica* and *E. coli* among the age groups

Age groups	Co-existence of both pathogens	
1-3 years	11 (39.28)	0.0015(13.04)
4-7 years	2(7.1)	
8-12	1 (3.57)	
13-16 years	1(3.57)	0.0083 (9.58)
17-20 years	3(10.71)	
>20 years	10 (35.71)	
Total	28 (21.53%)	

4.3.1. Co-existence of Two Pathogens Regarding Residency

The results in Figure (4-8) indicate that the co-existence of the two pathogens was higher in Al-Refai City compared to Al-Nasiriyah City (27(71.06%), 11(28.94%) vs). This variation was statistically significant ($P < 0.05$).

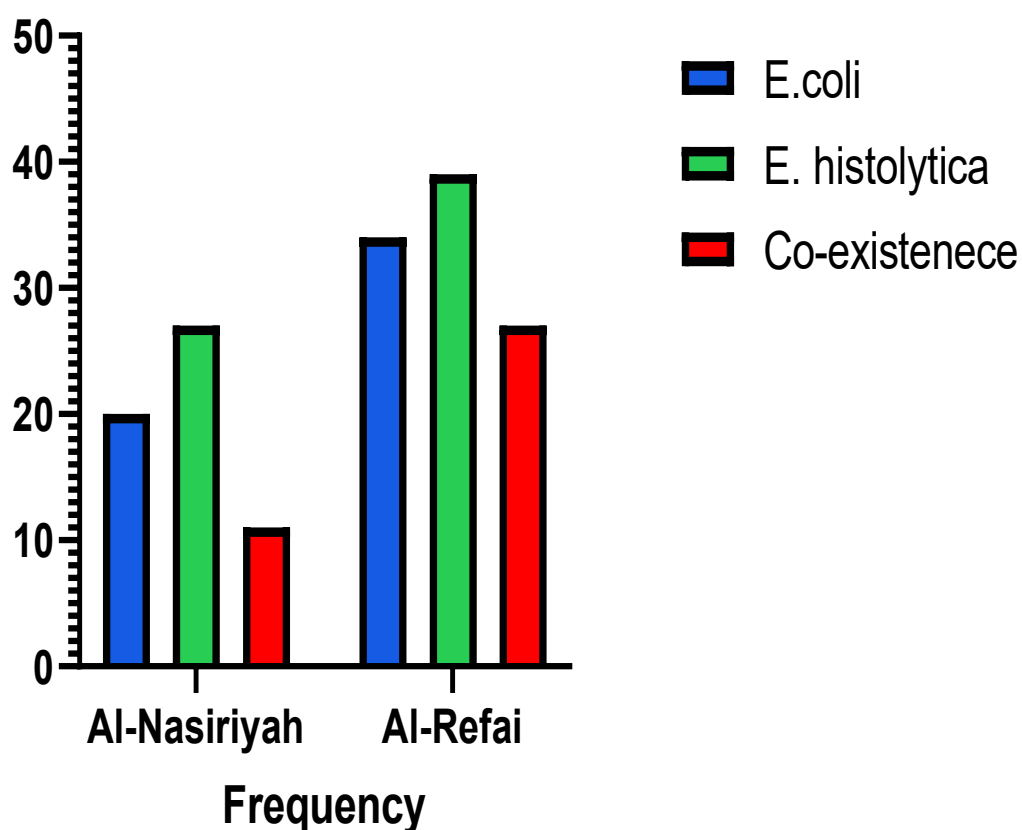


Figure (4-8): Co-existence of *E. histolytica* and *E. coli* in two different Cities

4.4. Results of the PCR Examination:

4.4.1. Conventional PCR Technique

The results that showed (6/12; 50%) of *E. histolytica* isolates showing positive results for *SSUrRNA* gene. The bands of this gene which determined the size of *SSUrRNA* gene, nearly 545bp, as shown in Fig. (4-10). The whole genomic DNA shown in Figure (4-9).

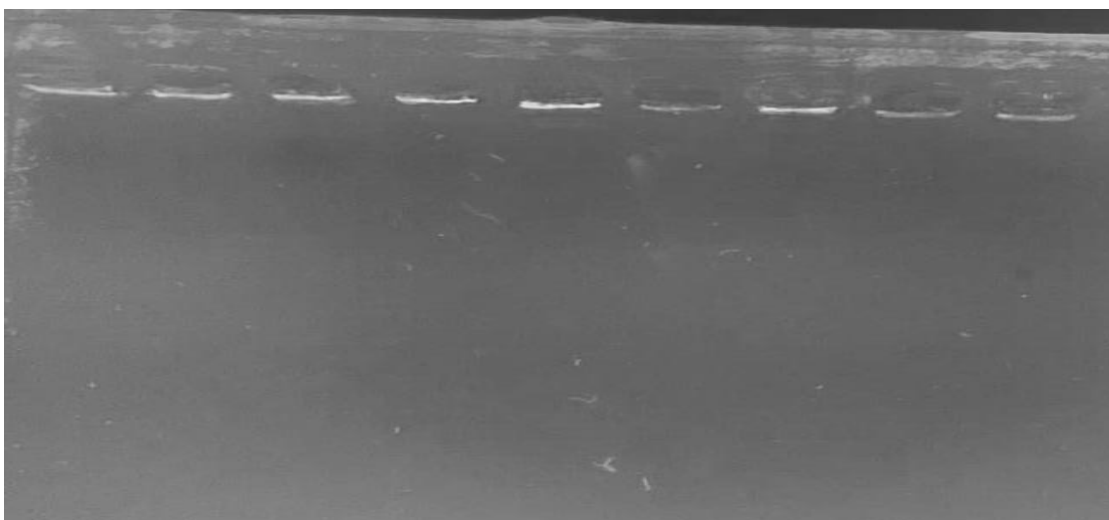


Figure (4-9): Agarose gel electrophoresis of genomic DNA of *E. histolytica*

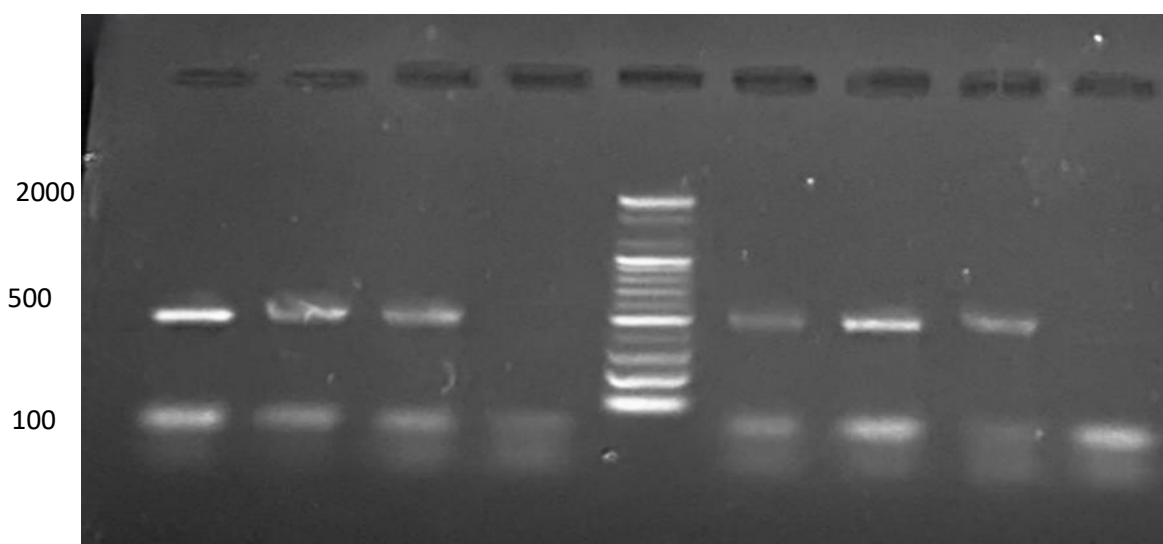


Figure (4-10): Agarose gel electrophoresis of *SSUrRNA* gene amplification for *E. histolytica*, with M representing the ladder, size of *SSUrRNA* gene, nearly 545bp and lanes 1-3 and 5-7 indicating positive results, while lane 4 serves as the negative control.

4.4.2. Real Time Technique

The real time PCR results recorded that half of DNA extracted from stool samples harbored *ehr* gene that used for detection *E. histolytica* (6/12; 50%), Figure (4.11) showed the amplification plots of *ehr* gene by real time PCR technique

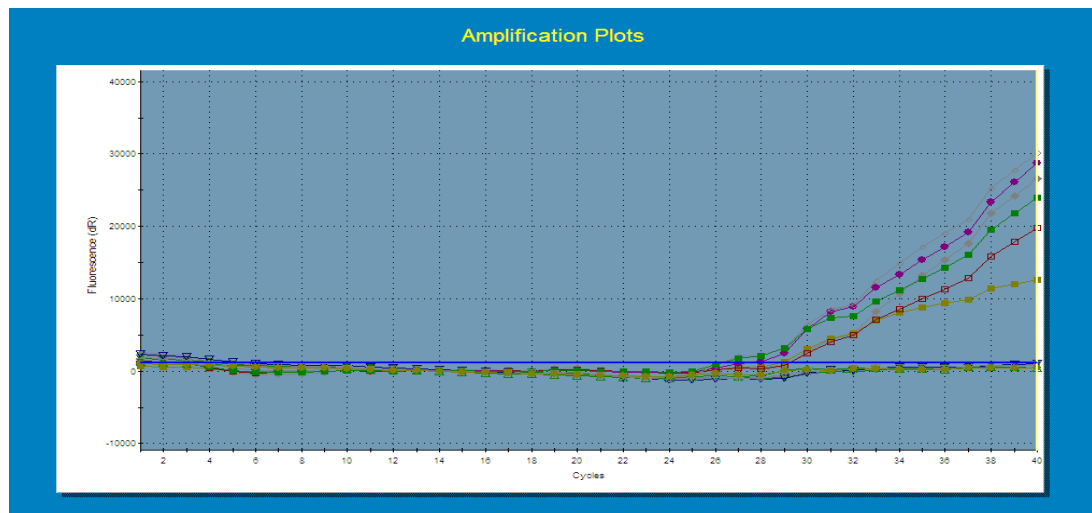


Figure (4-11): Amplification Plots of *ehr* gene in *E. histolytica*

While, results recorded that (1/24; 4.16%) of *E. coli* isolates giving positive results for *rfbE* gene, as in Fig. (4-12) represents the amplification plots of this gene, in this amplification plots showed one positive sample of *rfbE* gene, other sample as internal control.

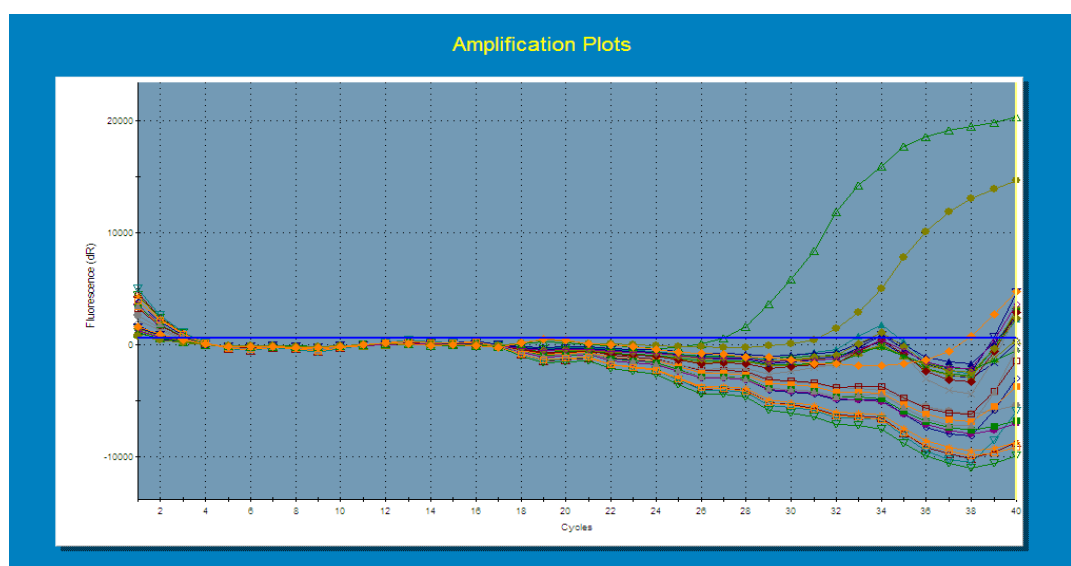


Figure (4-12): Amplification plots of *rfbE* gene in *E. coli*

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4.4.3. Confirmation of Morphological Diagnosis by Molecular Identification

PCR analysis was performed on 12 stool samples previously examined microscopically for *Entamoeba histolytica*. The results showed that 6 samples (50%) were positive.

Table (4-7): Confirmation of the results by techniques (Molecular and Microscopy) in the detection of *E. histolytica*

Type examination	Positive	Negative	Total
PCR	6(50%)	6(50%)	12

4.4.4. The Detection Rate of *E. histolytica* and *E. coli* based on a Molecular Approach

Based on PCR detection, this study's results (Table 4-8) showed significant differences ($P \geq 0.05$) in the detection rate of the two pathogens. High detection rate (50%) was observed for *E. histolytica* compared to only 4.16% for *E. coli* O157.

Table (4-8): Detection rates of *E. histolytica* and *E. coli* O157 from stool samples based on molecular detection

Microorganisms	No. of samples	Positive growth	Negative growth	P-value
<i>E. histolytica</i>	12	6(50) %	6(50) %	0.0028
<i>E. coli</i>	24	1 (4.16) %	23(58.47) %	

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Discussion

5.1. The Identification of *Entamoeba histolytica* Using Morphological Techniques.

The results represented indicate that the rate of isolated *E. histolytica* was (50.76%). This result aligns with a previous study conducted in Iraq. A study conducted found that the prevalence of *E. histolytica* infection was 47% in Basra city (Naser, 2023). In Erbil, the rate of *E. histolytica* was 41% among individuals with low incomes compared to those with moderate incomes (Mahmood and Bakr, 2020). Another study reported that the prevalence of *E. histolytica* infection was 75.0% (39/52) in Peninsular Malaysia (Nguir *et al.*, 2012). The current study disagrees with a study that found an incidence rate of *E. histolytica* infection of 17.5% in the Al-Fhood district of Thi-Qar (Al-Mosawi, 2016). Another study reported that the percentage of *E. histolytica* was 26.66% in Tikrit (Mahmood *et al.*, 2024). In Khartoum State, Sudan, 26% of *E. histolytica* infection was reported by Eltoum *et al.* (2023). The high infection rate could be increased by variations in testing methods, poor living conditions, family history of infection, unsanitary toilet facilities, and low levels of education (Nath *et al.*, 2015).

5.1.1. The Prevalence of *Entamoeba histolytica* Infections According to Sex

The prevalence of infection was greater in male (52.63%) than in females (48.15%); still, there was statistically significant differences. The present study, in agreement with the findings of the study done in Ethiopia by Roro *et al* (2022), illustrates a greater proportion of males than females. Another research reported that there was no significant variation in infection rate among males and females in Al-Anbar province, determining that *E. histolytica* was distributed fairly evenly throughout both sexes (Ali *et al*, 2023). In Pakistan, males were found to have a slightly greater prevalence of *E. histolytica* infection (22.36%) than females (20.9%) (Zahida *et al.*, 2010). Similar results are reported in a study in agreement with Hussein *et al.* (2022), males had the highest infection prevalence (28.2%), whereas females revealed the minimum prevalence (27.3%), However, a study carried out in Al-Rifai, located in Thi-Qar province, illustrated no statistically significance differences between male and female participants regarding *E. histolytica* infection rate. The present results vary from those

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done at Nishtar Hospital, Multan, Punjab, that found a greater prevalence in female patients (9.46%) as compared to males patients (8.15%) alone (Tasawar, 2013). Due to another results of study conducted by Naser (2023), the distribution of amoebiasis infection in females was more than males that showed 56% in females, as compared to 43% in males. In addition, statistically, there was no significant relationship found in the current study between the gender and prevalence of disease. According to Flaih *et al.* (2021), males had lower rate of infection than females in Al-Nasiriyah province in southern Iraq. Typically, females appear as more resistant to parasite diseases; furthermore, their immunity can be weakened in certain situations. In the present research, females presented a greater incidence of *E. histolytica*. This can result from differentiations in hormones in females within different stages of the reproductive cycle, that might enhance their immunity and facilitate the establishment of parasites that are opportunistic (Mazigo *et al.*, 2010).

5.1.2. The Prevalence of *Entamoeba histolytica* Infection Based on Residency

Demonstrates the association between geographical area and *E. histolytica* infection rates (Nasiriyah versus Al-Refai). The distribution of infection in Al-Refai city was 43.33% which was lower than that in Al-Nasiriyah city that was 67.50%, which illustrates statistically significant variation ($P < 0.05$).

According to the study conducted by Al-Kaeebi (2016) in the Al-Qadisiya province, the most rates of infection appeared in urban areas with poor health infrastructure and dense populations, particularly with regard to drinking water and sewage systems. Al-Nasiriyah is more in line with these requirements than Al-Rifai. According to the study, poor environmental conditions, lifestyle choices, a propensity to eat out, and general health conditions all functions a major role in distribution of intestinal parasites like *E. histolytica*. In the city of Wasit, another study by Ali and Flaaeh, (2022) found that represented no vital variation regarding to rate of infection between rural and urban areas. Intestinal parasite infections are frequent and constitute significant public health issues among impoverished and socioeconomically disadvantaged rural populations.

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Due to inadequate environmental sanitation and insufficient access to clean water (Ngui *et al.*, 2011).

5.1.3. *Entamoeba histolytica* Infection Occurrence Among Different Age Demographics

The highest infection rates were observed in children aged 1–3 years (40.90%) and adults over 20 years (42.42%). Indicating that age is a significant factor influencing susceptibility to infection. The findings of this study are in agreement with those reported by Salman *et al.* (2018), which documented a significant prevalence of *Entamoeba histolytica* infection among young children and older adults. This pattern indicates that age is an important factor influencing susceptibility, with both very young children and older adults being more vulnerable compared to intermediate age groups. These results align with the present study, highlighting the combined effect of age and gender on infection distribution. According to various research carried out in Malaysia by Noor Azian *et al.* (2007), adult persons were more likely than children to get amoebiasis. Furthermore, the prevalence of infection within this was significantly depending on poor personal hygiene. The children tend to exhibit unusual behaviors, like neglecting to wash hands before and after eating. They may also eat unprocessed foods, especially sugarcane and fruits, without washing. This behavior, as well as prevalent among adults.

The present study's findings do not align with those reported by Ail T. Nimmim Al-Damerchi (2017), in which the prevalence of infection was higher among adolescents; however, the difference compared to other age groups was not statistically significant. The current study not align with findings of Naser (2023), who discovered that infants which their ages less than one year are more susceptible to infections. This discrepancy may be attributed to variations in sample size, geographical location, hygiene and sanitation practices, or differences in diagnostic methods used between the studies. Such factors can influence exposure rates and the apparent distribution of infection across age groups.

5.2. The Detection Rate of *Escherchia coli* Based on Morphological Techniques

The results show that *E. coli* was found 54 out of 130 samples (41.53%) grew well on culturing media While the current study aligns with Al-Mashahedah and Dhahi (2025), who reported that the infection rates by *E. coli* was 57 (44.2%), data systems in children's hospital of Ibn Saif and general hospital of Al-Musayyib, located in Al-Musayib, city of Babil. According to findings of the study of Eybpoosh *et al.* (2021), in Iran, the documented infection rate illustrated positive *E. coli* cultures (95% CI: 72.6-77.3%). According to study of Al-Oebady (2017), 67 (89.3%) patients at the Samawah Teaching Hospital for Pediatrics and Gynecology in AL-Muthanna region have coliform bacteria. In Wasit, it was shown that the total infection rate with *E. coli* was 36.25% (Aifari, 2025). Insufficient water and sanitation facilities, along with inadequate personal hygiene, significantly contribute to the widespread presence of these conditions in developing regions (Petri *et al.*, 2008).

5.2.1. Prevalence of *Escherchia coli* Infections Depending on Patient Sex

Infection prevalence in female individuals was 57.41%, that is greater than the 30.26% which observed in males. The statistical investigations revealed a significant difference ($P < 0.05$).

This research aligns with results of study done of Altaie *et al.* (2025), who reported that *E. coli* was more prevalence in male than female participants (Altaie *et al.*, 2025). There was no statistically significant variation ($p = 0.356$). Another study reveals that *E. coli* infections are more common in women, which has significant public health ramifications. According to Al Mayahie (2014), adult women are more likely than males to have extraintestinal pathogenic *E. coli* in their normal stool flora, which may make them more vulnerable to extraintestinal infections. This finding may be due to a number of reasons, including as changes in immunological state, cultural roles, exposure to foodborne sources, and hygiene practices.

5.2.2. Prevalence of *Escherchia coli* Based on Habitation

The geographically distribution of *E. coli* O157 depend on habitation. The incidence of *E. coli* among Al-Nasiriya patients was greater (50%) compared to that of Al-Refai

patients (37.78%). The results showed a lack of statistical significance ($P > 0.05$). The current study aligns with study done by Zhou *et al.* (2021) in China, which found great rate of infection by *E. coli* in urban regions. Another study by Hasan and Najim (2024) reported emphasising its persistence in urban and peri-urban environments. These studies highlighted how poor sanitation systems, polluted water supplies, and deficient public health facilities contribute to sustained high rates of disease, especially across southern regions. The research collectively illustrates that environmental contact and availability of hygienic infrastructure significantly enhance *E. coli* prevalence. The current study, with its focus on identifying generic *E. coli* in the clinical samples, has the potential to enhance the management of significant infections by *E. coli*. The study of Hasan and Najim revealed dangerous strains, including O157:H7, along with their health-related effects. Variations in reported patient outcomes and infection rates could result from differences in the specific strains targeted and diagnostic scope. In their investigation, Mohamed and Habib (2023) examined *E. coli* in Arab countries' food distribution networks, identifying considerable levels of contamination in meat and dairy sectors, especially in areas where food safety monitoring and molecular detection systems are inadequate.

5.2.3. Prevalence of *Escherchia coli* Infections Based on Age Group

The results of the current study regarding the distribution of infection according to age groups. Among the six age groups, the highest prevalence was recorded in individuals older than 20 years, accounting for (40.74%), followed by children aged 1–3 years with (27.7%). Statistically, the distribution of infection among the age groups was highly significant ($p < 0.001$). This agrees with the study conducted by Al-Gallas *et al.* (2007) in Tunisia, that reported higher rates of diarrheagenic *E. coli* in both children and adults, due to the behavioral factors and environmental similarity. The current study agreement with findings of Zhou *et al.* (2020), which reported in China, adults had a greater prevalence of *E. coli* infection. The investigation done in Iran by Eybpoosh *et al.* (2021), documenting increased infection rates in two groups: children and adults. Adult susceptibility may stem from exposure to unsafe food and water,

potential disregard for food hygiene standards, and regular consumption of meals prepared outside home settings. These results contrast with Altaie *et al.* (2025) in Kirkuk Province, which whose results indicated that diarrheal disease occurrence peaked in children below 10 years old.

5.3. Age-Dependent Distribution of *Escherchia coli* and *Entamoeba histolytica* Infection.

Findings of this study revealed that out of 130 patients, (21.53%) were found to be positive for the co-existence of both *E. coli* and *E. histolytica*. The highest prevalence of co-infection was observed in the age groups 1–3 years (39.28%) and >20 years (35.71%), Statistical analysis showed a significant association between age groups and co-infection ($p<0.05$).

These results are partially consistent with the findings of Ali and Flaaieh (2022), who reported higher infection rates among children 27.8% compared to adults over 20 years 18.5%, which may be attributed to immature immunity and poor hygiene practices. Furthermore, the higher prevalence observed in adults over 20 years in the current study could be related to occupational exposure, lifestyle factors, or increased contact with contaminated food and water sources. Similar observations were reported in a study conducted in Salah Al-Din Province, Iraq, which showed that *E. coli* infection was more prevalent among children than older age groups (Sabry *et al.*, 2021). The findings of the present study align with the results reported by Hassen and Alsalim (2023), who demonstrated that *E. histolytica* infection alters the intestinal microbiota in children, this supports the present study's observation of the prevalence of *E. coli* among infected children, suggesting that *E. histolytica* infection may influence the gut microbial balance and facilitate bacterial over growth. This highlights the urgent necessity for more study in this region. These bacteria can infiltrate the human food supply chain from initial synthesis to final item consumption (Mohamed and Habib, 2023).

5.3.1. Coexistence of two Pathogens Concerning Residence

Findings of the study indicated that both pathogens coexisted more frequently in the of Al-Refai (71.6%) than in Al-Nasiriyah (28.94%), indicated that variation was

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statistically critical ($P < 0.05$). According to a study done by Ali and Flaaieh (2022), there was a strong relationship between the occurrence of *E. coli* and *E. histolytica*, with urban populations having a higher co-infection rate than rural regions. According to the researchers, *E. histolytica* may become more virulent because of the interaction between parasites and bacteria, particularly in those who live in poor environments or have compromised immune systems. Another study that conducted by Hassen and Alsalim, (2023) propose that the presence of co-infection of bacteria increase *E. histolytica* virulence via encouraging more invasive behaviors and modifying intestinal microbiota. Similarly, research done by El-Shazly *et al.* (2015) in Egypt, found that the co-infection rate was widely distributed among adult individuals in urban crowded area. Regional researches indicate that places with insufficient hygiene commonly report co-infection, with adult populations being higher affected.

5.4. Investigation via PCR

5.4.1. Verification of Microscopic Observations using PCR-Dependent Molecular Approach

12 stool samples which previously examined by microscope for *E. histolytica* were subjected to investigation. Among them 50% represented infected results.

Examinations corroborate the present results. According to Mehmet Tanyuksel *et al.* (2003) and Fotedar *et al.* (2007), PCR is the gold standard for identifying *E. histolytica* due to its specificity and sensitivity. In addition, PCR can facilitate early diagnosis and treatment of infection, thus, decreasing likelihood of *E. histolytica*-related issues. This developed approach illustrates how critical it is to integrate molecular methods into standard diagnostic methodologies.

5.4. 2. Identification of *Escherchia coli* and *Entamoeba histolytica* Using Molecular Method

Depending on molecular diagnosis by PCR approach for present examination, illustrated no significant difference ($P > 0.05$) in diagnosis of infections. *E. histolytica* illustrated high detection percentages (50%) whereas *E. coli* O157 showed only 4.16% of detection percentages.

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Another study disagrees with the findings of present research done by Alia and Flaaeih, (2022), that reported results that similar to those of an earlier study. This study illustrated that in stool samples from diarrheal Iraqi patients, *E. histolytica* was detected significantly more commonly (65%) than *E. coli* O157 (21.15%). Humans are at risk of contracting these infections from contaminated milk and other dairy products (Ayichew *et al.*, 2025). These results are consistent with earlier study showing that PCR is a more dependable technique than microscopy since it can identify several studies that indicate PCR's superior efficacy in detecting infections by bacteria (Jaiswal *et al.*, 2012, Mirelman *et al.*, 1997). The current findings are supported by other studies. For example, Tanyuksel *et al.* (2003) and Fotedar *et al.* (2007) represented, PCR technique has higher sensitivity than others. This development underlines how vital it is to incorporate molecular techniques into routine diagnostic processes.

Chapter Six

Conclusions and Recommendations

6.1. Conclusions

1-The study results indicate that the parasite *E. histolytica* and the bacterium *E. coli* are the primary agents responsible for diarrhea in the patient samples, highlighting the importance of accurate diagnosis to differentiate the causative pathogens and guide appropriate treatment.

2- Microscopic examination is a fundamental step in diagnosing intestinal infections, serving as a preliminary phase prior to molecular analyses for more precise identification of the causative agents.

3- Furthermore, residence, age, and gender were found to significantly influence infection rates, underscoring the importance of considering demographic factors when assessing disease prevalence.

4-The results indicate that culture media such as MacConkey and Sorbitol, along with microscopic examination, serve as preliminary detection steps for bacteria. However, accurate diagnosis requires advanced molecular methods, with real-time PCR being the optimal approach for precise identification of the causative agents.

6.2. Recommendations

1. Given the concordance between real-time PCR and conventional PCR results, it is recommended to rely on either method for diagnosis, considering the availability of equipment and laboratory expertise to ensure accurate and efficient application.

2. MacConkey Sorbitol media alone cannot be relied upon for the diagnosis of *E. coli* O157; instead, PCR should be employed as a precise diagnostic tool. Additionally, specific PCR probes can be used to compare this strain with other strains and identify genetic differences.

3. Further research and studies to understand the dynamics of co-infections

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الخلاصة:-

تشكل العدوى المعوية الناتجة عن مسببات مرضية طفيلية وبكتيرية مصدر قلق كبير للصحة العامة، خاصة في المناطق النامية. تعد *Entamoeba histolytica* الأوليات الممرضة المسؤولة عن التهاب القولون الأميبي، والذي قد يتطور أحياناً إلى مضاعفات معوية أو خارج معوية خطيرة. وفي المقابل، تعتبر *Escherichia coli* O157 بكتيريا معوية قادرة على التسبب بأمراض حادة في الجهاز الهضمي مع نتائج سريرية خطيرة. هدفت هذه الدراسة إلى فحص العلاقة بين العينات الإيجابية لكل من *E. coli* و *E. histolytica*

في الفترة من تشرين الأول 2024 وحتى شباط 2025، تم جمع 130 عينة من البراز من مرضى يعانون من الإسهال في مستشفى الرفاعي ومستشفى الحسين والمختبرات الخاصة في منطقتي الرفاعي والناصرية بمحافظة ذي قار. تراوحت أعمار المرضى بين سنة واحدة وأكثر من 20 سنة، وشملت كلا الجنسين. تم التعرف على *E. histolytica* عن طريق المسحة الرطب المباشرة، واستخدام تقنية PCR للكشف عن وجود الطفيلي عبر جينات *SSUrRNA* و *E.H.ssRNA* أما بالنسبة لتحديد *E. coli*، فتم الاعتماد على زراعتها على أوساط *MacConkey* و *EMB agar*، واستخدام *MacConkey sorbitol agar* كوسط تفريقي للتعرف على *E. coli* O157، غير قادرة على تخمير السوربيتول، مميزاً إياه عن باقي سلالات *E. coli*، مع إجراء اختبارات كيميوكيوية إضافية، واستخدام PCR للكشف عن وجود الجين *rfbE*.

أظهرت نتائج الدراسة أن *E. histolytica* وجدت في 66 من أصل 130 عينة، بنسبة قدرها 50.76٪، في المقابل تم الكشف عن *E. coli* في 54 من أصل 130 عينة بنسبة 41.53٪ بناءً على التعرف المظهري. وأظهر معدل الإصابة بـ *E. histolytica* حسب الجنس ارتفاعاً طفيفاً لدى الذكور (52.63٪) مقارنة بالإناث (48.15٪)، مع عدم وجود فروق ذات دلالة إحصائية. كما كانت نسبة الإصابة أعلى في منطقة الناصرية (67.50٪) مقارنة بمنطقة الرفاعي (43.33٪)، مع فروق ذات دلالة إحصائية ($P < 0.05$) وظهرت أعلى نسب للإصابة في الأطفال بين عمر 1–3 سنوات (40.90٪) والبالغين فوق 20 سنة (42.42٪)، مع وجود فرق إحصائي مهم.

بالمقارنة، *E. coli* كان معدل الإصابة أعلى لدى الإناث (57.41٪) مقارنة بالذكور (30.26٪)، وأظهرت التحليلات الإحصائية فرقاً ذا دلالة ($P < 0.05$) وسجلت الدراسة نسبة انتشار أعلى في مرضى منطقة الناصرية (50٪) مقارنة بالرفاعي (37.78٪)، إلا أن هذه الفروق لم تكن ذات دلالة إحصائية ($P > 0.05$) وبلغت أعلى نسبة انتشار بين الأفراد فوق 20 سنة (40.74٪)، تلتها فئة الأطفال من عمر 1–3 سنوات (27.7٪)، مع دلالة إحصائية قوية بين الفئات العمرية ($P < 0.001$).

وجد 28 عينة (21.53٪) إيجابية لوجود كلا الممرضين معاً. وسجلت أعلى نسبة للتزامن في الإصابات بين الفئات العمرية 1–3 سنوات (39.28٪) وفوق 20 سنة (35.71٪)، أظهر التحليل الإحصائي وجود ارتباط كبير بين

الفئات العمرية العدوى المشتركة. ($P < 0.05$) كما كانت معدلات تواجد الممرضين معاً أعلى في منطقة الرفاعي (71.06%) مقارنة بالناصرية (28.94%)، مع اختلاف ذو دلالة إحصائية. ($P < 0.05$)

تم أيضاً فحص 12 عينة براز تم فحصها مسبقاً مجهرياً لـ *E. histolytica* باستخدام تقنيات conventional PCR و real time PCR ، وأظهرت النتائج إيجابية 6 عينات منها (50%) لـ *E. histolytica*. أما *E. coli* *O157* في حين لم يتم الكشف باستخدام conventional PCR ، ولكنه تم تحديده في عينة واحدة من أصل 24 (4.16%) عبر real time PCR. وأظهرت الدراسة وجود فروق مهمة في نتائج الإصابة بين الممرضين ($P \geq 0.05$).



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وزارة التعليم العالي والبحث العلمي
جامعة الشطرة كلية الطب البيطري
فرع الاحياء المجهرية



الكشف الجزيئي للعدوى المشتركة بين الاميبا الحالة للنسيج و الأ شريكيا القولونية في قضائين بمحافظة ذي قار

رسالة ماجستير مقدمة الى مجلس كلية الطب البيطري- جامعة الشطرة وهي جزء من متطلبات الحصول على شهادة الماجستير في اختصاص علوم الطب البيطري (الاحياء المجهرية)

قدمت من قبل:

زينب رائد جواد الحميداوي

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بأشراف

أ.م.د نثيلة رشيد حميد

أ.م.د عبد العزيز صالح عبد العزيز الشمري