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Department of Biology

Molecular Detection of Some Parasitic and Bacterial Causes of Diarrhea Among Patients in Babylon Province

A thesis

Submitted to the Council of the College of Sciences for Women, University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master in Biology

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1444 هجري **2022 ميلادي**

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيم

﴿ وَمَا يَعْلَمُ تَأْوِيلَهُ إِلَّا اللَّهُ وَ الرَّاسِخُونَ فِي الْعِلْمِ
يَقُولُونَ آمَنَّا بِهِ كُلٌّ مِنْ عِنْدِ رَبِّنَا ﴾

صدق الله العظيم

سورة ال عمران / الآية7

Dedication

To My Dears…………………

Parents............................

Husband..............................

Baby...

Brothers...

Sisters..

Best Friends..

And to all people whom I love, I dedicate this work................

Sura

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Thanks, and appreciation to Allah the Almighty for all the blessings he bestowed upon me . Thanks, appreciation and gratitude to the dear supervisors, Prof. Dr. Ahmed Khudhair Al-Hamairy , Dr. Zainab Hashim Al-Zubaidy , for their support and guidance me . appreciation and gratitude go to the dean and staff of the college of science for women, University of Babylon .Finally, I'd like to thank my friends and everyone who supported me with good words.

Sura

Supervisors Certification

We certify that this thesis entitled " **Molecular Detection of Some parasitic and Bacterial Causes of Diarrhea among patients in Babylon province** " was prepared by **Sura Mohammed Jassim**, under our supervision at the Department of Biology College of Science for Women, University of Babylon , as a partial fulfillment of the requirements for Degree of Master in Science of Biology .

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الخلاصة

أجريت الدراسة الحالية خلال الفترة من أكتوبر 2021 إلى فبراير 2022 ، حيث اشتملت هذه الدراسة على فحص 100 عينة براز (أطفال ، بالغين ، ذكور وإناث) بطريقة الفحص المجهري المباشر ، و 36 عينة إيجابية للطفيليات ، و 26 عينة إيجابية تم فحصها لبكتريا الاشريكية القولونية باستخدام تقنية تفاعل البوليميراز المتسلسل (PCR). لمرضى الإسهال والمرضى المراجعين في مستشفى بابل للولادة والأطفال وكذلك بعض المراكز الصحية والمختبرات الخاصة في محافظة بابل وبعض نواحيها . وللفئات العمرية أقل من سنة إلى 21 سنة فأكثر. أظهرت الدراسة الحالية أن معدلات الإصابة بالطفيليات المسببة للإسهال (الاميبا الحالة للنسيج , الجيارديا) بلغت 37٪ موزعة كالتالي (35٪ و 2٪ على التوالي) بطريقة المسحة المباشرة ومعدلات الإصابة بالإسهال المسببة للبكتيريا(الاشريكية القولونية) كانت(26٪) باستخدام الزرع على ماكونكي أجار وباستخدام تفاعل البوليمر المتسلسل (PCR) كانت معدلات الإصابة بالطفيليات (37.5٪) (الاميبا الحالة للنسيج , الجيارديا) موزعة على النحو التالي (35.41٪ ، 2.08٪ على التوالي) وكانت معدلات الإصابة ببكتيريا (الاشريكية القولونية) (26.04٪).

أظهرت نتائج الفحص المجهري لعينات الطفيليات المسببة للإسهال أن أعلى معدلات الإصابة كانت بين المناطق الريفية (43.1٪) مقارنة بالمدينة (30.6٪). وكانت أعلى معدلات الإصابة بين الذكور (38.4٪) مقابل الإناث (35.4٪). وكانت أعلى معدلات الإصابة كانت (50٪) في الفئة العمرية (6-10) سنوات ، بينما كانت معدلات الإصابة منخفضة (10٪) في الفئة العمرية (21 سنة فأكثر). كما تبين أن أعلى معدلات الإصابة في وجود الحيوانات في منازل المصابين كانت (38.4٪) ، مقابل عدم وجود حيوانات في تلك المنازل (36٪). أظهرت هذه الدراسة تبايناً في معدلات الإصابة حسب المستوى التعليمي لرب الأسرة ، ووجد أن أعلى معدلات الإصابة كانت لرب الأسرة الحاصل على تعليم ابتدائي ، حيث كانت نسبة الإصابة (50٪) بينما انخفضت معدلات الإصابة لدى رب الأسرة الحاصل على تعليم أكاديمي (10٪). أما بالنسبة للتوزيع الشهري للعدوى ، فقد سجلت الدراسة أن أعلى نسبة

إصابة كانت في أكتوبر 2021 عندما كانت (45٪) ، بينما كانت أقل نسبة إصابة (31.2٪) في فبراير (2022).

في الدراسة الحالية ، تم استخدام تقنية تفاعل البوليميراز المتسلسل (PCR) باستهداف الجين 18srRNA للاميبا الحالة للنسيج والجيارديا وجين 16srRNA لبكتريا الاشريكية القولونية ، والتي تم من خلالها فحص 96 عينة للفحص المجهري للطفيليات المسببة للإسهال (الاميبا الحالة للنسيج ، الجيارديا) ، وبلغت معدلات الإصابة (37.5٪).) وتوزعت على النحو التالي (35.41٪ ، 2.08٪ ، على التوالي). أما بالنسبة للبكتيريا ، فقد تم فحص 96 عينة للبكتيريا المسببة للإسهال (الاشريكية القولونية) ، وبلغت معدلات الإصابة (26.04٪). بالاعتماد على تقنية تفاعل البوليميراز المتسلسل ، ظهرت أعلى معدلات الإصابة في المناطق الريفية للطفيليات والبكتيريا المسببة للإسهال ، حيث سجلت أعلى نسبة من الطفيليات (41.1٪) ، بينما سجلت أعلى نسبة إصابة بكتيرية عند (28٪). بينما انخفضت النسبة في الحضر للطفيليات والبكتيريا حيث سجلت أقل نسبة طفيليات (33.33٪) بينما سجلت أقل نسبة للبكتيريا (23.91٪). اعتمادًا على تقنية تفاعل البوليميراز المتسلسل ، ظهر أعلى معدل إصابة عند الذكور (39.2٪) ، بينما انخفض معدل الإصابة عند الإناث (35.5٪) للطفيليات. أما بالنسبة للبكتيريا فقد سجلت الدراسة زيادة في معدل الإصابة عند الإناث (31.7٪) ، بينما انخفض معدل الإصابة بالعدوى عند الذكور (21.81٪) ، وسجلت هذه الدراسة أعلى نسبة إصابة في الفئة العمرية (6-10) سنة وبلغت (50٪) بينما كان الانخفاض في معدل الإصابة في الفئة العمرية (21 سنة فأكثر) وكان (10٪) للطفيليات . أما بالنسبة للبكتيريا فقد سجلت الدراسة أيضا زيادة في معدل الإصابة في الفئة العمرية (6-10) وصلت إلى (35.29٪) بينما انخفضت الدراسة في الفئة العمرية (21 سنة فأكثر) حيث وصلت أيضا إلى (10٪) . أما بالنسبة للمعايير الأخرى للطفيليات فكانت أعلى معدلات الإصابة في وجود الحيوانات في المنازل (39.4٪) ، بينما انخفضت معدلات الإصابة بغياب الحيوانات في المنازل (36.2٪). ووجدت الدراسة أن أعلى معدلات الإصابة كانت لرب الأسرة الحاصل على تعليم ابتدائي (50٪) ، بينما انخفضت معدلات الإصابة مع رب الأسرة الحاصل على تعليم أكاديمي (10٪). وسجلت أعلى معدلات الإصابة في أكتوبر 2021 (45٪) , بينما سجلت الأشهر المتبقية معدلات متشابهة حيث سجلت (35.2٪ ، 35.4٪ ، 35.6٪ ، 35.7٪) (يناير 2022 ، نوفمبر 2021 ، فبراير 2022 ، ديسمبر 2021 ، على

التوالي) .أما بالنسبة لنوع الإصابة بالطفيليات والبكتيريا ، فقد كانت الإصابة المفردة أعلى نسبة في هذه المجموعة (81.13٪) ، حيث شكل طفيلي الأميبا الحالة للنسيج (60.46٪) ، وطفيلي الجيارديا (2.32٪)، والبكتيريا(37.2٪) للعدوى المفردة. أما الإصابة الكلية المزدوجة فقد بلغت (18.86٪) حيث شكل طفيلي الأميبا الحالة للنسيج مع الإشريكية القولونية (90٪) بينما طفيلي الجيارديا مع الإشريكية القولونية (10٪) . لم يتم تسجيل أي إصابة ثلاثية في هذه الدراسة. استنتج من خلال الدراسة الحالية أن انتشار الطفيليات المسببة للإسهال في محافظة بابل كان مرتفعا جدا من خلال الكشف بالطرق الميكروسكوبية والجزيئية (طريقة تفاعل البوليميراز المتسلسل) وكانت المناطق الريفية بها معدلات إصابة أعلى من المناطق الحضرية.

Summary

The current study was conducted during the period from October 2021 to February 2022, this study included the examination of 100 stool samples microscopically by direct smear method (37%) samples positive for parasites and (26%) samples positive for *Escherichia coli* and then examined the positive results by using Polymerase Chain Reaction (PCR) technique. For peoples with diarrhea attending to Babylon Maternity and Children Hospital, as well as some health centers and private laboratories in Babylon province and some of its districts. The current study showed that the infection rates of diarrhea-causing parasites(*Entamoeba histolytica* , *Giardia lamblia*) was (37%) , distributed as follows (35% and 2%, respectively) by direct smear methods , and the infection rates of diarrhea-causing by bacteria(*Escherichia coli*) was (26%)by using cultured on the MacConky agar whereas by using polymear chain reaction (PCR) the infection rates for parasites (*E.histolytica* , *G.lamblia*) were (37.5%) , distributed as follows (35.41%, 2.08% , respectively), And the infection rates of *E. coli* was (26.04%) .

The results of the microscopic examination of the samples for parasites causes diarrhea showed that the highest infection rates was among rural areas (43.1%) compared with urban area(30.6%). The highest infection rates in males(38.4%) compared to females (35.4%). The highest infection rates was (50%) with age group (6-10) years, while the lowest infection rates (10%) with age group (21 and over) years. The highest rates of infection with presence of animals in the

houses of patients (38.4%), compared with absence of animals in those homes (36%) . This study showed a variation in the prevalence rates according to the level of education of the head of the family, the highest rates of infection for head of the family with a primary education (50%), while the infection rates decreased for the head of the family with academic education (10%). As for the monthly distribution of infection, the study recorded that the highest percentage of infection was in October 2021 (45%), while the lowest percentage of infection was (31.2%) in February 2022.

In the current study, the polymerase chain reaction (PCR) technique was also used by targeting the gene 18srRNA for the *E.histolytica* , *G.lamblia* and gene 16srRNA for the *E.coli*, through 96 samples which were examined for microscopic examination of the parasites that cause diarrhea (*E.histolytica*,*G.lamblia*), the infection rates were (37.5%) and distributed as follows (35.41%, 2.08% , respectively). As for 96 samples were examined for the *E.coli* that cause diarrhea, and the infection rates were (26.04%) in routine examination of bacteria. Depending on the PCR technique, the highest rates of infection were shown in rural areas for parasites and *E.coli* that cause diarrhea, where the highest percentage of parasites recorded(41.1%), while the highest percentage of *E.coli* infection was(28%). The percentage decreased in urban areas for parasites and *E.coli*, where the lowest percentage of parasites recorded (33.33%), The lowest percentage of *E.coli* (23.91%).

Depending on the PCR technique, the highest infection rates were shown in males (39.2%), while the infection rate decreased in females (35.5%) for parasites. As for *E. coli* , the study recorded an increase in the infection rate in females (31.7%), while the rate of infection decreased Infection in males (21.81%), this study recorded the highest infection rate in the age group (6-10 years), (50%), while decreaseing in the infection rate was in the age group (21 years and over) (10%) for parasites. As for *E.coli*, the study also recorded an increase in the rate of infection in the age group (6-10) (35.29%), while the study decreased in the age group (21 years and over) (10%). For parasites, the highest infection rates were in the presence of animals in homes (39.4%), while the infection rates decreased in the absence of animals in houses 36.2%. The study found that the highest rates of infection were for those who have a head of the family with a primary education (50%), while the rates of infection decreased with the head of the family who obtain an academic education level (10%), and the highest infection rates were recorded in October 2021 (45%).) The remaining months recorded similar rates, as they recorded (35.2%, 35.4%, 35.6%, 35.7%) (January 2022, November 2021, February 2022, December 2021, respectively).

As for the type of infection with parasites and *E.coli*, the single infection was the highest percentage in this group (81.13%), the *E.histolytica* constituted (60.46%).The *G.lamblia* constituted (2.32%), and the *E.coli* accounted for (37.2%) for the single infection. Whereas the double infection (18.86%).The parasite *E.histolytica* with *E.coli* constituted (90%), while the parasite *G.lamblia* with *E.coli* constituted (10%). No triple infection was recorded in this study. Through the current study, it was concluded that the prevalence of parasites that cause diarrhea in Babylon province was very high through detection by microscopic and molecular methods (PCR method), compared with previous studies and rural areas had higher rates of infection than urban areas.

Eight random positive samples of the PCR product in the current study were sent to Macrogen Company in South Korea for the purpose of determining the DNA sequence of parasites (*E.histolytica* , *G.lamblia*) and bacteria (*E.coli*),and this study was identified and genetically recorded in the NCBI gene bank .The results of the study showed that all the transmitted isolates are identical to the NCBI gene bank isolates.

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Abbreviations	**Meanings**
aEPEC	Atypical Enteropathogenic E. coli
AIEC	Adherentinvasive E. coli
ALA	Aminoleveulinic acid
bp	base pair
CWPs	cyst wall proteins
D.W	Distilled water
DAEC	Diffuselyadhering E. coli
DNA	Deoxyribonucleic Acid
E.C-F	*Escherichia coli* Forward
E.C-R	*Escherichia coli* Reverse
E.H-F	*E.histolytica* Forward
E.H-R	*E.histolytica* Reverse
EAEC	Enteroaggregative E. coli
EDTA	Ethylene Diamine Tetra Acetic Acid
EHEC	Enterohaemorrhagic E. coli
EPEC	Enteropathogenic E. coli
ETEC	Enterto-xigenic E. coli
G.L-F	*G.lamblia* Forward
G.L-R	*G.lamblia* Reverse
GEMS	Global Enteric Multicenter Study
GD	Genomic DNA
HIV	human immunodeficiency virus
MAC	Mackonkey's agar
NIH	National Institute of Health

NLM	National Library of Medicine
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonuclease Nucleic Acid
TBE	Trise-Borate EDTA
tEPEC	Typical Enteropathogenic E. coli
UPEC	Uropathogenic E.coli

1. Introduction:

Diarrheae is major health problem in many parts of world and cause high fatality rate. Generally, more than three million deaths occur each year due to diarrhea in infants and children in developed countries. The most common form of gastrointestinal infection is the rapid development of frequent intestinal evacuations of a more or less fluid character. Amoebic diarrheae is caused by some protozoan parasite such as *Entamoeba histolytic*, *Giardia lamblia*. It is transmitted in areas where poor sanitation allows contamination of drinking water and food with faeces in these areas (Al-Haboobi, 2014).

Diarrheae is the passage of unusually loose or watery stools, at least three times in 24 hours. However, it is the consistency of stools rather than the number that is most important (WHO,2013). There are three main forms of acute childhood diarrhea, all of them are potentially life-threatening and require different treatment courses (acute watery diarrhea, bloody diarrhea, and persistent diarrhea) (WHO.2014).

Etiologies of diarrhea could be bacterial, viral, or parasitie. People living with human immunodeficiency virus (HIV) as well as children who are malnourished or have impaired immunity are most at risk of life-threatening diarrheae (Nkenfou *et al*.2013; Shah *et al*.2016; WHO 2017). There are two major protozoan pathogens responsible for diarrhea, *Entamoeba histolytica*, *Giardia lamblia* (Stark *et al*.,2011). *Entamoeba histolytica*, *G. lamblia* are the causative agents of amebiasis, giardiasis, respectively. These organisms are among the most common intestinal protozoa associated with diarrheae in developed countries (Davis *et al*.,2002; Fletcher *et al*.,2012).

Escherichia coli is the most important bacterial cause of diarrheae in children. Several pathogenic and nonpathogenic factors are predisposed to diarrheae (Izzo *et al*., 2011; Bartels *et al*., 2013). Single primary pathogen or co-infection can predispose to the development of diarrheae. Other factors such as nutritional factors, hygiene conditions and environmental factors could contribute to diarrheae (Fernandez *et al*., 2009)

Any epidemiologic risk factors such as age group, gender, consumption of vegetation, recent travel, crowded living conditions or animal contacts, recent medication use, underlying conditions such as HIV, and immunocompromised patients (Pour *et al*., 2013).

The parasites live in the intestines of infected acquired through the fecal-oral route as a result of direct person-to-person contact (such as hand-to-mouth contact) , also diarrheal disease caused by consumption of contaminated food or drinks by a variety of pathogens including parasites or by personal contact with the carrier or a contaminated surface, dangerous places where transmission of diarrheal disease may be halted on hands, in water and in foods.(Okhuysen , 2001; Karanis *et al* ., 2007). The present study determined that the prevalence of bacterial and parasitic agents of diarrhea in stools of individuals and the sequencing gene, phylogenetic relationship for these organisms (*Entamoeba histolytica , Giardia lamblia , Escherichia coli*).

1.2. Aims of the present study:

1. Detection of parasitic diarrhea agent by direct smear methods and detection of *E. coli* as bacterial causative agent of diarrhea by culture and other methods and molecular detection of *E. histolytica* and *G. lamblia* and *E. coli* by PCR through specific primers.
2. Determine the genotyping sequences of *E. histolytica*, *G.lamblia*, *E.coli* and registration in NCBI and detect the accession No. and drawing the phylogenetic tree of these organisms above.

2. Literatures Review:

2.1. Historical View:

2.1.1. *E. histolytica:*

In 1855 Lamble found amoeba in the stool of a child with diarrhea (Dhawan., 2012). The description of parasitic amoeba for the first time was by Loch in 1875 in stool of a person infected with dysentery in Russia, and then isolated amoeba injected in the intestines of a dog, he noted infected mucous layer of the colon dog and cause ulcers containing the amoeba, and examined the gut patient after its death , and fired on the amoeba in those ulcers (Roberts and Janovy, 2005).

In 1890, Osler examined the case infected with amoeba and noted the existence of trophozoite with the presence of blood and a few mucus in the stool of an infected person with fever and enlargement of the liver, then in 1891, Concilman and Lafleur found that the amoeba had a role in the invasion histological and the amoeba is responsible for the infection to amoebic dysentery and amoebic liver abscess (Dhawan., 2012).

Then each of Quinek and Ross in 1893 discovered the cyst phase of the parasite while in 1903, schaudinn suggested name *E.histolytica* that has for its ability to attack and tissue analysis (Singh and Petri., 2001). It proved all of Walker and Sellards in 1913 to cyst phase is contagious phase of the parasite, Dobell demonstrated the life cycle of *E.histolytica* in 1925 (Tanyuskel and Petri., 2003).

At least six types of amoeba proven that they parasitse on the human namely: *E.histolytica , E.dispar, E.coli, E. hartmani , E. polecki and E. moshkovskii* (John and Petri, 2006) .

2.1.2. Classification of *E. histolytica:* (Zeibig, (2014)

Kingdom: Protista

Sunbkingdam: Protozoa

Phylum : Sarcomastigophora

Subphylum : Sarcodina

Super class : Rhizopoda

Class : Lobosea

Subclass : Gymnamobia

Order : Amoebida

Suborder : Tubulina

Genus : *Entamoeba*

Species : *E. histolytica*

2.1.3. Morphology:

Entamoeba histolytica occurs in four forms, trophozoite, precyclic, cystic, and metacyst stages. (Chakraborty, 2004; Paniker's, 2018). The stage common recoginzed in the feces are trophozoite and cyst, but only trophozoite are present and invade in the tissues (Zeibig, 2014).

2.1.3.1.Trophozoite:

Trophozoite is the vegetative feeding phase of the parasite (Chakraborty, 2004). It is a facultative arrow that metabolizes glucose as the main energy source. The trophozoite is measured in 20-30 μm. In cytoplasm, there's a central nucleus called the karyosome.Some soluble lysosomes and organelles perform the same function as mitochondria called mitosomes. Researchers thought that mitosomes are debris resulting from the breakdown of the mitochondria organell (Despommier *et al*. 2017). Endoplasm contains food vacuoles containing erythrocytes sometimes leukocytes and tissue residues that the parasite swallowed from the host's body (Paniker's, 2018).

2.1.3.2.The Cyst:

The diameter of the cyst is 10-15 μm. *Entamoeba histolytica* infection occurs when ingesting cysts (Guerrant *et al* . 2011). It has a cyst wall to protect it from the environment condition, it's resistant to dehydration, even some chemicals such as (chlorinated compounds , fluorides) can live in water for a month, while those in stool can live in dry lands for more than 12 days, and can withstand temperatures of up to 50 C°(Bogitsh *et al*. 2012) . It contains four or fewer nuclei Initially, it has one nucleus in the precyst stage, then it multiplies by binary fission and becomes two nuclei, then four nuclei, the adult cyst contains four nuclei. Immature cysts may supplement their growth outside when they're excreted through the feces(Chakratborty, 2004; Guerrant *et al*. 2011; Bogitsh *et al*. 2012).

2.1.4. Life Cycle:

Entamoeba histolytica passes its life cycle in one host, humans. The infection is transmitted by swallowing cysts in contaminated food or water, which passes through the stomach without damage due to the cyst wall which is resistant to gastric juice (Paniker's 2018), it reaches the ileum where its exception occurs, the cyst wall shattered due to the alkaline medium of the caecum or lower part of the ileum (Guerrant *et al*, 2011).This results in the metacystic where the mitotic divided produces it causes the nucleus division first, followed by the cytoplasm then there will be eight small amoebulae. Each amoeba can evolve into trophozoites. There are small size trophozoites that feed on bacteria .The large ones feed on erythrocytes and attack the epithelial cells of the mucosal epithelium of the large intestine (caecum or colon) causing amoebiasis .Some develop into cysts in the bowel lumen that passed into the feces to repeat the cycle (Chakratborty 2004; Bogitsh ., *et al*. 2012 ;Baker 2017)

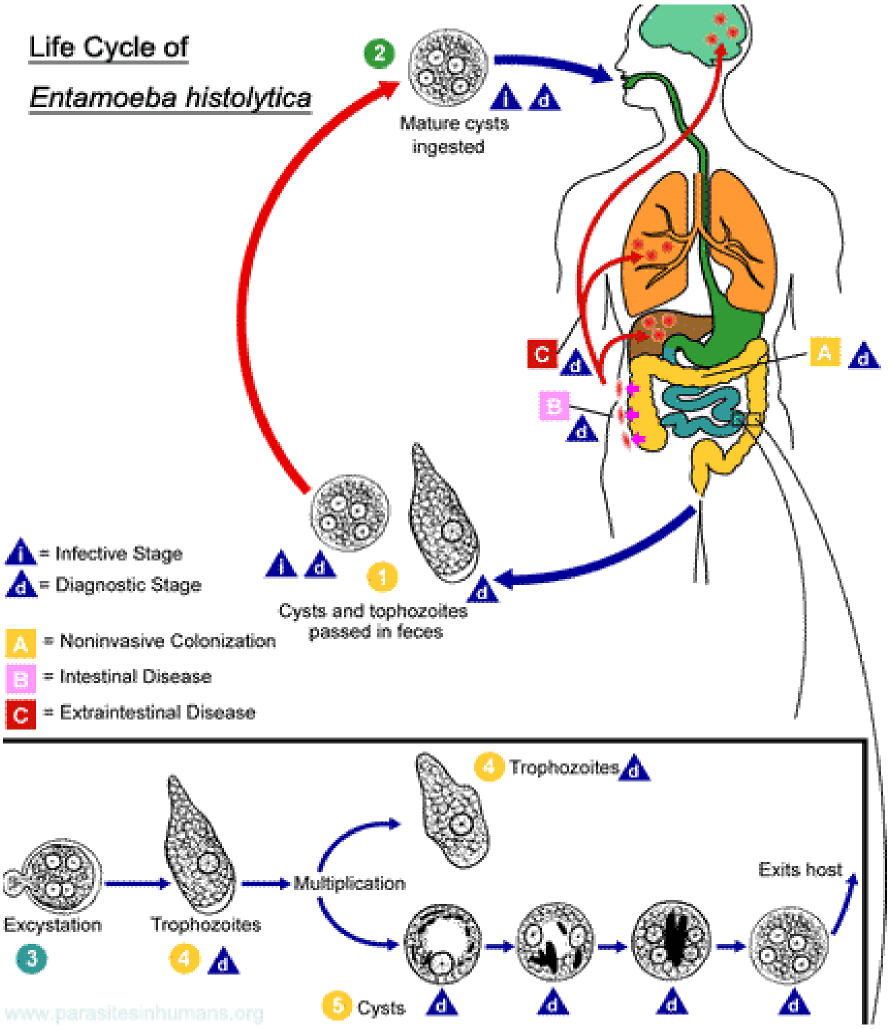


Figure (1): Life cycle of *E. histolytica* (Singh&Petri,2001)

2.1.5. Pathogenesis and Clinical Features:

Entamoeba histolytica causes intestinal and extra intestinal amoebiasis, the lumen-dwelling amoebae do not cause any illness, they cause disease only when trophozoites invade the intestinal tissues. The trophozoite penetrates the epithelial cells in the colon, aided by its movement and histolysin, a tissue lytic enzyme, and cysteine protease as well as amoeba pores. Mucosal penetration produces discrete ulcers with pinhead centre and raised edges. Sometimes the invasion remains superficial and heals spontaneously. The ulcers are multiple and are confined to the colon, being most numerous in the caecum and recto-sigmoidal region. The intervening mucous membrane between the ulcers remains healthy.The amoebic ulcer is flask shaped in cross-section. Multiple ulcers may coalesce to form large necrotic lesions with ragged and undermined edges and are covered with brownish slough. The ulcers generally do not extend deeper than submucosal layer. Amoebae are seen at the periphery of the lesions and extending into the surrounding healthy tissues, clinical manifestations are diarrheae, vague abdominal symptoms and dysentery (Rohela Mahmud *et al*. 2017) . This may resemble bacillary dysentery. The ulcers may involve the muscular and serous coats of the colon, causing perforation and peritonitis. Blood vessel erosion may cause haemorrhage. Deep ulcers form scars and may lead to strictures and partial obstruction. A granulomatous pseudotumoral growth may develop on the intestinal wall from a chronic ulcer. This amoebic granuloma or amoeboma may be mistaken for a malignant tumour. The incubation period for intestinal amoebiasis varies from 1 to 4 months. Liver involvement is the most common extraintestinal complication of intestinal amoebiasis. About 5–10% of patients with intestinal amoebiasis will develop amoebic liver abscess (ALA). ALA arises from haematogenous spread of amoebic trophozoites from colonic mucosa or by direct extension. Often ALA patients do not present with bowel symptoms (Rohela Mahmud *et al*. 2017).

2.1.6. Treatment:

1. Luminal amoebicides: Diloxanide furoate, iodoquinol, paromomycin and tetracycline act in the intestinal lumen but not in tissues.

2. Tissue amoebicides: Emetine and chloroquine are effective in systemic infection, but less effective in the intestine.

3. Both luminal and tissue amoebicides: Metronidazole (750–800 mg three times daily for five–ten days), tinidazole and ornidazole act on both sites. Carriers should also be treated because of the risk of transmitting the infection to others.

Paromomycin or iodoquinol should be used in these cases. Although metronidazole and tinidazole are both luminal and tissue amoebicides, neither of them reach adequate levels in the gut lumen, therefore, patients with ALA should also receive treatment with a luminal agent to ensure eradication of infection, Paromomycin (25–35 mg/kg/day, divided into three doses for seven days) is the drug of choice. (Rohela Mahmud *et al*. 2017)

2.1.7. Prevention and Control:

1. Boil drinking water.

2. Wash fruits and vegetables in clean water before eating.

3. To detect and treat carriers and prohibit them from food handling.

4. Health education. (Rohela Mahmud *et al*. 2017)

2.2. Giardia Lamblia:

The first observation of *Giardia* within 330 years ago, *Giardia lamblia* discovered in 1681 by Antonie Van Leeuwenhoek, when he examined a sample of his own diarrheal stool (Adam., 2001). But the real study of this parasite was in 1859 by Vilem Dusan Lambel ,who ranked the *Giardia* which was isolated from human and was called it cercomonas intestinals. Raphael Blanchard proposed the name *Lambli*a *intestinalis* within 1888 so as to honor Lambl, it was not until 1915 that the name *G.lamblia* was introduced, the name was selected so as to commemorate the work of the French Giard, as well as Lambl, (Ford., 2005). There are six different species of *Giardia* based on both molecular analysis and morphological (Adam., 2001). Five from these species are host specific where, *G.muris* infects mice, *G.agilis* are specific to voles and amphibians, *G.microti* infects mustkrats , *G.ardea* and *G.psittaci* are found in birds whereas the sixth species, *Giardia* infected a great range of diverse mammals, as well as humans (Caccio *et al*., 2005) .

2.2.1. Classification of *G.lamblia*: (Zeibig , 2014) .

Kingdom : Protista

Sub kingdom : Protozoa

Phylum : Sarcomastigophora

Sub Phylum : Mastigophora

Class :Zoomastigophora

Order : Diplomonadida

Suborder: Diplomonadina

Family : Hexamitidae

Genus : *Giardia*

Species :*G. lamblia*

2.2.2. Morphology of *G.lamblia*:

It exists in two forms:

1. Trophozoite

2. Cyst

2.2.2.1. Trophozoite:

The trophozoite of *Giardia* measures (12-15) µm in length with around (5-9) µm width (Souza *et al*., 2004). It has been distinguished by tear drop shape or pear bisect length-wise(bilaterally symmetrical), (Adam.,2001).The movment of parasite within the host intestine involves propulsion around its own axis, which assist four pairs of flagella, the giardial trophozoite have two nuclei, each one contains a central karyosome (Benchimol, 2005). The fibral adhesive disc present on the ventral side of the trophozoite is a *Giardia* specific attribute (Elmendorf *et al*., 2003). It is considered a virulence factor since it is used in attachment to the host epithelium, in addition it is necessary for the parasite as a means to avoid elimination due to intestinal peristalsis (Elmendorf *et al*., 2003; Muller and von Allmen, 2005). The components of the adhesive disc include members of a *Giardias*- specific protein family of protein the giardins, such as; β, γ giardin along with α and β-tubulin, (Elmendorf *et al*., 2003; Davids *et al.*, 2008). Microscopy imaging of the ventral adhesive disc showed microribbons extending to the cytoplasm from a wall of microtubule which are spread evenly along the surface of the disc in a spiral layer (Benchimol., 2004; Sant'Anna *et al*., 2005). Certain typical eukaryotic organelles exist in cytoplasm of *G.lamblia* like lysosmal vacuoles plus ribosomel and glycogen granules while Golgi complex is absent in trophozoite and appears in cyst (Hehl & Marti., 2004).

2.2.2.2. The Cyst of *G. Lamblia*:

The cyst measures up to 12 µm in length with (8-10) µm in width (Erlandsen *et al*.,1996). The immature cyst contains two nuclei and two intermediate bodies, while the mature contains four nuclei and the bodies of four centrist, cytoplasm slightly away from the wall of the cyst, leaving a clear void (Adam, 1991). Cyst wall integrity has been challenged in several in vitro studies where it has been documented to sustain stress such as exposure to a certain extent irradiation and to water disinfection agents (Belosevic *et al*.,2001; Sundermann and

Estridge, 2010). In the enviroment ,the *Giardia* parasite is rigorously protected inside a cyst wall, a hardy capsule consisting of a sugar plus protein meshwork while the main constituent of the carbohydrate moiety is a β(1-3)-N-acetyl-d-galactosamine homopolymer, the remaining 40% constitutes cyst wall proteins (CWPs) to which the carbohydrates form a strong interaction (Sun *et al*., 2003; Davids *et al*., 2006). The oval shaped cysts are responsible for the movement of the disease from one host to another (Adam.,1991).

2.2.3. Life Cycle of *G.lamblia:*

G. lamblia occurs through two major metabolic states through its life cycle : first the vegetatively replicating trophozoite and second the non-motile, inactive cyst (Adam., 2001). In addition, there are two intermediate phases :the excyzoite and encyzoite stages, the excyzoite is the stage where the parasite speedily evacuates from its protective crypt or the cyst wall also transforms into four replicating trophozoites (Bernander *et al*., 2001). Whereas the encyzoite phase is where the trophozoite gradually rebuilds its protective covering and meta morphosizes reverse into a state of hibernation (Reiner *et al*., 2008). The actively metabolizing, motile form (trophozoite) lives within the jejunum also duodenum and multiplies via binary fission way, Trophozoites that are swept into the fecal stream mislay their motility round up and excreted as resistant, latent cysts. The cyst stage is adequately hardy to survive host-to-host transfer, Giardiasis infection is caused during ingestion of mature cysts (infective dose varies from 10-100 cyst) by way of contaminated food or water. Exposure of cysts to acidity of host stomach plus body temperature stimulate the excystation, with the appearance of trophozoites which speedily multiply and attach to the host small intestinal villi through a disk that exists on ventral surface of an active stage (Meyer., 1990).

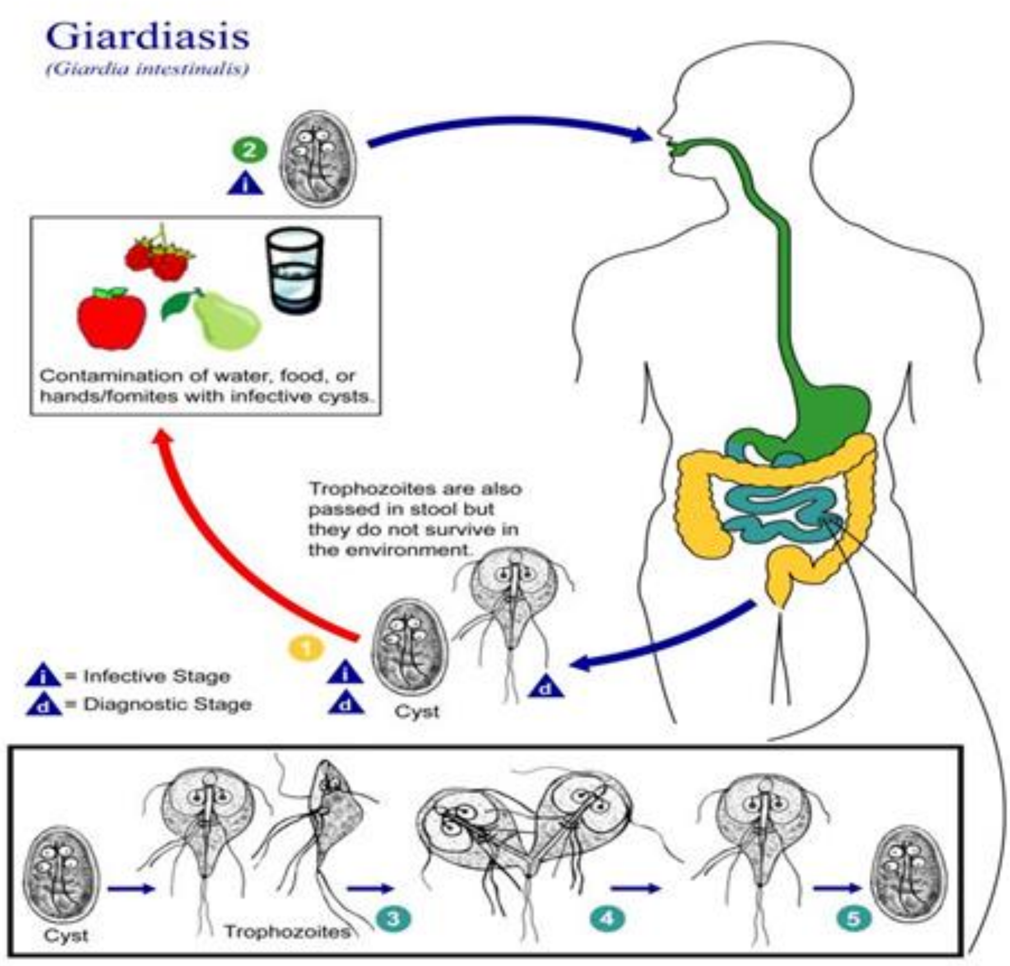


Figure (2-2): life cycle of *Giardia lamblia* (Adam, 2001)

2.2.4. Pathogenesis and Clinical Features:

Pathogenesis of *Giardia* is thought to be connected with various factors such as the number of organisms ingested, the specific strain ingested , non antibody protective factors in the gastrointestinal tract, and the immune response of the host (Meyer., 1990). Lectin, a protein on the trophozoite covering, recognizes specific receptors on the intestinal cell and may be responsible for the tight attachment between *Giardia* with the villi which is followed through: first mucosal scratch second mechanical obstruction that is caused only in the presence of numerous organisms, and third the conjugation of bile salts (Washington *et al*., 2006). In some cases, the infection is asymptomatic parasite satisfactory and the person with the disease holder Carrier. Studies indicated that inflammatory mast cells may interfere with duodenal growth of *G. lamblia* trophozoites. Other inflammatory cells, as well as CD8+ T cells, contribute to villus-shortening and crypt hyperplasia. Inflammation results are in an increased return rate of intestinal mucosal epithelium. Immature replacement cells have less functional surface area with less digestive and absorptive ability (Meyer., 1990). The length of the incubation period is (1- 3) weeks usually, depends at least partially on the number of cysts that are ingested. Normal hosts that are infected with giardiasis may have some or all of the following symptoms: foul-smelling stools and loose, excessive flatulence, steatorrhea (fatty diarrhea), malaise, abdominal cramps, fatigue and weight loss or a coeliac-disease-like syndrome (Washington *et al*., 2006).

2.2.5. Treatment:

Metronidazole (250 mg 3 times daily for 5 days) or tinidazole is the drug of choice. Paromomycin can be given to symptomatic pregnant woman**(**Rohela Mahmud **,** 2017).

2.2.6. Prevention and Control:

1. Proper faecal disposal

2. Personal hygiene

3. Boiling of drinking water

4. Filtration of drinking water

5. Wash fruits and vegetables with clean water before eating

6. Health education (Rohela Mahmud, 2017)

2.2.7. Disease distribution:

Intestinal parasitic infections are endemic worldwide and have been described as constituting the greatest single worldwide cause of illness and disease, poverty, illiteracy, poor hygiene, lack of access to potable water and hot humid tropical climate are the factors associated with intestinal parasitic infections (Mehrajm .,2008). Study of Cook *et al*. (2009) retrospectively analyzed demographic factors that may affect the prevalence of intestinal parasites among Guatemalan school children in the Palajunoj Valley of Guatemala, Clinical visits were performed on 10586 school children aged 5-15 years over a four-year , during which 5705 viable stool samples were screened for infection with the following parasites *G. lamblia*, *E. histolytica*. The average overall prevalence of infection for specific parasites were *E. histolytica* 16.1%, *G. lamblia* 10.9% . Wegayehu *et al*. (2013) made this study for assessing the magnitude and pattern of intestinal parasitism in highland and lowland dwellers in Gamo area, South Ethiopia. Out of the total examined (858) , 342 (39.9%) were found positive for intestinal parasite. The prevalence of *E. histolytica* was the highest 98 (11.4%) and (10.6%) for *G. lamblia*. A study was conducted by Firdu *et al*. (2014) to determine the prevalence of *G. lamblia,* and *E. histolytica* in diarrheal children at Yirgalem Hospital in Ethiopia. A total of 230 children participated in the study .The prevalence of *G.lamblia, and E. histolytica* was 15.65%, and 4.35% respectively in children with diarrhea and 1.74%, and 1.74% in those without it, respectively. Mohammed *et al*. (2009) has collected 900 faece samples from the city of Kassala in South Sudan for children aged from six months to 13 years ,results of the examination showed that parasitic infections rates were 12.3% for *G. lamblia* and 0.4% for *E. histolytica* .Al- Kaisy *et al*. (2008) studied factors that are affecting the epidemic of *E. histolytica* and *G. lamblia* , this study included stool samples from 220 patients attended hospitals of Al - Khalis and Baladruz and suffered from diarrhea, the percentage of infections of *E. histolytica* and *G. lamblia* were (70.5% , 29.5% respectively). Jaeffer (2011) studied the prevalence of *G. lamblia* and *E. histolytica* which were screened among 513 children aged less than 12 years in Al-Shulaa and Al-Khadimya - Baghdad, to evaluate the relationship of some factors (gender, age, grade and type of drinking water) with the prevalence of these two intestinal protozoa parasites. The study revealed that the total rate of *(G.lamblia* and *E. histolytica*) infection was (13.64%), the infectivity rate of *G.lamblia* was higher than the infectivity rate of *E.histolytica* that was (10.72%) and (2.92%) respectively. Al -Hamairy *et al*. (2013) recorded through the study spread of intestinal parasites and their relationship to anemia in the Al-doullab village, Babylon province, which by examining stool specimens were 389, the infection rate is 36.7% for *E. histolytica* and 26.1% for *G. lamblia.* AL-Kubaisy *et al* .(2014) studied the impact of risk factors on the parasitic diarrhea and to determine the parasitic profile among children in Baghdad-Iraq, a total number of 2033 cases were included in the study. The estimated prevalence rate of parasitic diarrhea was(22%). *G. lamblia* was found to be the most prevalent parasite with an infection rate of (45.54%) followed by *E. histolytica* (23.44%). In an epidemiological study to investigate the prevalence of intestinal parasites among the people of some primary schools in the district Mahaweel Babylon province, Al-Mamori (2000) has examined the 2116 males and females , the proportion of the total incidence of intestinal parasites was (61.7%) included (12%)of the *E. histolytica* and (9.9%) for *G. lamblia* .

2.3.Escherichiacoli:

2.3.1.HistoricalView:

In 1885, the German-Austrian pediatrician Theodor Escherich discovered organism in the feces of healthy individuals. He called it Bacterium coli commune because it is found in the colon. Early classifications of prokaryotes placed these in a handful of genera based on their shape and motility (at that time Ernst Haeckel's classification of bacteria in the kingdom Monera was in place) (Farrar J, *et al*. (2013) , Haeckel (1867), Escherich (1885).Bacterium coli was the type of species of the now invalid genus Bacterium when it was revealed that the former type species ("Bacterium triloculare") was missing (Breed, Conn 1936). Following a revision of Bacterium, it was reclassified as Bacillus coli by Migula in 1895 (Migula 1895) and later reclassified in the newly created genus Escherichia, named after its original discoverer , Castellani, Chalmers (1919) .

2.3.2. Classification of *E.coli:* (Migula,1895).

Domain : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Enterobacteriales

Family : Enterobacteriaceae

Genus : *Escherichia*

Species : *E.coli*

2.4.2. General Characterization of *E.coli:*

Escherichia is the cylindrical rods shape it exists singly or in pairs belong to the family of Enterobacteriaceae, no motile or motile by peritrichous flagella, oxidase negative, aerobic and facultative anaerobic, formed acid and gas from most fermentable carbohydrates to give one of the characteristics of diagnosis , do not produce H2S. Most strains of *Escherichia coli* ferment Lactose, but fermentation may be absent or delayed in *E.blattae*, *E.hermannii*, *E.fergusonii* and *E.vulneris* (Imamovic *et al.,* 2018). Escherichia has several species mainly *E. blattae*, *E. albertii*, *E. hermanii*, *E. fergusonii*, *E. senegalensis*, *E. coli* and *E. vulneris* which are the major cause of infections in human, through biochemical reactions that may differentiate between these species (Beloin and Roux, 2010). *Escherichia coli* is a gram negative bacteria,(0.5 –0.7) µm in diameter and (1.0–3.0)µm in length, non-spore forming rod, with some having prominent polysaccharide capsule which provides resistance against many host defense mechanisms, (Ejrnaes, 2011). Enterobacteriaceae are the most of catalase positive. A common feature in the Enterobacteriaceae, which helps to distinguish them from other related bacteria, is the lack of cytochrome C oxidase with exceptions such as Plesiomonas spp (Van and Balmefrezol, 2007). Growth of *E. coli* may be in the narrow range of (37-42) C°, most strains are may be able to grow over a wide range of temperature approximately15- 40C° and can grow within a pH range of approximately (5.5–8.0) (Bedenić *et al*., 2012). *Escherichia coli* becomes a very common laboratory organism because scientists able to grow it quickly on both simple media and complex. *Escherichia coli* are able grow in presence of air using oxygen as terminal electron receptor (aerobically) or without air through the use of fermentative metabolism, the ability to grow both anaerobically and aerobically (Baqer, 2013). *Escherichia coli* can be classified based on pathotype into intestinal pathogenic *E.coli* and extra intestinal pathogenic *E. coli* (Carlos *et al*., 2010). Intra intestinal pathogenic *E.coli* cause diarrhea that can be classified into Enteropathogenic

E.coli EPEC, Diffuselyadhering *E.coli* DAEC , Enterto-xigenic *E.coli* ETEC, Adherentinvasive *E.coli* AIEC, Enterohaemorrhagic *E.coli* EHEC, and EAEC Enteroaggregative *E. coli* (Nowrouzian and Oswald, 2012) .*Escherichia coli* is ordinarily found as party the normal micro flora in the human gastrointestinal tract and is elaborately involved in the lives of humans, these bacteria can be easily grown in *vitro* and used in genetics for the ease of manipulating their genes, making them one of the best studies of prokaryotic model organisms, they are characterized as opportunistic pathogens are classically associated with host disorder such as a result of immunocompromising illness, surgery and catheterization (Nowrouzian and Oswald, 2012; Mezaal and Abbas, 2015). *Escherichia coli* bacterial is composed of pathogenic and non-pathogenic strains, Non- pathogenic strains which may be evolve into pathogenic variables and vice versa, most extra intestinal infections associated with *E. coli* are caused by commensal strains that become pathogenic by adaptation stratagems or the acquisition of virulence determinants (Sáez-López *et al.*, 2016).The commensal *E.coli* are able to colonize the gastrointestinal tract and were well adapted to conditions in the large intestine (Bailey *et al.*, 2010). Some physiological studies about *E. coli* had demonstrated the ability of these organisms to adapt themselves to their different habitats (Cohen, 2017).

2.5.3. Pathogensis of *E.coli*:

It is a Gram negative bacterium It is Catalse positive, Non spore forming, (Gillpsie and Hawkey, 2006).This microorganism has diameter that ranges between 2-6 mm, and its leanth is 1.1-1.5 mm, its motile with peritrichous flagella and fimbriae (Alhilali and Almuhana, 2011). Some strain possess poly saccharide Capsule composed of K.antigen (Schaechter,2009). It usually grows on ordinary media to form , low convex, circular, smooth, opal colonies after 18-24hours culturing on nutrient agar, and a red large colonies on macConkey agar (Alhilali and Almuhana, 2011).The optimum temparture for their growth range between 15-45^{o}C The most specific biochemical characters that distinguish *E.coli* from other enterobacteriaceae species yield positive results to indol test, methyl red test and their ability to ferment many carbohydrate with the production of gas while it gave negative results to Vogas –proskaure test, urease and H2S production (Arora and Arora , 2008). *Escherichia coli* involved three types of serotype according to their antigen (somatic, capsular and flageller antigens) which include O serotype which refers to *E.coli* that possess somatic antigen, H serotype that refer to *E.coli* that possess flagellar antigen and K serotype which refer to *E.coli* with capsular antigen. All these antigens have been characterized by heat stability.(Harvey *et al*., 2007). The natural habitat of *E.coli* is an intestinal tract of human and animals consequently the source infection is stool contaminated of water and foods (Bando *et al*., 2009).The existence of *E. coli* indicates that there exist indicate that is fecal contamination of water sources, drinking water and food. *E. coli* usually colonizes the digestive system of human infants within a few hours after birth and its human host usually coexist with mutual benefit for decades (Marrs and Zhang, 2015).

2.3.4. Epidemiological review of *E.coli*:

Endemic diarrhea is considered a common public disease around the world wide, particularly in developed countries. It is a world/ks major cause of mortality and morbidity, it is likely to account for almost18 percent or 1.9 million deaths annually among kids under three years old, among which almost 40% take place in the Africa (Eijk *et al.*, 2010). *Escherichia coli* has been highlighted as a significant bacterial etiologic factor for this disease (Kaper *et al*., 2004). Patho-types of diarrhea-genic *E.coli* is considered a major bacterial cause of children diarrheain developing countries (Nguyen *et al*., 2005), EPEC strains are considered some the most consistent causes of diarrhea in children in developing countries (Trabseulsietal., 2002). The Atypical EPEC strains proved to have an association to children's endemic diarrhea and adults' outbreaks of diarrhea in the industrialized and developing countries. Seemingly, this pathotype has showed up and it is a leading cause of infantile diarrhea in many countries (Bando *et al*., 2009). The prevalence of EPEC infections varies between epidemiological studies on the basis of differences in study populations, age distributions, and methods (serotyping, adherence patterns, and presence of the eae or conserved LEE genes) used for detection and diagnosis.In addition, differences in geographic regions, periods of time and socioeconomic class may also contribute to differences in the epidemiology of EPEC-induced diarrheal disease. Lack of discrimination between tEPEC . Diarrhea due to tEPEC decreases with age, and infections in adults are rarely reported. This apparent resistance in adults and older children has been attributed to the loss of specific receptors with age or development of immunity. Six For many decades, studies conducted worldwide have shown that tEPEC serotypes are strongly associated with diarrhea in children < one year of age, mainly in poor children in urban centers.6,12,15 The association with diarrhea was particularly strong in infants less than six months of age. Studies in Brazil, Chile, Mexico, and South Africa, showed that 30-40% of infantile diarrhea cases were due to tEPEC serotypes.15,112,114 However, the epidemiology of EPEC infections has shifted. In numerous developing countries, where the prevalence of EPEC infection had been high until the 1990s, recent studies have not identified a significant association between tEPEC and infantile diarrhea. In Brazil, 92% of EPEC isolates collected from children between 2001 and 2002 were atypical, However other studies still report tEPEC being more prevalent than aEPEC as a cause of diarrhea. In addition, in some less developed areas (Africa and Asia), tEPEC are still some of the most important enteropathogens.

2.3.5. Diarrheagenic *Escherichia coli:*

E. coli strains that cause diarrhea may therefore be considered as three groups, as follows.

(1) Enterotpxigenic *E. coli* (ETEC) Common serogroups: 06, 08, 015, 025, 027, 063, 078,0115, 0148, 0153, 0159,0167.

(2) Entero-invasive *E. coli* (EIEC) Common serogroups: O28ac, 0112ac, 0124, 0136, 0143, 0144, 0152, 0164.

(3) Enteropathogenic *E. coli* (EPEC) Common serogroups: 026, 055, 086, 0111, 0114, 0119, 0125, 0126, 0127, 0128,0142.

The Six categories of E.coli associated with the diarrhea disease, which classified according to their serotype and have different pathogenesis (Benenson, 1995). include: Enteroaggregative *E.coli* (EAEC) It can adhere to human epidermoid carcinoma (HEP-2)cells producing an aggregative adhesion (AA)pattern (Nataro *et al*., 1993) and does not secret heat labile and heat stable toxin.The great majority of (EAEC) carry a 60 MDa plasmid containing a gene that confer AA and a gene encoding EAEC heat stable enterotoxin-1(EAST-1). Other virulence factors include putative hemolysin toxins, various types of fimbeiae and outer membrane protein that may be involved in the adhesion process (Law and Chart, 1998).Enterohemorrhagic *E.coli* (EHEC) It was firstly recognized as a human pathogen in 1982 causes many disease like hemolytic uremic syndrome (HUS) and bloody which diarrhea and non-bloody diarrhea (Varma *et al*., 2003) because of its ability to produce shiga toxin it was also Known as verocytotoxins (Caprioli *et al*., 2005). Enterohemorrhagic *E.coli* EHEC in the most serious pathogens among the developing countries(Meng &Schroeder 2007; Ochoa *et al*.,2008). Enteroinvasive *E.coli*(EIEC) It resembles shigella in many characters like the capacity to invade and proliferate within epithelial cells and cause eventual death of the cell (Sansonetti,1992). The invasive property of EIEC and shigella was dependent on the presence of large plasmid coding for the production of several outer membrane protein included in invasiveness, which antigenically homologous in shigella (Keskimaki, 2001). Enterotoxigenic *E.coli*(ETEC) It cause watery diarrhea and occasionally a pattern similar to that caused by vibrio cholera . It was responsible for the high mortality rate of many children under 5 years. In the developing countries (Mackie and MacCartney, 1996). Enterotoxigenic *E.coli* ETEC may cause severe diarrhea similar to vebriosis in adult living in area where the organism is common, such as in India and Bangladesh (KESkimaki, 2001). Some strain of enterotoxigenic *E.coli* can secreted of heat labile enterotoxin(LT) and heat stable enterotoxin (ST) In which the former one was in structure and function similar to cholera Toxin that expressed by produced vibrio cholera (Eisinghorst and weitz,1994).

Diffusely Adherent *E. coli* (DAEC) They Are notorious by their pattern of adherence to HEp-2 cells. They explained the characterization and cloning of a surface fimbria in the mentioned strains, which intervenes the (DA) diffusely adhesion phenotype. Yet, the plasmid and chromosomal genes that control this type of adherence were described. Few clinical or epidemiological studies allow enough description of the clinical syndrome related with DAEC infection. In a study, most patients with DAEC suffered from watery diarrhea with no blood or stool leukocytes (Poitrin *et al*., 1995). Levine *et al*. (1996) denoted that the associated risk of DAEC related with diarrhea was raised with ages from 1 to 4-5 years in Santiago.

Enteropathogenic *E.coli* (EPEC) Enteropathogenic *E. coli* is a significant class of diarrheagenic *E. coli* that are related to diarrhea in infants of the developing world. It is sticks to the mucosal cells of the small intestine which affecting the epithelial microvilli . which is often self-limited, but could be chronic (Dedeićljubović *et al*.,2009). The formation of attaching to and affacemnt (A/E) lesions is necessary for those microbes to result in diarrhea (Khan *et al*., 2008). TEPEC is having both the intimin gene (eaeA) and bundle-forming pilus gene (bfp) for E. coliattaching and eroding. (Fujihara *et al*., 2009)

Table (2-1): Classification of *Escherichia coli* Associated With Diarrhea

E.coli	**Epidemiology**	**Diarrhea**	**Mechanism**
EHEC	Hemorrhagic colitis and hemolytic uremic syndrome in all ages and thrombotic thrombocytopenic purpura in adults	Bloody or non bloody diarrhea	Cytotoxin production and adherence
EPEC	Acute and chronic endemic and epidemic diarrhea in infants	Watery	Adherence effacement
ETEC	Infantile diarrhea in developing countries and travelers' diarrhea	Watery	Adherence, enterotoxin production
EIEC	Diarrhea with fever in all ages	Bloody or not	Adherence, invasion of mucosa
EAggEC	Chronic diarrhea in infants	Watery	Adherence
DAEC	diarrhea was raised with ages from 1 to 4-5 years	Watery or non bloody diarrhea	adherence to HEp-2 cells

2.3.6. Virulence Factors of *E. coli*:

Virulence factors means the ability of microorganisms to establish itself and replicate within specific host species, and that enhance the microbe's potential to cause disease (Samuel and Reeves, 2003). Important virulence factors of *E. coli* confer resistance to effects on the host defenses. In addition, virulent bacteria are able to produce molecules that actively inhibit the immune response of the host, thereby enhancing bacterial persistence and tissue damage which affected them, the genes encoding virulence factors of UPEC are localized to chromosomal gene clusters called "pathogenicity islands" (Alizade *et al*., 2017). The different virulence factors are in concord, their expression turn on or off during the course of the infection and can be regulated by environmental signals (Rezai *et al*., 2015)

Virulence factors of *E. coli* are divided into two groups:

• Virulence factors associated with the surface of bacterial cell (adherence factors)

• Virulence factors, which are secreted and exported to the site of action (Toxins) (Alizade *et al*., 2017)

2.3.7.Normal Microbiota:

E.coli belongs to a group of bacteria informally known as coliforms that are found in the gastrointestinal tract of warm-blooded animals Brenner DJ, *et al*.(2005). *E.coli* normally colonizes an infant's gastrointestinal tract within 40 hours of birth, arriving with food or water or from the individuals handling the child. In the bowel, *E. coli* adheres to the mucus of the large intestine. It is the primary facultative anaerobe of the human gastrointestinal tract (*Todar 2007)* . As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals Evans (2007).

2.3.8. Identification of the *E.coli* by molecular method (PCR):

Despite the apparent species-specific nature of 16S rRNAs, recently found that the *Escherichia coli* ribosome is able to accommodate foreign 16S rRNA (Kitahara *et al*., 2012). Namely, using *E. coli* Δ7, a null mutant of the rRNA (ribosomal RNA) operon, as a host strain, we have shown that various 16S rRNA genes, including those from a different phylogenetic class (i.e. Betaproteobacteria), were able to complement growth. The lowest identity of functional 16S rRNA gene to that of *E. coli* was as low as (80%), implying that hundreds of simultaneous nucleotide changes are permitted in the maintenance of ribosome function. The basis for this high mutability is the conservation of the RNA secondary structures, which is consistent with a previous finding that 16S rRNA is typically recognized by ribosomal proteins via salt bridges between phosphate oxygen atoms of the RNA backbone, but nucleotide bases are not strictly discriminated (Brodersen *et al.,* 2002). Furthermore, insertion/deletion is allowed in some RNA helices (e.g., h6, 10, and 17) that are not involved in protein binding. Understanding the sequence and structural variations of 16S rRNA that are accommodated in the E. coli ribosome should be helpful for our understanding of the evolution of rRNA and the sequence–structure–function relationships of the ribosome (Eden *et al*., 1991)

2.3.9. Preparation of Culture and Diagnostic Media:

The following culture media used in the current study were preparation and used according to :

2.3.9.1. MacConkey Agar Medium:

The manufacturer recommendations procedure for preparing MacConkey agar medium was followed. It was utilized to distinguish Gram negative bacteria from gram positive bacteria (MacFaddin , 2000).

2.3.9.2.Blood Agar Medium :

The manufacturer recommendation procedure for preparing blood agar media was followed. This medium was used to promote bacterial isolation and to assess bacteria’s ability to determine the type of Hemolysis (Russell *et al* ., 2006).

2.3.10. Biochemical test:

Escherichia coli can be recovered easily from clinical specimens on general or selective media at 37°C under aerobic conditions. *The E. coli* in stool are most often recovered on MacConkey or eosin methylene-blue agar, which selectively grow members of the Enterobacteriaceae and permit differentiation of enteric organisms on the basis of morphology .Enterobacteriaceae are usually identified via biochemical reactions. These tests can be performed in individual culture tubes or by using test “strips” which are commercially available. Either method produces satisfactory results. For epidemiologic or clinical purposes, *E. coli* strains are often selected from agar plates after presumptive visual identification. However, this method should be used only with caution, because only about 90% of *E. coli* strains are lactose positive; some diarrheagenic *E. coli* strains, including many of the EIEC strains,are typically lactose negative .The indole test, positive in 99% of *E. coli* strains, is the single best test for differentiation from other members of the Enterobacteriaceae. Methyl red Test, Methyl red-voges proskauer broth was inoculated with a fresh bacterial culture and incubated at 37C° for 24 hr. When, a few drops of methyl red solution were added and mixed. The result was read immediately, positive test was bright red (Igbeneghu and Lamikanra, 2014).

2.3.10. 1. Gram stain:

The Gram staining method is named after Hans Christian Gram, the Danish bacteriologist who originally devised it in 1844, and is one of the most important staining techniques in microbiology. It is almost always the first test performed for the identification of bacteria. The primary stain of this method is Crystal Violet, which can be sometimes substituted with equally effective Methylene Blue. , gram-positive cells will be stained purple and gram-negative cells will be stained pink.

2.3.10. 2. Methyl red:

Methyl Red (MR) reagent is an indicator solution used to indicate the ph of the broth culture in the methyl red test. The test is useful for differentiating among members of the Enterobacteriaceae. The methyl red test is a quantitative test that measures the amount of acid produced by different bacterial species. The result of this test for *E.coli* is positive and the bacteria appear pink .

2.3.11. Molecular Detection Methods:

Diarrheagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed. Indeed, molecular methods remain the most popular and most reliable techniques for differentiating diarrheagenic strains from nonpathogenic members of the stool flora and distinguishing one category from another. Substantial progress has been made both in the development of nucleic acid-based probe technologies as well as PCR methods.

3. Materials and Methods:

3.1. Materials:

3.1.1. The Equipment and Instruments that used in this study was listed in table (3-1):

Table (3-1). Equipment and instruments:

No.	Equipment and instrument	Company	Origin
1	Autoclave	HIRAYAMA	Japan
2	Centrifuge	Bioneer	South Korea
3	Cotton	Dunya	Iraq
4	Cloves	CMA	Jordan
5	Disposable Syringe 5ml	Sterile E0.	China
6	Disposable plastic petri dishes	Bio-Hit	Finland
7	Eppendorf tubes	Bioneer	South Korea
8	Gel electrophoresis	Shandod Scientific	UK
9	Hood	Bio Lab	South Korea
10	Incubator	Mammert	Germany
11	Light microscope	Olympus	Japan
12	Micropipettes 0.5-10,5-50, 100-1000	Slamid	USA
13	Oven	Mammert	Germany
14	Plastic containers	Sterile EO.	China
15	Plain tube	CMA	Jordan
16	Refrigerator	Royal	Japan
17	Swap	Afco	Jordan
18	Sensitive Balance	Sartorius	Germany
19	Slides and cover sides	Superestar	India
20	Thermocycler PCR	MyGene	South Korea
21	Tips (Different sizes)	Jippo	Japan
22	Water bath	GFL	Germany
23	Vortex	Cyan	Belgium

3.1.2. The Chemicals Materials:

Table (3-2). the Chemical materials that used in this study was listed in table (3-2):

No.	Chemical	Company	Origin
1	Agarose	HBD	England
2	Alcohol	BDH	England
3	Brain Heart Infusion Broth	Himedia	India
4	Blood agar	Himedia	India
5	Crystal violate	BDH	England
6	DNA ladder market	Bio lab	UK
7	DNA Ioading dye	Promega	USA
8	Ethidium Bromide	Himedia	India
9	Glycerol	BDH	England
10	Genomic DNA extraction kit	Favorgen	Taiwan
11	Iodine	BDH	England
12	Maconkey agar	Difco	USA
13	Nuclease – free - water	Promega	USA
14	Normal saline	BDH	England
15	PCR pre mix (master mix)	Bioneer	Sout hKorea
16	Primers	Bioneer	South Korea
17	Safranine	BDH	England
18	TBE buffer		

3.1.3. The Kits:

Table (3-3). List of kits:

No.	kits	Company	Country
1	DNA Extraction Kit for parasite	Bioneer	South Korea
	-Proteinase K -Stool Lysis buffer (SL) -Binding buffer (ST) -Washing buffer 1 (W1) -Washing buffer 2 (W2) -Elution buffer (E) -GD column -Collection tube 2ml		
2	DNA Extraction Kit for Bacteria	Bioneer	South Korea
	-GT Buffer -GB Buffer -Washing buffer 1 (W1) (Add Ethanol) -Proteinase K (Add ddH2O) -Elution Buffer -GD Columns -2ml Collection Tubes		

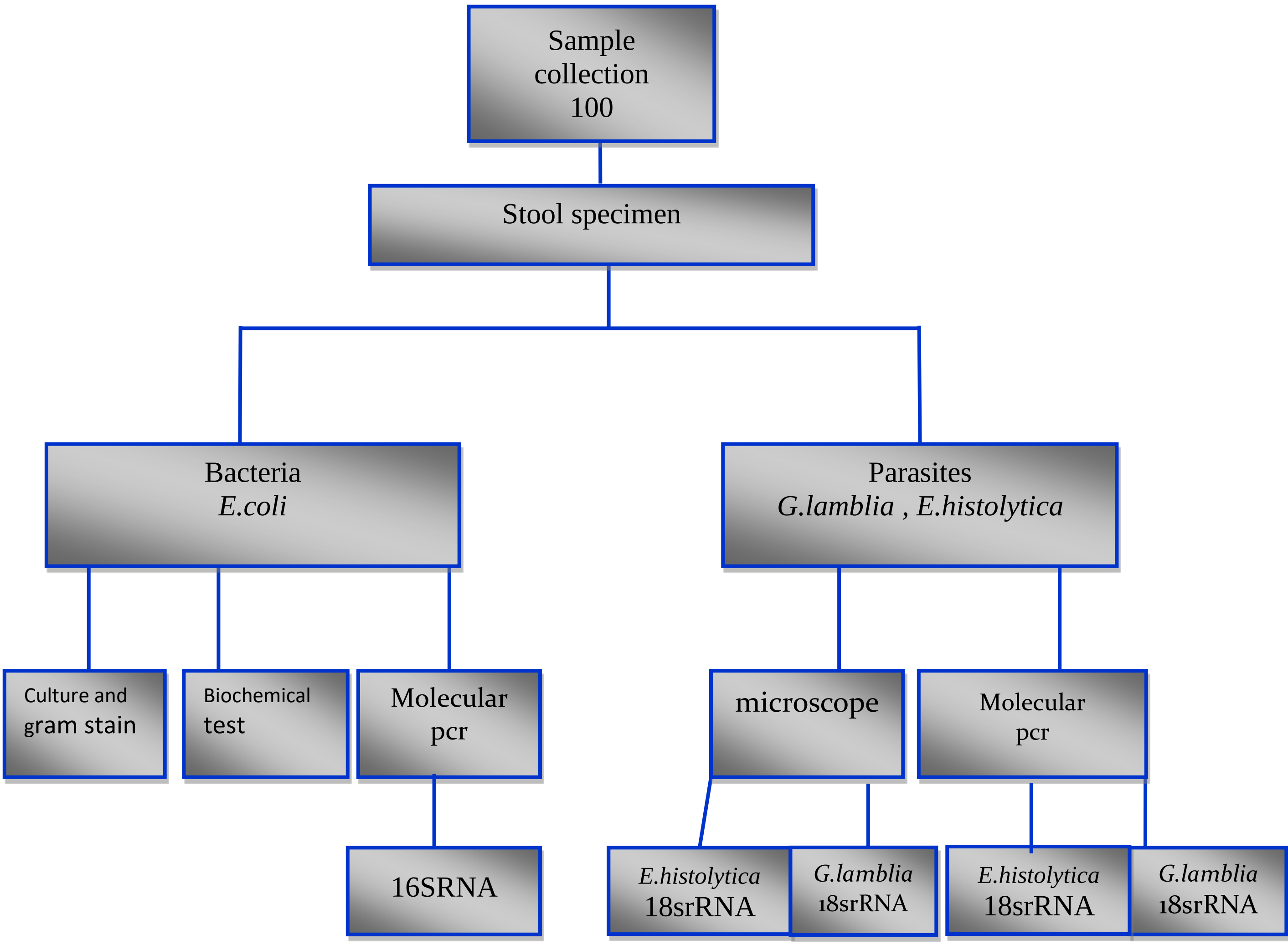


Figure (4): Scheme shows the present study.

3.2. Methods:

3.2.1. Samples Collection:

A total 100 stool samples were collected from some patients who suffer from diarrhea infection based on symptoms, these samples were collected from October 2021 till February 2022 , diagnosed by the physion and stool microscope examination, the age groups ranged from less than one year to 65 years the samples were collected from AL-Hilla city, Babylon maternity and children hospital, private laboratories, and transferred to the advance parasitology and bacteriology laboratories in the College of Science for Women in Babylon University. Samples were placed in the sterilized plastic containers and with a tight lid to keep samples moisture and prevent from being dry. Also a questionnaire sheet has been taken , it has some information from patients in terms of residence area, Genders , age groups, the presence of animals in the houses or not, educational level, and data or month that were collected with it the samples. Then examined samples during a period not exceeding half an hour from the time they were obtained by optical microscope by using a direct smear methods for *G.lamblia* , *E.histolytica* . As for bacteria, it was examined by culture dishes. For parasites positive samples have been saved by adding formalin at a concentration of (37%) and diluted to 10%. As for bacteria positive samples have been saved by adding glycerol. After that made an extracting DNA process and subjected to polymerase chain reaction technique.

3.2.2.1. Sample Examination for Parasites:

3.2.2.1.1. Microscopic Examination:

All the samples were observed macroscopically color, consistency and presence of blood or mucus for visible parasites. The fresh fecal samples were examined for protozoa and helminthes by direct wet mount examination by using normal saline (0.9%) and lugols iodine as well as after concentration by formol-ether sedimentation concentration technique. Direct smears of stool samples were prepared for parasite

3.2.2.1.2. Direct Smear Method:

Stool samples were examined by the preparation of direct smear methods by using clean glass slides , with a small drop of normal saline (0.9%) or lugols iodine stain put on slide glass well mixed with small portion of feces by using wooden stick, putting the slide covers, and examined the sample under power enlarge 40X and 100X (Tanyuksel&petri.,2003).

3.2.2.2. Molecular Diagnosis for Parasite:

3.2.2.2.1. Genomic DNA Extraction of Stool to Detect the *E.histolytica* and *G.lamblia*:

Genomic DNA from stool samples were extracted by stool DNA Extraction Kit , Bioneer. South Korea, and done according to the company instructions in the following steps:

1. A 200 mg of the 96 stool single positive sample examined by direct smear method of *G.lamblia*, *E.histolytica*. was transferred to sterile 1.5ml microcentrifuge tube, then 20µl of proteinase K and 400 µl Stool lysis buffer (SL) was added and mixed by vortex then incubated at 60 C° for 10 minutes.
2. Then, the tube was placed in centrifuge at 12,000 rpm for five minutes.
3. The supernatant was transferred in a new tube and 200µl binding buffer was added to each tube.
4. The tubes incubated over again for 10 min. at 60 C°.
5. 100 µl isopropanol was added and the samples were mixed lightly by vortex for about five seconds, then spin down for 10 seconds to reduce the liquid clinging to the walls and lid of the tube.
6. DNA filter column was placed in a two ml collection tube and transferred all of the mixture (including any precipitate) to column. After that it is centrifuged at 8000 rpm for five minutes. The two ml collection tube containing the flow-through were discarded and placed the column in a new two ml collection tube.
7. 500µl W1 buffer were added to the DNA filter column, then centrifuge at 10000 rpm for 30 seconds. The flow-through was discarded and placed the column back in the two ml collection tube.
8. 500µl W2 Buffer (ethanol) was added to each column. Then centrifuged at 8000 rpm for 30 seconds. The flow-through was discarded and placed the column back in the two mili liter collection tube.

9. All the tubes were centrifuged again for one minute at 12000 rpm to dry the column matrix.
10. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 μl of pre-heated elution buffer were added to the center of the column matrix.
11. The tubes were let stand for at least five minutes to ensure the elution buffer was absorbed by the matrix. Then they were centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

3.2.2.2.2. Primers for Parasite:

Two PCR primers were designed in the present study for detection *G. lamblia*, *E. histolytica*. based subunit for parasite 18ribosomal rRNA gene by using NCBI-Genbank (M54878.1, X64142.1, respectively). Two primers plus design online. These primers were provided from Bioneer company, South Korea

Table (3-4). The primers used in the present study:

Primer	Sequence		Size
G.lamblia	F	GTTGAAACGCCCGTAGTTGG	574
	R	CTCGCTCGTTGTCGCAATG	
E.histolytica	F	ACGAGGAATTGGGGTTCGAC	204
	R	CACCAGACTTGCCCTCCAAT	

3.2.2.1.3.2. PCR Reaction Mixture for Parasites:

The polymerase chain reaction (PCR) was used to amplify fragments of DNA using Maxime PCR PreMix Kit (*i-Taq*) (Intronbio/Korea). The template DNA, forward and reverse primers, and RNase-free water were added to PCR tube, containing *i-Taq* DNA polymerase, dNTP mixture, and reaction buffer mixed together as in table (3-5)

Table (3-5). Contents of the PCR Reaction Mixture:

Contents of reaction mixture	Volume (μl)
Master Mix	12.5
Template DNA	8.5
Forward primer	2
Reverse primer	2
Total	25

3.2.2.1.3. PCR Thermocycler Conditions for Parasite:

PCR thermocycler conditions by using convential PCR thermocycler system as in the following table (3-6):

Table (3-6). The PCR construction conditions:

PCR STEP	Temp	Time	Repeat
Initial Denaturation	95C°	5min	1
Denaturation	95 C°	30 sec	35 cycle
Annealing	57 C°	30 sec	
Extension	72 C°	55 sec	
Final extension	72 C°	5 min	1

3.2.2.1.4. PCR Product Analysis:

The PCR products of positive samples were analyzed by agarose gel electrophoresis in following these steps:

1- 1.5% Agarose gel was prepared by using 1X TBE and dissolving in water bath at 100 C° for 15 minutes, after that, left to cool 50 C°.

2-Then 3 microliter of Ethidium bromide stain were added into agarose gel solution.

3-Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidification for 15 minutes at room temperature. Then the comb was removed gently from the tray and 10µl of PCR product is added in to each comb well and five ml of (100bp Ladder) in one well.

4-The gel tray was fixed in electrophoresis chamber and filled by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for one hour.

5-PCR products were visualized by using UV transilluminator and then photography of bands that reveals by digital camera.

3.2.2.2. Sample Examination For *Escherichia coli*:

3.2.2.2.1. Isolation and Identification of *E. coli*:

All stool samples collected were initially inoculated on sterile blood agar , and incubated at 37 C° for 18-24 hr. Thereafter, colonies from the blood agar were inoculated on MacConkey agar, Brain Heart Infusion Broth and incubated over night at 37 °C. The pink coloured colonies grown on MacConkey agar.

3.2.2.2.2. Preparation of Culture Media for *E.coli* :

Preparation of Culture and Diagnostic Media. The following culture media used in the current study.

1.Mackonkey's agar (MAC):

This medium was prepared by weight (51.5 gm.) of powder medium , dissolving in 1 litter (L) of D.W and heated to boiling with frequent agitation to completely dissolve the medium, then sterilized by autoclave at 121 °C for 15 minutes, then cooled to 55ºC and poured into petri dishes and leave until it solidifies ,this petri dishes incubated in an incubator at 37 Cº for 24 hours to reveled any contaminated medium (Hinenoya, Atsushi, *et al*, 2020).

2.Brain Heart Infusion Broth:

This medium is used to activate, grow and as stock culture for isolates *E. coli*; it is prepared by dissolving (37 gm.) of powder medium in 1L of distilled water, heated to boiling until completely dissolve and adding 20 % glycerin, then pouring to sterile test tubes and sterilizing by autoclave (Arafa *et al.*, 2020).

3.Blood Agar:

Blood agar was prepared by weight (40 gm.) of powder medium , dissolving in (1) L of distilled water and heated to boiling with frequent agitation to completely dissolve the medium, followed sterilized by autoclave at 121C° for15 minutes. After sterilization cooling the medium to 45°C and added the 5% human blood and pour into sterile petri dishes, and leave until it solidifies. Incubated in an incubator at

37C° for 24 hours to revel any contaminated medium (Igbeneghu and Lamikanra, 2014).

3.2.2.2.3. Genomic DNA Extraction for *E.coli*:

DNA of *E.coli* was extracted according to the manufacturer's (protocol, adopted by the manufacturer of DNA extraction kit Geneaid)

Step 1. Cell Harvesting/Pre-lysis:

a) Bacterial cells (up to 1 x 109) were transferred to a 1.5 ml microcentrifuge tube .

b) Bacterial cells centrifuged for one minute at 14-16,000 xg and the supernatant was then discarded.

c)Two hundred µl of GT Buffer was added to the tube and resuspended the cell pellet by shacking vigorously or pipetting.

d)The suspension was incubated at room temperature for five minutes Lysis Step of the Cultured Cell Protocol was then proceeded.

Step 2. Cell Lysis:

a) Two hundred µl of GB Buffer were added to the sample and vortexed for five seconds.

b) It was incubated at 70 ºC for 10 minutes or until the sample lysate is clear during incubation, the tube was inverted every three minutes. At this time, the required (Elution Buffer (200 µl per sample) was incubated at 70 ºC (for Step five DNA Elution.

Optional Step. RNA Degradation:

If RNA free genomic DNA is required,. After 70 ºC incubation, five µl of RNase A (10 mg/ml) were added to the sample lysate and mixed by vortex. Then, it was incubated at room temperature for five minutes

Step 3. DNA Binding:

a) Two hundred µl of absolute ethanol were added to the sample lysate and vortexed immediately if precipitate appears, break it up by pipetting.

b) The GD Column was placed in a two ml Collection tube.

c) All of the mixture (including any precipitate) was transferred to the GD column.

d) Centrifuged at 14-16,000 xg for two minutes.

e) The two ml collection tube containing the flow-through was discarded and the GD column was placed in a new two ml Collection tube.

Step 4. DNA Washing:

a) Four hundred µl of W1 Buffer were added to the GD column

b) Centrifuged 14-16,000 xg for 30 seconds.

c) The flow-through was discarded and the GD column was placed back in the two ml Collection tube.

d) Six hundred µl of Wash Buffer (ethanol added) were added to the GD column.

e) Centrifuged at 14-16,000 x g for 30 seconds.

f) The flow-through was discarded and the GD column was placed back in the two ml collection tube.

g) Centrifuged again for three minutes at 14-16,000 xg to dry the column matrix.

Step 5. DNA Elution:

Standard elution volume is 100 µl. If less sample is to be used, the elution volume (30-50 µl) was reduced to increase DNA concentration.

If higher DNA yield is required, the DNA elution step was repeated to increase DNA recovery and the total elution volume to approximately 200 µl

a) The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube.

b) One hundred µl of preheated Elution Buffer or TE were added to the center of the column matrix.

c) Tube was let stand for 3-5 minutes or until the Elution Buffer or TE is absorbed by the matrix.

d) Centrifuged at 14-16,000 xg for 30 seconds to elute the purified DNA.

3.2.2.2.4.Preparations of Primer for *E.coli*:

The primer pair (Macrogen/Korea) (Table 3-9) used in this study were dissolved using sterile ddH_2O. Stock solution(100pmol/µl) was prepared by adding ddH_2O to the vial containing lyophilized primer while working stock of 10 pmol/µl was made by mixing 10 µl of the stock primer and 90 µl of ddH_2O.

Table (3- 7). Primers Sequences of 16SrRNAgene:

Primer name	Sequence 5-3		Reference
F	AGAGTTTGATCCTGGCTCA		(Loy *et al.*, 2002)
R	GGTTACCTTGTTACGACTT		

3.2.2.2. 5. PCR Reaction Mixture for *E.coli*:

The polymerase chain reaction (PCR) was used to amplify fragments of DNA using Maxime PCR PreMix Kit (*i-Taq*) (Intronbio/Korea). The template DNA, forward and reverse primers, and RNase-free water were added to PCR tube, containing *i-Taq* DNA polymerase, dNTP mixture, and reaction buffer mixed together as in table (3-10)

Table (3-8). Contents of the Reaction Mixture:

Contents of reaction mixture	Volume (µl)
Master Mix	12
Template DNA	4
Forward primer (10 pmol/µl)	2
Reverse primer (10 pmol/µl)	2

Nuclease free water	Up to 20 ul

3.2. 2.2.6. PCR Condition for *E.coli*:

The Conventional PCR was used to amplify a target DNA by using specific primer pairs. PCR typically consists of three consecutive steps (denaturation, annealing, and elongation) of repeated cycles to get PCR product (amplicon).

The PCR thermal cycling conditions are mentioned in table (3-9). The size was of PCR products (5 µL) analyzed in 1.5% (w/v) agarose gel by electrophoresis using 1×TBE buffer and visualized by staining with simply safe dye. The Product size was determined by comparison with sizer100 bp DNA ladder (Intronbio/Korea).

Table(3-9). PCR conditioned for amplification of 16S rRNA gene:

PCR STEP	Temp	Time	Repeat
Initial Denaturation	95C°	5min	1
Denaturation	95 C°	30sec	35cycle
Annealing	55 C°	45sec	
Extension	72 C°	45sec	
Final extension	72 C°	5min	1

3.2.2.2.7. Agarose Gel Electrophoresis:

Agarose gel electrophoresis is the most effective way of separating DNA fragments. The concentration of agarose in a gel depends on the sizes of the DNA fragments need to be separated, ranging between (0.5%-2%) A (0.7%) gel was used to obtain good separation of genomic DNA (5-10 kb) after extraction while (1.5%-2%) was used to gain good resolution for small fragments of PCR product (0.2-1 kb). However, the specific weight of agarose was added to 100 ml of 1×TBE buffer and then melted in microwave until the solution becomes clear. Once the agarose was cooled to 50-55°C, 5µl of simply safe dye (10 mg/ml) was added to 100 ml of melting agarose gel to get final concentration 0.5µg/ml (Sambrook & Rusell, 2001).

The agarose was poured in the gel tray with sealed ends, comb placed properly, and then left to dry. The samples were loaded in a separate well of the gel, with marker in one well. Electrodes were connected correctly and the run was applied according to the gel percentage and size of gel, (The time of agarose gel electrophoresis is 45 minute for genomic DNA and 1 hour and 30 minute for PCR DNA sequencing of 16sRNA of tested isolate).

3.2.2.2.8.Biochemical Test :

Morphological diagnosis by Microscope examination

Gram stain :

E.coli appear under microscope in pink/red color and gram negative .

Methyl red :

E.coli appear under microscope in red color and MR positive .

3.2.2.2.8.1 Reagents Used in Methyl Red (MR):

1-MRVP broth (pH 6.9) Ingredients per liter of deionized water: buffered peptone = 7.0 gm , glucose= 5.0 gm dipotassium phosphate = 5.0 gm

2-Methyl red solution 0.02%

a. Dissolve 0.1 g of methyl red in 300 ml of ethyl alcohol, 95%
b.Add sufficient distilled water to make 500 ml
c. Store at 4 to 8 degree C in a brown bottle. Solution is stable for 1 year.

3.2.2.2.8.2. Procedure of Methyl Red (MR) Test:

1-Prior to inoculation, allow medium to equilibrate to room temperature.

2-Using organisms taken from an 18-24 hour pure culture, lightly inoculate the medium.

3-Incubate aerobically at 37 degrees C. for 24 hours.

4-Following 24 hours of incubation, aliquot 1ml of the broth to a clean test tube.

5-Reincubate the remaining broth for an additional 24 hours.

6-Add 2 to 3 drops of methyl red indicator to aliquot.

7- Observe red color immediately.

3.2.2.2.8.3. Reagents Used in Gram Staining:

1-Crystal Violet, the primary stain

2-Iodine, the mordant

3-A decolorizer made of acetone and alcohol (95%)

4-Safranin, the counterstain

3.2.2.2.8.4. Procedure of Gram Stain:

(Gephardt *et al*, 1981, Feedback from ASMCUE participants, ASMCUE , 2005)

1. Flood air-dried, heat-fixed smear of cells for one minute with crystal violet staining reagent. Please note that the quality of the smear (too heavy or too light cell concentration) will affect the Gram Stain results.

2. Wash slide in a gentle and indirect stream of tap water for two seconds.

3. Flood slide with the mordant: Gram's iodine. Wait one minute.

4. Wash slide in a gentle and indirect stream of tap water for two seconds.

5. Flood slide with decolorizing agent. Wait 15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.

6. Flood slide with counterstain, Safranine. Wait 30 seconds to one minute.

7. Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.

8. Observe the results of the staining procedure under oil immersion using a Bright field microscope. At the completion of the Gram Stain, gram-negative bacteria will stain pink/red and gram-positive bacteria will stain blue/purple.

3.2.2.3.Gene Sequencing Analysis :

The DNA samples for 16srRNA and 18srRNA genes with primers (R + F) were sent to the company of Macrogen Inc (South Korea , Geumchen,Seoul). The results were analyzed according to the program BLASTN Available on the site NCBI to know the sequence and number of nitrogenous bases .

3.2.2.3.1. Sequence Editing and Analysis by Bio Edit for Parasites and Bacteria :

Bio Edit (Sequence Alignment Editor version 4.7.8) a software of the Department of Microbiology, North Carolina State University (Hall, 1999) was used as the general tools for sequence editing and analysis e.g., for locating the putative ORFs in all reading frames, analyzing nucleotide composition, amino acids composition and codon usage ORF for performing multiple sequence alignment.

4. Result and Discussion:

4.1. Prevalence of parasitic diarrheic agents in the present study:

The present study shows overall percentage of infection of *E.histolytica* and *G.lamblia* (37%). A similar proportion almost to many studies, including finding, Mahmud (2006) in Al-Sowera, as the percentage rate of infection about (34.6%) Rahi *et al*., (2013) in the Al-Kut city, since the percentage of infection is (33.83%). The current study recorded (37%) a rate less than the record Salman (2014) in Kirkuk city, where the rate of infection (52.07%), and for the record Shakir *et al.*(2014) in Baghdad city, as was the prevalence of intestinal parasites among children under five years of age (84.16%). While the study recorded the rates of infection more than it has ever, Jaeffer (2011) in Baghdad, as the rate of infection record was (13.64%), as well as Al-Kubaisy *et al.*(2014), where the rate of infections among children in Baghdad province record, reaching 22%. The difference in rates of infection recorded in the current study and previous studies that were mentioned above may be carried out to the difference in the level of sanitation, personal hygiene, residential density, geographic location, climatic conditions, the total number screened, test methods,(Al-Ibrahimi , 2013).

4.2. Isolation and Identification of *Escherichia coli:*

Out of 100-hundred stool specimens collected from patients suffering from severe diarrhea in Babylon province specimens gave positive *E.coli* culture on macConky agar appear as a small , circular pink colonies , flat, dry, with lactose fermenter bounded by darker pink colour as a result of bile salts deposition appeared when cultured on MacConky agar. Out of 100 (26%) samples were positive for *E.coli* by using culture on MacConky agar. While (25%) specimans showed positive results for *E.coli* by using PCR technique out of 26 Further identification of *E.coli* isolates has been carried out depending on microscopic and biochemical test. The results of the Gram stain showed a Gram –ve. A wide range of bacterial pathogens was detected. Bacterial *E.coli* were isolated from 25 (25%) of 100 stool samples and 75 (75%) was other bacteria. *E.coli* bacteria were isolated according to several criteria , including gender were isolated (males 12 from 59 and females 13 from 51),according to table (10).According to the residence area were isolated (urban area 11 from 47 and rural area 14 from 53). These rates of detection of bacterial pathogens in patients are shown in Table (8). While according to the age groups isolated (less than one year) 4 from 15 while (1-5 years) were 8 from 32, (6-10years) were 6 from 17,(11-15years)were 4 from 14 , (16-20 years) were 2 from 12 and (21 and more)1from 10) these results of detection of bacterial pathogens in patients are shown in Table (12) . The results of this study referred to a high percentage of the distribution of *E.coli* in the rural area and in the both sex male and female and in the age group (6-10), suffering from severe diarrhea. This study agree with Mitra *et al*., (2011) showed that 178/313 isolates (25%) were belong to EPEC have been isolated from stool samples which were collected from children. This study disagree with Jafari *et al.* (2009) found that,70 (12.6%) of 555 isolates obtained from patient with diarrhea were identified as EPEC. Also, EPEC represented the main causes while it is associated with (16.6%)and (5.2%) in Tunisia respectively (Scaletsky *et al*., 2002; Vidal *et al*., 2005; AIGallas *et al*., 2007).

4.3. Prevalence of complicated infection parasites and bacteria:

The present study showed in difference of the overall percentage of infection (37%) with diarrhea parasites and the total percentage of infection bacteria (26%). this is illustrated in table (4-1) .

Table (4-1): Prevalence of infection parasites and bacterial

Kind of parasite and bacteria	Examined No.	Infected No.	(%)	Total
E.histolytica	100	35	35	37
G.lamblia		2	2	
E.coli		26	26	26

This study disagree with that recorded by Beena Uppal *et al.*(2015) where he scored the rates of infection for parasites (4.78%) and the rates of infection for bacteria (8.90%).

4.1.1. Prevalence of parasitic infection according to residence area using direct microscopic examination :

This table (4-2) showed the highest rates (43.1%) of infection was with all studies of parasite that were seen among rural areas patients, when compared with the low rate (30.6%) in urban area .The present study showed difference in the rates of parasitic infections,according to the residential area, the highest rates for *E.histolytica* was in the rural areas (41.1%) and lowest in the urban areas (28.5%) while the highest rates of *G. lamblia* was in the urban areas (2%) and lowest in the rural areas (1.96%) with non-significant differences ($p > 0.05$)

Table (4-2): Prevalence of infection parasitic according to residence area by using direct microscopic examination .

Residence Area	Examind No.	Infected No.	Parasite that cause diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec No.	(%)	Infec No.	(%)	
Urban areas	49	15	14	28.5	1	2	30.6
Rural areas	51	22	21	41.1	1	1.96	43.1
Total	100	37	35	35	2	2	37
Statistical analysis			$p = 0.779$ $x2 = 0.078$		$p = 0.77$ $x2 = 0.078$		Pvalue =0.194 $x2calculated = 1.681$ $x2tabulated = 3.841$
			Non-significant differences ($p > 0.05$)		Non-significant differences ($p > 0.05$)		Non-significant differences ($p > 0.05$)

This study agrees with Al-Kubaisy *et al.* (2014) where he scored the highest rate of infection in the rural area, reaching 50.9% in Baghdad . This study disagrees with what recorded by Al-Moussawi (2012) as the rates of infection in the rural area (79%) amounted to 15% higher than in the cities area (12.36%). The reason for the high incidence of infection in rural areas is due to several factors, including the lack of clean drinking water availability, and the lack of guidance and counseling by the authorities concerned , lower health and cultural level of the rural population , the lack of hospitals and health centers in those areas, as well as use of animal waste and human feaces and sometimes as an organic fertilizer for the growth of plants and vegetables.

4.1.2. Prevalence of parasitic infection according to residence area using by PCR technique :

This table reveals the rates of parasitic infections, according to the residence area, the highest rates of infection were in the rural area (41.1%) while the low rates of infection in the town (urban area) (33.3%), and the highest rates infection of *E.histolytica* were in the rural areas (39.2%) but *G.lamblia* were in the urban area (2.2%), whereas the lowest rates of infection for E.histolytica were in the urban area (31.1%) but G.lamblia were in the rural area (1.9%) . It has been observed as non-significant differences in infection rates according to residence area ($P > 0.05$)

Table (4-3): Prevalence of parasitic infection according to residence area by using PCR technique .

Residence area	Examind No.	Infected No.	Parasites that cause diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec. No.	(%)	Infec. No.	(%)	
Urban area	45	15	14	31.1	1	2.2	33.33
Rural area	51	21	20	39.2	1	1.9	41.1
Total	96	36	34	35.4	2	2	37.5
Statistical analysis				$p = 0.40735$ $x2calculated = 0.686$ $x2tabulated = 3.841$ Non-significant differences (p>0.05)		$p = 0.9287$ $x2calculated = 0.008$ $x2tabulated = 3.841$ Non-significant differences (p>0.05)	$p = 0.4283$ $x2calculated = 0.6274$ $x2tabulated = 3.841$ Non-significant differences ($p > 0.05$)

The present study showed difference in the rates of infection with parasitic infections, according to the residence area, the highest rate of infection of parasites was in the rural area (41.1%) while the low rates of infection in the town (urban area) (33.3%) . This study agrees with what was recorded by Al-Khafaji (2013) in Al-Qadisyai as the rates of infection parasites in the rural area (62.9%) higher than in the urban area (37.9%). The reason for the high incidence of infection in rural areas is due to several factors, including the lack of clean drinking water availability, and the lack of guidance and counseling by the authorities concerned , lower health and cultural level of the rural population , the lack of hospitals and health centers in those areas, as well as use of animal waste and human feaces and sometimes as an organic fertilizer for the growth of plants and vegetables.

4.1.3. Prevalence of Infection parasitic according to the gender using direct microscopic examination:

This table reveals the prevalence of parasitic infections in both sexes (37%). The highest rate of infection was in males 38.4% while the lowest rate of infection was in females (35.4%).The highest rates of *E.histolytica* were in females (36.5%) and lowest rates in males (33.3%), while the highest rate of *G.lamblia* were in males (2%) and lowest in females (1.9%). Has been observed non-significant differences in infection rates between two gender of parasites kinds at the $(p > 0.05)$ and as it illustrated in table (4-4) .

Table (4-4): Prevalence of infection parasite according to the gender .

The sex	Examind No.	Infected No.	Parasites that cause diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec. No	(%)	Infec. No.	(%)	
Males	52	20	19	36.5	1	1.9	38.4
Females	48	17	16	33.3	1	2	35.4
Total	100	37	35	35	2	2	37
Statistical analysis				$p = 0.905$ $x2 = 0.014$		$p = 0.905$ $x2 = 0.014$	$p = 0.752$ $x2calculated = 0.099$ $x2tabular = 3.841$
				Non-significant differences $p > 0.05$		Non-significant differences $p > 0.05$	Non-significant differences ($p > 0.05$)

The results show the percentage of parasitic infections in both sexes were (37%).The present study agrees with the study of Ali Malaa *et al* (2020) in karbala where he scored the highest rates of infection in male (11.17%) and lowest rates in females (9.41%) , and agrees with study of Abdulsadah. Rahi & Lara Majeed ,(2019) in Wasit where he scored the highest rates of infection in males (34.5%) and lower than in females (23%), and agree with study of Kubaisy *et al.* (2014) in Baghdad, where he scored the highest rate of infection in males (58.5%) and lower than in females (41.5%) , and also disagrees with the study of Jaeffer (2011) in Baghdad, where he scored the highest rate of infection (15.35%) in females and (12.28%) in males. The difference in the rate of infection between males and females may be due to the fact that males are more active and they have contact with the external environment factors at play and of being in working group in the communities. This makes them more relevant pathogens sick of females as males eat well and drink in public places or from street vendors, in addition to the nature of anarchism and a lack of attention to personal hygiene and hand washing hands which increase the chances of being infected either for *E.histolytica* and *G.lamblia* and the absence of significant differences between male and females could be due to portability and having the same opportunity to infection in both gender (Al-Mamouri, 2000).

4.1.4. Prevalence of infection parasitic according to gender by using PCR technique:

This table showed the highest rates of infections were in males (39.2%) while the lowest rates of infections in females (35.5%) . The highest rates of infection of two parasites were in males (37.2%) for *E.histolytica* and in females (2.2%) for *G.lamblia* , Whereas the lowest rates of infection were in females for *E.histolytica* (33.3%) and in males for *G.lamblia* (1.9%) . It has been observed as non-significant differences in infection rates between the gender at the ($p > 0.05$) that illustrated in Table (4-5) .

Table (4-5): Prevalence of infection parasites according to gender .

The Sex	Examind No.	Infected No.	Parasite that cause diarrhea				Total percentage of infection (%)
			E.histolytica		*G.lamblia*		
			Infec No.	(%)	Infec No.	(%)	
Males	51	20	19	37.2	1	1.9	39.2
Females	45	16	15	33.3	1	2.2	35.5
Total	96	36	34	35.4	2	2	37.5
Statistical analysis				$p = 0.688483$ $x2calculated = 0.1607$ $x2tabulated = 3.841$		$p = 0.9287$ $x2calculated = 0.00801$ $x2tabulated = 3.841$	$p = 0.71164$ $x2calculated = 0.1366$ $x2tabulated = 3.841$
				Non-significant differences $p > 0.05$		Non-significant differences $p > 0.05$	Non-significant differences $p > 0.05$

The present study agree with Al-Warid (2010) in Baghdad as it did not find any significant differences between genders as the percentage ratios for parasites infection between males and females (14.98%) and (14.58%), respectively, as well as incompatible with Koffi *et al*. (2014) as it did not find any significant differences between the gender although he found the highest rates of infection among females (65.36%) and the lowest rates of infection among males (60.13%) .The reason that males are more active and they have contact with the external environment factors at play and of being in working group in the communities. This makes them more relevant pathogens sick of females as males eat well and drink in public places or from street vendors, in addition to the nature of anarchism and a lack of attention to personal hygiene and hand washing hands which increase the chances of being infected either for *E.histolytica* and *G.lamblia*

4.1.5. Prevalence of infection parasitic according to the age groups by using direct microscopic examination:

Table (4-4) showed the highest rates of infection were focused in the age group(6-10) which was (50%). While the decline in the rates of infection was observed in the age group (21 and more) (10%) , and the highest rates of infection according to age groups (1-5) were (45.7%) and lowest rates of infection in the age group (21 and more) were (10%) for *E.histolytica* , while the G.lamblia record the highest rates of infection in the age group (6-10) were (12.5%) and the lowest rates of infection (0%) respectively in the age groups (less than one year , 1-5 , 11-15 , 16-20 , 21and more) , has been observed non-significant differences in infection rates among different age groups in the ($p > 0.05$) and as it is illustrated in table (4-6) .

Table (4-6): Prevalence of infection parasite according to age group.

Age group	Examind No.	Infected No.	Parasite that cause diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec. No.	(%)	Infec. No.	(%)	
Less than one year	16	4	4	25	0	0	25
1-5	35	16	16	45.7	0	0	45.7
6-10	16	8	6	37.5	2	12.5	50
11-15	12	5	5	41.6	0	0	41.6
16-20	11	3	3	27.2	0	0	27.2
21and more	10	1	1	10	0	0	10
Total	100	37	35	35	2	2	37
Statistical analysis				$p = 0.1757$ $x2 = 7.664$		$p = 0.000828$ $x2 = 583.278$	$p = 0.222$ $x2calculated = 6.974$ $x2tabulated = 11.07$
				Non-significant differences $p > 0.05$		Significant differences ($p > 0.05$)	Non-significant differences ($p > 0.05$)

The present study recorded prevalence of parasitic infections in different age groups from age-less than one year to age 21 and more.

The highest rates of infection (50%) were in the age group (6-10), while the lowest rates of infection(10%)was in the age group (21 and more). This study disagrees with what was recorded in the study of Al-Ibrahimi (2013) in Al-Diwaniya province which reached the highest rate of infection in the age group (2-4) years (13.87%), while the lowest rate of infection in the age group (8-10) were (1.52%). The high incidence of intestinal parasites in (6-10) age group may be due to the fact that this group belongs to the primary school and the students at this stage are more active and moving with little attention to their personal hygiene and taking into account health conditions. The reason for the high infection in the age group (6-10) may be because the nature of this parasite opportunistic as it affects children and people with little immunity, as well as the spread of artificial feeding that may be a source of infection .

4.1.6. Percentage of infection parasitic according to age groups by using PCR technique:

Table (4-7) shows the parasitic infections in different age groups from age-less than one year to age 21 and over, the highest rates of infections of parasites (50%)were in the age group (6-10) and the lowest rate of infections of parasites(10%) were in the age group (21 and over). The highest rates of infection for *E.histolytica* were in the age group(1-5) as (46.8%) , but the *G.lamblia* were in the age group (6-10) as (12.5%), whereas the lowest rates of infection for *E.histolytica* were in the age group (21and more) as (10%) but the *G.lamblia* were (0%) in the age groups (less than one year, 1-5, 11-15, 16-20, 21 and more).It has been observed as non-significant differences in infection rates among different age groups in the ($p > 0.05$) that are illustrated in table (4-7)

Table (4-7): Prevalence of infection parasites according to age groups.

The age	Examind No.	Infected No.	Parasite that causes diarrhea				Total percentage of infection (%)
			E.histolytica		*G.lamblia*		
			Infec. No.	(%)	Infec. No.	(%)	
Less than one year	15	4	4	26.6	0	0	26.6
1-5	32	15	15	46.8	0	0	46.8
6-10	16	8	6	37.5	2	12.5	50
11-15	12	5	5	41.6	0	0	41.6
16-20	11	3	3	27.2	0	0	27.2
21andmore	10	1	1	10	0	0	10
Total	96	36	34	35.4	2	2	37.5
Statistical analysis				$p = 0.3347$ $x2calculated = 5.717$ $x2tabulated = 11.07$ Non-significant differences $p > 0.05$		$p = 0.0694$ $x2calculated = 10.212$ $x2tabulated = 11.07$ Significant differences $p < 0.05$	$p = 0.2340$ $x2calculated = 6.8242$ $x2tabulated = 11.07$ Non-significant differences $p > 0.05$

This study is compatible with Al-Warid (2010) in Baghdad, who scored the highest rate of infection parasites in less than ten years age group was 55.04%. This study is incompatible with Ngosso *et al*.(2015) in Dar Es Salaam, Tanzania who scored the highest prevalence of diarrhea (29.6%) in the age groups of (12-23) months, followed by (24-60) months were 15.6%,then (6-11)months were 8% and (least 0-5 months) were (2.4%). The reason for the high infection in the age group (6-10) may be because the nature of this parasite opportunistic as it affects children and people with little immunity, as well as the spread of artificial feeding that may be a source of infection .

4.1.7. Prevalence of infection parasitic according to the presence of animals in the houses or not by using direct microscopic examination:

Table (4-8) showed the highest rates of infection were in the presence of animals (38.4%) while the low rate of infection where in the places without animals (36%). And the highest rate of infections was in the presence of animal in the houses of *E.histolytica* (35.8%), and *G.lamblia* record the highest rates of infection (2.5%), while the lowest rates of infection were in the those not having animals in their houses for *E.histolytica* (34.4%) and for *G.lamblia* (1.6%) . it also has been observed non-significant differences in infection rates in ($p >$ 0.05) as it illustrated in table (4-8) .

Table (4-8): Prevalence of infection parasitic according to the presence of animals in the houses or no.

Animals existence in the houses or not	Examind No.	Infected No.	Parasite that causes diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec No.	(%)	Infec No.	(%)	
Found	39	15	14	35.8	1	2.5	38.4
Not found	61	22	21	34.4	1	1.6	36
Total	100	37	35	35	2	2	37
Statistical analysis				$p = 0.779$ $x2 = 0.0784$		$p = 0.779$ $x2 = 0.0784$	$p = 0.8087$ $x2calculated = 0.0585$ $x2tabulated = 3.841$
				Non-significant differences ($p > 0.05$)		Non-significant differences ($p > 0.05$)	Non-significant differences ($p > 0.05$)

The present study showed difference in the rates of infection with parasitic infections, according to the presence of animals in the houses (Such as birds, cats, dogs and sheep or cattle) or not. The highest rate of infection(38.4%) was in the presence of animals , while the lowest rate of infection (36%) was when there were no animals but there are no significant differences between two groups.This study agrees with the study of Al-Morshidy (2007) in Hilla city who scored the highest rate of infection in those who have animals in their houses at a percentage of infection (76.6%), and the lowest rate of infection in those who do not have animals (30.8%). Due to the increase of the rate of infection of intestinal parasites in patients who have animals in their houses the role of these animals in the increase of rate of infection of humans is clear, because directly transferred by contact or indirectly by eating food contaminated with opportunistic parasites transferred to it by the animals in this case plays all from not washing hands and introduced into the mouths and not good for cooking food plays an important role in the transmission of infection (Al-Morshidy, 2007).

4.1.8. Prevalence of infection parasitic according to the presence of animals in the houses or no using PCR technique :

Table (4-9) reveals the rates of parasitic infections, according to the presence of animals in the houses such as (birds, cats, dogs and sheep and cattle) or not. The highest rates of infection parasite (39.4%) were those who have animals in their houses, and the lowest rates of infection (36.2%) were those who don't have animals in their houses . The highest rate of infection focused in the *E.histolytica* and *G.lamblia* (36.8% , 2.6%) , respectively in those that have animals in their houses , whereas the lowest rate of infection in the *E.histolytica* and *G.lamblia* (34.4% , 1.7%) in those that don't have animals in their houses, with non-significant differences in infection rates according to the presence of animals ($p > 0.05$).

Table (4-9): Prevalence of infection parasites according to the presence of animals in the houses or no .

The presence of animals In the houses or not	Examind No.	Infected No.	Parasite that causes diarrhea				Total percentage of infection (%)
			E.histolytica		*G.lamblia*		
			Infec. No.	(%)	Infec. No.	(%)	
Found	38	15	14	36.8	1	2.6	39.4
Not found	58	21	20	34.4	1	1.7	36.2
Total	96	36	34	35.4	2	2	37.5
Statistical analysis				$p = 0.81314$ $x2calculated = 0.0558$ $x2tabulated = 3.841$		$p = 0.7608$ $x2calculated = 0.09267$ $x2tabulated = 3.841$	$p = 0.7464$ $x2calculated = 0.10453$ $x2tabulated = 3.841$
				Non-significant differences $p > 0.05$		Non-significant differences $p > 0.05$	Non-significant differences $p > 0.05$

This study did not agree with Al-Warid (2010) in Baghdad, he has recorded did not notice that there was no significant difference between the presence or absence of animals in homes, (19.49%) and (13.89%), respectively. The reasons Due to the increase of the rate of infection of intestinal parasites in patients who have animals in their houses the role of these animals in the increase of rate of infection of humans is clear, because directly transferred by contact or indirectly by eating food contaminated with opportunistic.

4.1.9. Percentage of infection parasitic according to educational level using direct microscopic examination :

This study showed a difference in parasitic infections rates, according to the level of education the head of household. The present study indicated the highest rates of infection for those with parents who are primary educational (50%) Although convergence in the rates between the educational level of parents (not educated, primary, secondary, academic) as the percentage rate of infection (35 %, 50 %, 36.3%, 10%,respectively). The highest rates of infection were in the primary educational level for *E.histolytica* and *G.lamblia* as (42.8% , 7.1%) respectively. Whereas the lowest rate of infection was (10%) in the academic educational level for *E.histolytica* and it was (0%) in the (not educated , secondary , academic) educational levels for *G.lamblia*. It has been observed non-significant differences in the incidence rates by level of education ($p > 0.05$) that are illustrated in table (4-10) .

Table (4-10): Prevalence of infection parasitic according to educational level of patients.

Educational level	Examind No.	Infected No.	Parasites that casues diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec No.	(%)	Infec No.	(%)	
Not educated	40	14	14	35	0	0	35
Primary	28	14	12	42.8	2	7.1	50
Secondary	22	8	8	36.3	0	0	36.3
Academic	10	1	1	10	0	0	10
Total	100	37	35	35	2	2	37
Statistical analysis				$p = 3.175$ $x2 = 0.7777$		$p = 1.8 \times 10$ $x2 = 47.120$	$p = 0.1557$ $x2calculated = 2.1024$ $x2tabulated = 7.815$
				Non-Significant differences $p > 0.05$		Non-Significant difference $p > 0.05$	Non-significant differences $p > 0.05$

As for the relationship of infection with intestinal parasites under study which the educational level of the head of the household. This study is disagrees with AL- Kubaisy *et al*. (2014) in Baghdad, who scored the highest rate of infection for those with a high level of education increased by (54.7%) and lower than those of pupils with educational and illiterate level by (45.3%). The high rate of infection with parasites that cause diarrhea by level of education among parents with non-literate (illiterate) could be due to a lack of awareness of the health of the family. They do not realize the danger of living pollutants which may be due to the low cultural level of the family. The results of the current study differ from many previous studies, but the convergence ratios and high rate of infection with parents who obtained an academic level may be due to several reasons, including the living situation of the country led to the neglect of health which is important side and pay attention to look for opportunities to work , or may be attributed to the volume of samples in the present study .

4.1.10. Percentage of infection parasitic according to the education level by using PCR technique:

Table (4-11) shows the parasitic infections rates, according to the level of education the head of household, and found that the highest rate of infection of parasites for those who got primary education level (50%). The lowest rate of infection of parasite was with the head of the family who get academic education level(10%). There is convergence in the percentage rates between the educational level of parents (primary, secondary , not educated , academic) as the percentage rates , (50%, 38%, 35.8%, 10%, respectively).The highest rate of infection focused in the *E.histolytica* and *G.lamblia* (42.3%, 7.6%) respectively in the primary educational level, while the lowest rates of infection recorded in *E.histolytica* were in the academic educational level (10%) , but the *G.lamblia* were (0%) in the educational levels (not educated , secondary, academic).It has been observed as non-significant differences in infection rates by level of education in ($p > 0.05$) .

Table (4-11): Prevalence of infection parasites according to education level by using PCR technique .

Educational level	Examind No.	Infected No.	Parasite that causes diarrhea				Total percentage of infection (%)
			E.histolytica		*G.lamblia*		
			Infec No.	(%)	Infec No.	(%)	
Not educated	39	14	14	35.8	0	0	35.8
Primary	26	13	11	42.3	2	7.6	50
Secondary	21	8	8	38	0	0	38
Academic	10	1	1	10	0	0	10
Total	96	36	34	35.4	2	2	37.5
Statistical analysis				$p = 0.3294$ $x2calculated = 0.6095$ $x2tabulated = 7.815$		$p = 0.1386$ $x2calculated = 5.286$ $x2tabulated = 7.815$	$p = 0.17136$ $x2calculated = 1.7792$ $x2tabulated = 7.815$
				Non-significant differences $p > 0.05$		Non-significant differences $p > 0.05$	Non-significant differences $p > 0.05$

This study is consistent with the study of Al-Warid (2010) in Baghdad, who scored the highest rate of infection for obtaining the educational level of primary was(45.8%) and lower than un-employed by (10.09%). The reasons may be attributed to the volume of samples in the present study or may be The high rate of infection with parasites that cause diarrhea by level of education among parents with non-literate (illiterate) could be due to a lack of awareness of the health of the family. They do not realize the danger of living pollutants which may be due to the low cultural level of the family.

4.1.11. Prevalence of infection parasitic according to the collecting sample monthly using direct microscopic examination :

The results of the current study also showed that there were infection rates between months, the highest total percentage of infections in October (45 %) , while the lowest percentage was in February (31.2%). The highest rates of infection were at October (2021) for *E.histolytica* (45%) , But *G.lamblia* was in the February (2022) as (6.2%) . Whereas the lowest rates of infections were in the February 2022 (25%) for *E.histolytica but G.lamblia* was (0 %) in the (October 2021 , November 2021 , December 2021) , with non-significant differences ($p > 0.05$) .

Table (4-12): Prevalence of infection parasitic according to the collecting sample monthly.

Months	Examind No.	Infected No.	Parasite that causes diarrhea				Total percentages of infection(%)
			E.hstolytica		*G.lamblia*		
			Infec No.	(%)	Infec No.	(%)	
October 2021	20	9	9	45	0	0	45
November 2021	32	12	12	37.5	0	0	37.5
December 2021	14	5	5	35.7	0	0	35.7
January 2022	18	6	5	27.7	1	5.5	33.2
February 2022	16	5	4	25	1	6.2	31.2
Total	100	37	35	35	2	2	37
Statistical analysis				$p = 0.719915$ $x2calculated = 2.086168$ $x2tabulated = 9.488$		$p = 0.4083$ $x2calculated = 3.982$ $x2tabulated = 9.488$	$p = 0.9255$ $x2calculated = 0.89324$ $x2tabulated = 9.488$
				Non significant differences $p > 0.05$		Non significant differences $p > 0.05$	Non significant differences $p > 0.05$

The results of the current study showed that there were differences in infection rates among months of the years . The highest rates of infection at October 2021 (45%), followed in November 2021 (37.5 %). While the lowest rates of infection were in February 2022 (31.2%).The convergence or similarity of the current study of what was recorded by Hussein *et al.*(2011)where the rates of infection ranged between November and October monthly rate (61.4% and 60.4% respectively). This study disagrees with what was recorded by Al-Moussawi (2012) in Babylon province, with the highest rates of infection in May (22.97%), while the lowest percentage of infection in January (3.88%). The high infection of intestinal parasites in the summer months may be due to the availability of appropriate conditions of temperature and humidity and the nature of the climate in Iraq, which is hot in summer and mild during most of winter period. It may contribute much in prevalence in the region and constitute the presence of insect vectors like (flies) and increase its numbers which is a vector for infectious mechanically phases in these times of the year to fit the weather conditions as well as increasing drinking water, juices and ice cream that may be made of non-sterile water plays a big role in prevalence of intestinal parasites (Hussein *et al.*,2011).

4.1.12. Prevalence of infection Parasitic according to the collecting sample monthly using PCR technique:

Table (4-13) showed the highest rates of infections parasites in the month of October 2021were (45%), and the lowest rates of infections of parasites were in January 2022 (35.2%) . The highest rates of infection recorded for *E.histolytica* were in the October 2021 as

(45%), and the G.lamblia record in the February 2022 as (7.1%), while the lowest rates of infection recorded for *E.histolytica* were in February 2022 as (28.5%) and the *G.lamblia* recorded were (0%) in (October 2021, November 2021, December 2021), with non-significant differences in infection rates to the collecting sample monthly in ($p > 0.05$) .

Table (4-13): Prevalence of parasites infection according to the collecting sample monthly .

Months	Examind No.	Infected No.	Parasite that causes diarrhea				Total percentages of infection (%)
			E.histolytica		*G.lamblia*		
			Infec. No.	(%)	Infec. No.	(%)	
October 2021	20	9	9	45	0	0	45
November 2021	31	11	11	35.4	0	0	35.4
December 2021	14	5	5	35.7	0	0	35.7
January 2022	17	6	5	29.4	1	5.8	35.2
February 2022	14	5	4	28.5	1	7.1	35.6
Total	96	36	34	35.4	2	2	37.5
Statistical analysis				p=0.045 x2calculated=9.725 x2tabulated=9.488		$p = 0.3616$ $x2calculated = 4.3425$ $x2tabulated = 9.488$	$p = 0.8998$ $x2calculated = 1.0642$ $x2tabulated = 9.488$
				significant differences p<0.05		Non-significant differences $p > 0.05$	Non-significant differences $p > 0.05$

The results also showed observed variation in infection rates . This study does not agree with Al-Warid (2010) in Baghdad who found that the maximum positive infection rate was seen in two months, May and July 2009 which was (25.7%) and (19.35%) respectively. The reasons high infection of intestinal parasites in the summer months may be due to the availability of appropriate conditions of temperature and humidity and the nature of the climate in Iraq, which is hot in summer and mild during most of winter period.

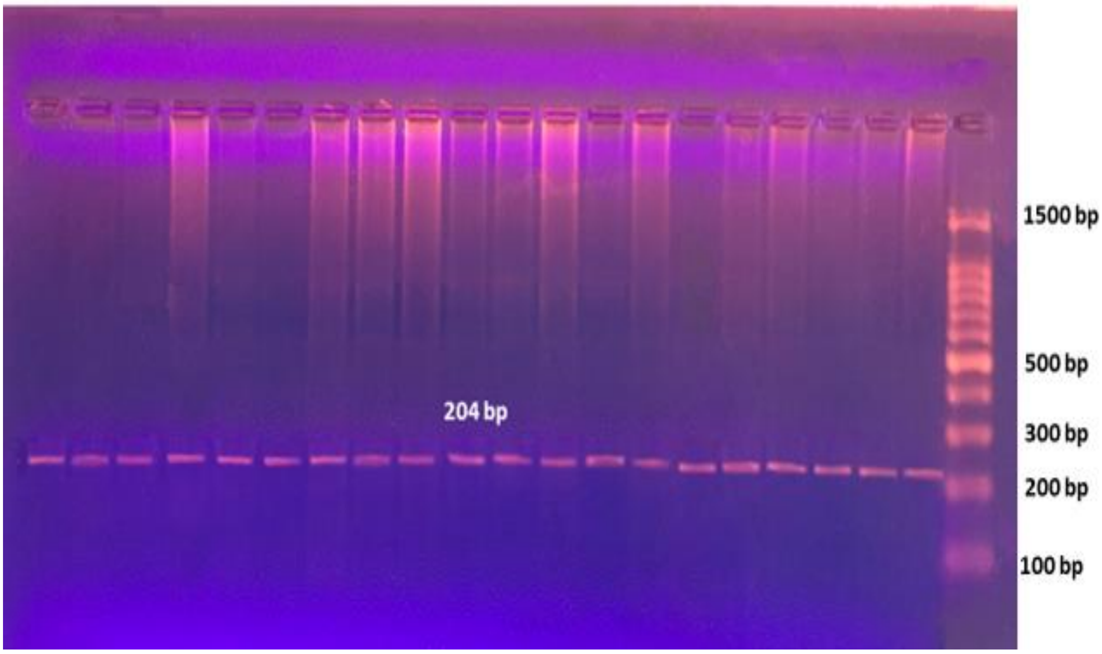


Figure (4-1): Agarose gel electrophoresis image that show the PCR product analysis of 18s_RNA gene from genomic DNA of human stool samples. Where M: Marker (100-1500bp), lina (1-20) positive samples for *E.histolytica* at 204 bp .

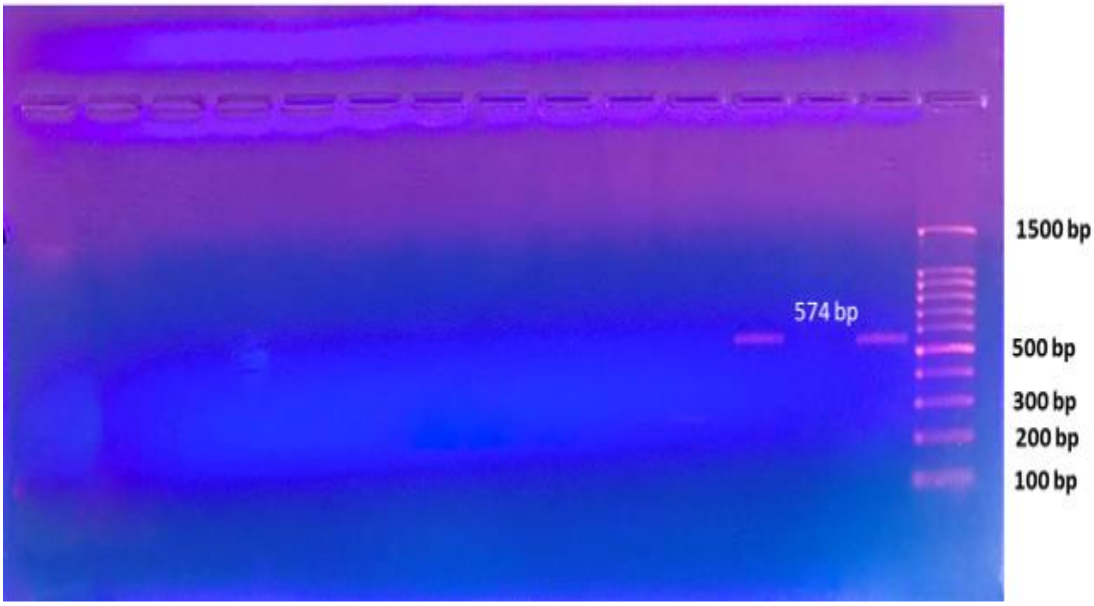


Figure (4-2): Agarose gel electrophoresis image showed the PCR product analysis of 18s_RNA gene from genomic DNA of human stool samples. Where M: Marker (100-1500bp), lina (1-2) positive samples for *G.lamblia* at 574 bp .

4.2.1. Molecular Study of Bacteria *E.coli* :

4.2.1.1. Percentage of Infection bacterial according to residence area by using PCR technique :

The present study showed difference in the rates of infection with bacterial infections, according to the residential area, the highest rate of infection bacteria was in the rural area (28 %) while the low rate of infection in the town urban area (23.91%) .

Table (4-14): Prevalence of infection bacterial according to residence area .

Residence Area	Examind No.	Infected No.	Bacteria that cause diarrhea		Total percentages of infection (%)
			Escherichia coli		
			Infec No.	(%)	
Urban area	46	11	11	23.91	23.91
Rural area	50	14	14	28	28
Total	96	25	25	26.04	26.04
Statistical analysis					$p = 0.6485$ $x2calculated = 0.2077$ $x2tabulated = 3.841$
					Non-significant differences $(p > 0.05)$

This study disagree with Cho, Seung-Hak, *et al*.(2006), this study recorded the highest rate in urban area (20.1%), while the lowest rate was recorded in rural area (12.8%).

4.2.1.2. Percentage of infection bacterial according to gender by using PCR technique :

This table show the highest rates of infections *E.coli* were in the females (31.7%) and the lowest rates of infections were in males (21.81%).

Table (4-15): Prevalence of infection bacterial according to gender.

The sex	Examind No.	Infected No.	Bacteria that causes diarrhea		Total percentage of infection (%)
			Escherichia coli		
			Infec. No.	**(%)**	
Males	55	12	12	21.81	21.81
Females	41	13	13	31.7	31.7
Total	96	25	25	26.04	26.04
Statistical analysis					$p = 0.2747$ $x2calculated = 1.1927$ $x2tabulated = 3.841$
					Non-significant differences ($p > 0.05$)

This study disagree with Mitra *et al* (2011), where the highest rate of females was recorded (58.82%) , and the rate of males was recorded (41.18%) . And this study agree with Shen, Hongwei, *et al*.(2016), the highest rate was recorded in females (56.1%),and the lowest rate was recorded in males (43.9%).

4.2.1.3. Percentage of infection bacterial according to age groups using PCR technique:

Table (16) shows the bacterial infections in different age groups from age-less than one year to age 21 and over . The highest rate of infection bacteria (35.2%) was in the age group (6-10) , and the lowest rate of infection bacteria (10%) was in the age group (21 and more). While in the other age groups (less than one year , 1-5 , 11-15 , 16-20) the following percentages were recorded (26.6% , 25% , 28.5% , 16.6%)respectively.

Table (4-16): Prevalence of bacterial infection according to age groups.

The age	Examind No.	Infected No.	Bacteria that causes diarrhea		Total percentage of infection (%)
			Escherichia coli		
			Infec. No.	(%)	
Less than one year	13	4	4	30.7	30.7
1-5	30	8	8	26.66	26.66
6-10	17	6	6	35.2	35.29
11-15	14	4	4	28.5	28.57
16-20	12	2	2	16.6	16.66
21 and more	10	1	1	10	10
Total	96	25	25	26.04	26.04
Statistical analysis					$p = 0.7242$ $x2calculated = 2.842$ $x2tabulated = 11.07$
					Non-significant differences

				(p>0.05)

This study disagree with Nazek AL-Gallas, *et al*.(2007) in Tunisia, who scored the highest rate of infection bacteria *E.coli* in children (70.4%) and in the adult scored the highest rate of infection bacteria *E.coli* (16.4%). Also disagree with Dutta, Subarna, *et al*.(2013),the highest rate was recorded in 21 and more (41.93%),while the lowest rate was recorded in less than one year -15 years (3.7%).

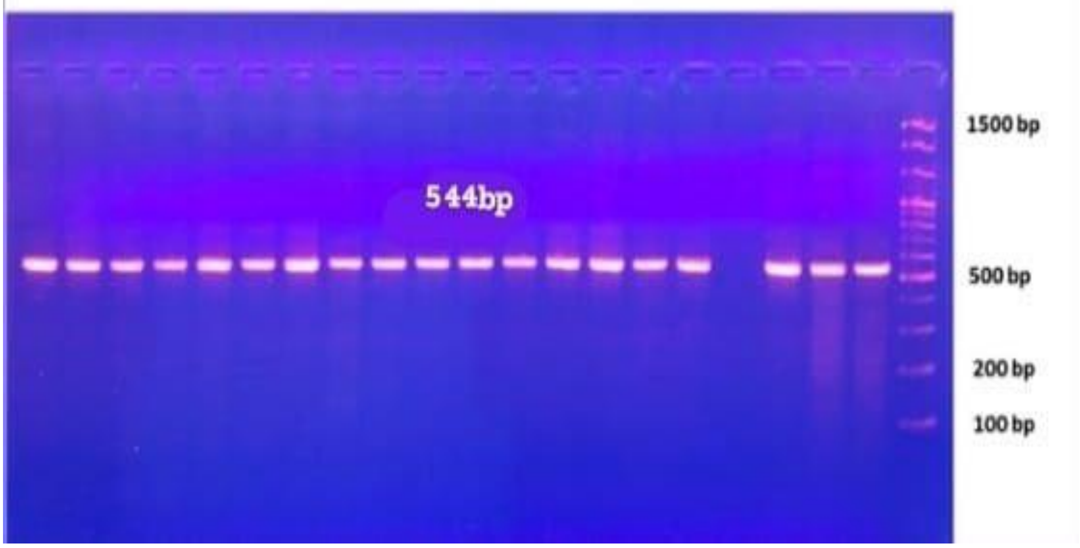


Figure (4-3): Agarose gel electrophoresis image that show the PCR product analysis of 16s_RNA gene from genomic DNA of human stool samples. Where M: Marker (100-1500bp), lina (1-19) positive samples for *E.coli* at 544bp .

Table (4-17) : type of parasite and bacterial infection quantity and rates in the Babylon province .

Type of infection	Infected No.	%	Type of parasites and bacteria	Infected No.	%
Single	43	81.13	*E.histolytica* *G.lamblia* *E.coli*	26 1 16	60.46 2.32 37.2
Double	10	18.86	*E.histolytica* + *E.coli* *G.lamblia* +*E.coli*	9 1	90 10
Triple	-	-	-	-	-
Total	53			53	

Table (4-17) shows the correlation between parasite and bacterial. some samples showed twice(parasite and *E.coli*) result where in this table some samples had parasites and bacteria in the same sample, the table above shows 10 double sample that resulted in parasite and bacteria in the same sample, where 9 sample had (*E.histolytica*, *E.coli*) and one sample had *(G.lamblia* , *E.coli*).Table above also shows 43 single sample that resulted 26 sample (*E.histolytica*) and 1 sample (*G.lamblia*) and 16 sample (*E.coli*). The reason of that it's because of that whenever a person becomes infected with the parasite , the immunity of the infected person will decrease. The decrease in his immunity enhances the person of bacteria *E.coli* .

4.4. Sequencing Study:

4.4.1. DNA Technics Sequencing :

DNA sequencing method was done for confirmative detection and a phylogenetic relationship of parasites (*E.histolytica*, *G.lamblia*) and bacteria (*E.coli*), which is based on 18SrRNA gene for parasites and 16SrRNA gene for bacteria by analyzing Phylogenetic tree using NCBI BLASTn programs . The purified 18SrRNA and 16SrRNA gene samples of PCR products were sent to Bioneer Company in Korea for DNA sequencing using 18SrRNA and 16SrRNA forwarding primers . The results of DNA sequencing should be firstly examined to confirm the nucleotide sequences and closed relationships with others world strains, test used to confirm was through using NCBI- Blast-query nucleotide-online, it was perfect program and gave the exact results of identity percentage with other world strains and they were ranged from (83-94%) for 16 sRNA and 18sRNA gene, whereas sequencing results suggest that the 16SRNA and 18SRNA sequences are in general highly conserved, however, the prevalence of the gene was (100%) from all isolation according (Shehata, 2013).

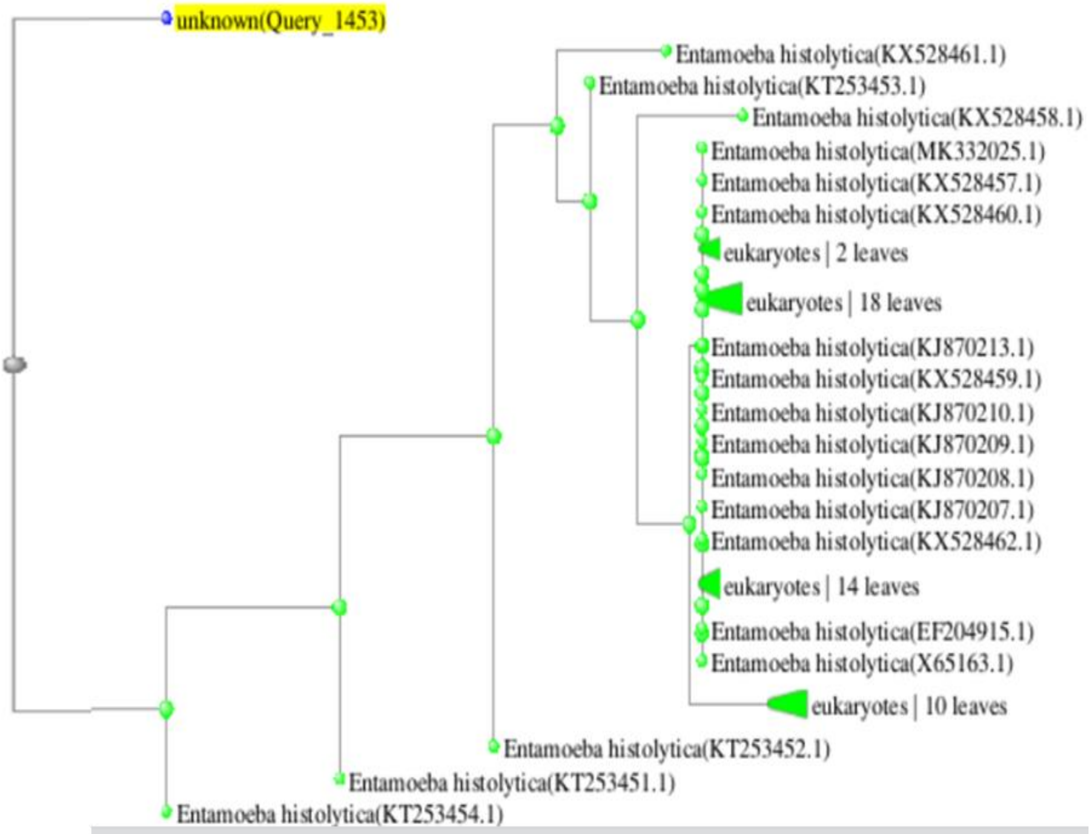
Table (4-18): Gene bank describe the accession number of the sequences used in phylogenetic tree .

Name	Genbank Accession number	Score	Gaps	Identities	countries
Escherichia coli isolate No.1	ID: CP088400.1	727 bits(805)	5/490	94%	China
Escherichia coli isolate No.2	ID: MF449423.1	718bits(795)	4/503	92%	Iran
Escherichia coli isolate No.3	ID: KC165758.1	754 bits(835)	3/496	94%	Mexico
Giardia lamblia isolate No.1	ID: U09491.1	50/bits(54)	0/37	89%	Pakistan
Giardia	ID: U09492.1	96.9bits(106)			

lamblia isolate No.2			0/83	86%	Iran
Entamoeba histolytica isolate No.1	ID: KX528462.1	130bits(143)	0/119	84%	Azerbaijan
Entamoeba histolytica isolate No.2	ID: KX528458.1	44.6 bits(48)	17/155	84%	cairo
Entamoeba histolytica isolate No.3	KX528462.1	123bits(135)	0/120	83%	Azerbaijan

4.4.2. Genetic Tree For *E.histolytica* :

Figure (4-9) shows the analysis of the genetic tree based on the gene sequence of the local *E.histolytica* isolates , which are used in the analysis of genetic relationships. The genetic tree was built using the maximum likelihood method and the tree method (Tamura-Nei model tree method) as the amoebic gene in isolates of local group1 and 3 was genetically identical with the Azerbaijani isolate while isolate 2 was genetically identical to Egyptian isolation.



Figure(4-4): Genetic tree of the samples isolated for *E.histolytica* the current study and its comparison with other global isolates recorded in NCBI.

4.4.3. Genetic Tree For *G.lamblia* :

Figure (4-10) shows the analysis of the genetic tree based on the gene sequence of the local *G.lamblia* isolates, which are used in the analysis of genetic relationships. The genetic tree was built using the maximum likelihood method and the tree method (Tamura-Nei model tree method) as the gene in isolates of local group 1 was genetically identical with the Pakistan isolate while isolate 2 was genetically identical to Iran isolation.

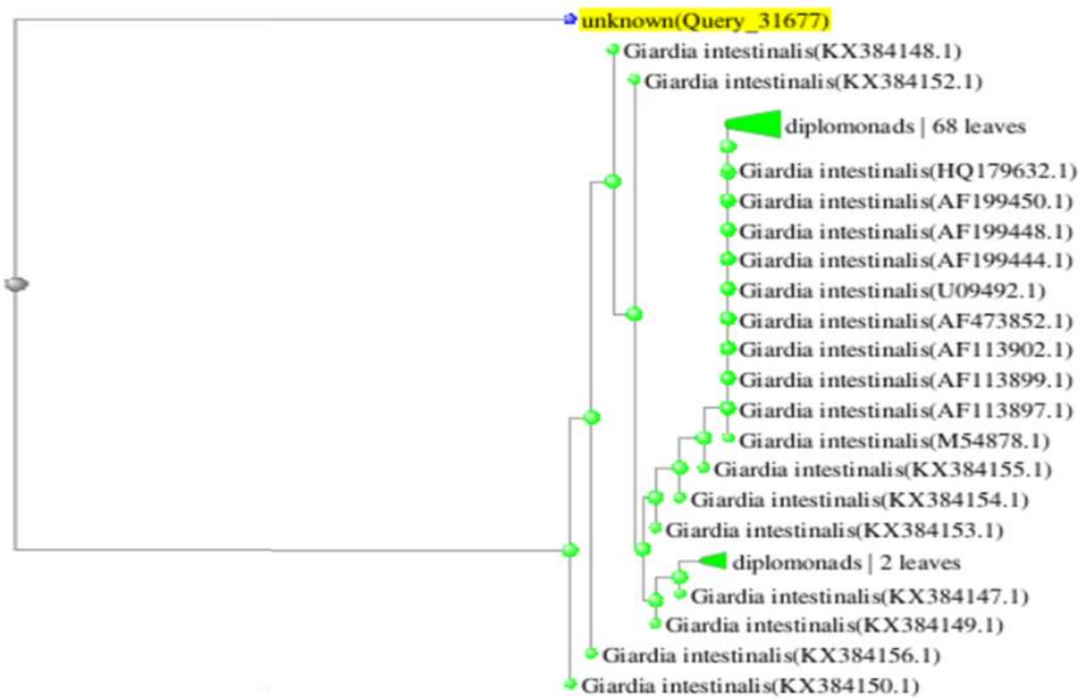


Figure (4-5): Genetic tree of the samples isolated for *G.lamblia* the current study and its comparison with other global isolates recorded in NCBI.

4.4.4. Genetic Tree For *E.coli* :

Figure (4-16) shows the analysis of the genetic tree based on the gene sequence of the local *E.coli* isolates, which are used in the analysis of genetic relationships.The genetic tree was built using the maximum likelihood method and the tree method(Tamura–Nei model tree method) as the gene in isolates of local group 1 was genetically identical with the china isolate while isolate 2 was genetically identical to Iran isolate and the isolate 3 was genetically identical to Mexico isolation .

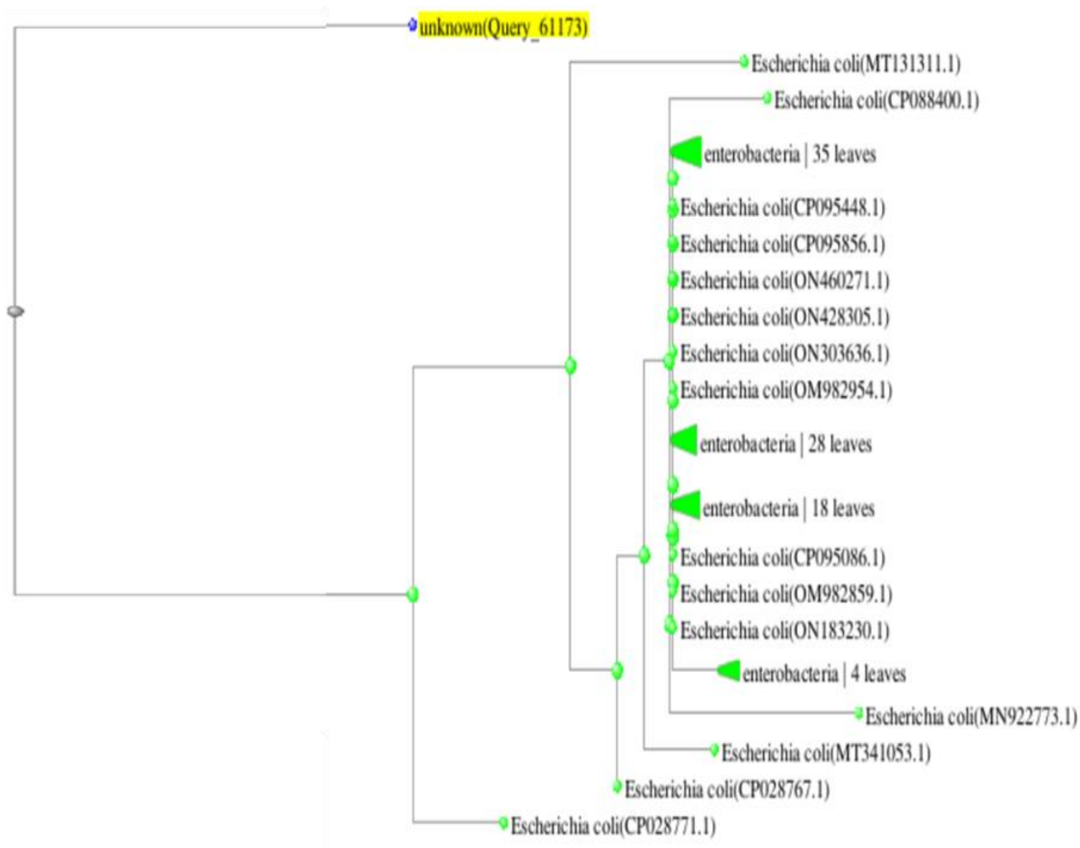


Figure (4-6):Genetic tree of the samples isolated for *E.coli* in the current study and its comparison with other global isolates recorded in NCBI.

Conclusions:

In light of the results , the study concluded :

1-There was a high prevalence of E.histolytica and E.coli and less G.lamblia in Babylon province by using microscopic examination and molecular methods.

2- There was non-significant relationship between the infection and the sex , age group ,residence area, monthly distribution and educational level, as well as the presence of animals in the house.

3- The PCR technique is more accurate than direct smear method to determine the two parasites and culture method to determine *E.coli* .

4- The *Giardia lamblia* parasites were the lowest prevalence from *Entamoeba histolytica* and its infection focusing on the little age group.

5- The single infection the highest rates when using direct smear method and culture method and PCR technique comparison with double infection.

6-The genetic sequence samples for parasites and bacteria showed that for the first and second isolates *E.histolytica*, they were identical to the Azerbaijan and Egyptian study , the third isolate was identical to the Azerbaijan study, G.lamblia parasite first isolate was identical to the Pakistan study, and the second isolate was identical for the Iran study, as for *Escherichia coli* for the first and second isolate identical to the China and Mexico study, while the third isolate was identical to the Iran study.

Recommendations:

1- Spreading health awareness about following the correct protocols to use antibiotics which must be under physician's supervision.

2- In the microscopic examination, it is necessary that the positive samples for other technologies subject to make sure you have parasites in stool samples.

3- Using another advanced technology, such as real-time –PCR to detect the parasites and *E.coli* determine the gene is that responsible for virulence factor of parasites and *E.coli* study of the important enzyme (virulence factor)that possesses diarrheal parasites

4- Study pathogenesis for these parasites and bacteria for the purpose of understanding the mechanism of the disease and the development of effective drugs against the parasite and bacteria .

5- Studies on the possibility of finding an effective vaccine against this parasites diarrhea

6- Drawing the genetic tree for all the genes of the studied and unstudied virulence factors in Iraq, identifying them and comparing them with international studies to decipher the pathogenicity codes of parasites and bacteria and their control.

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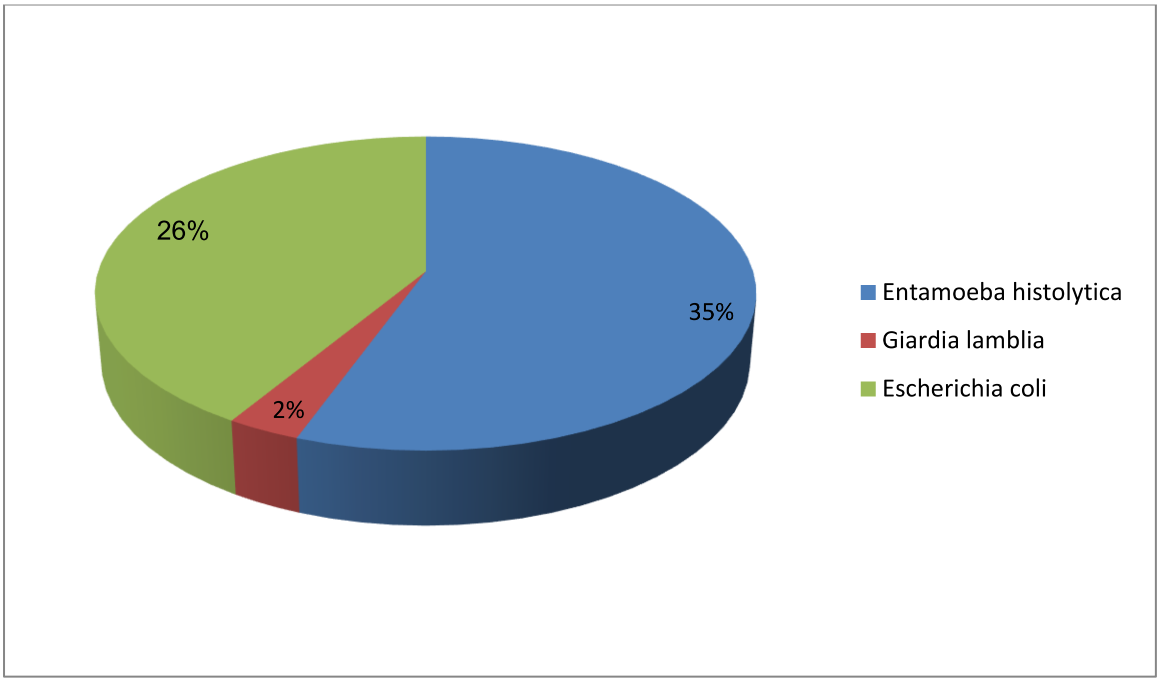
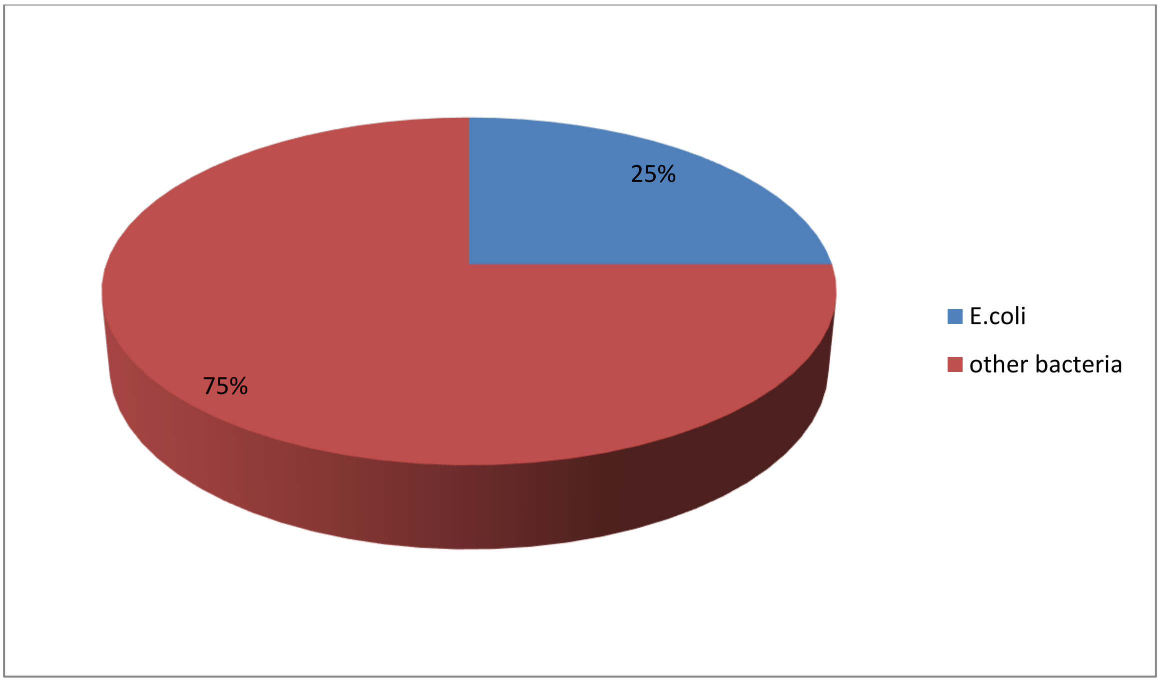
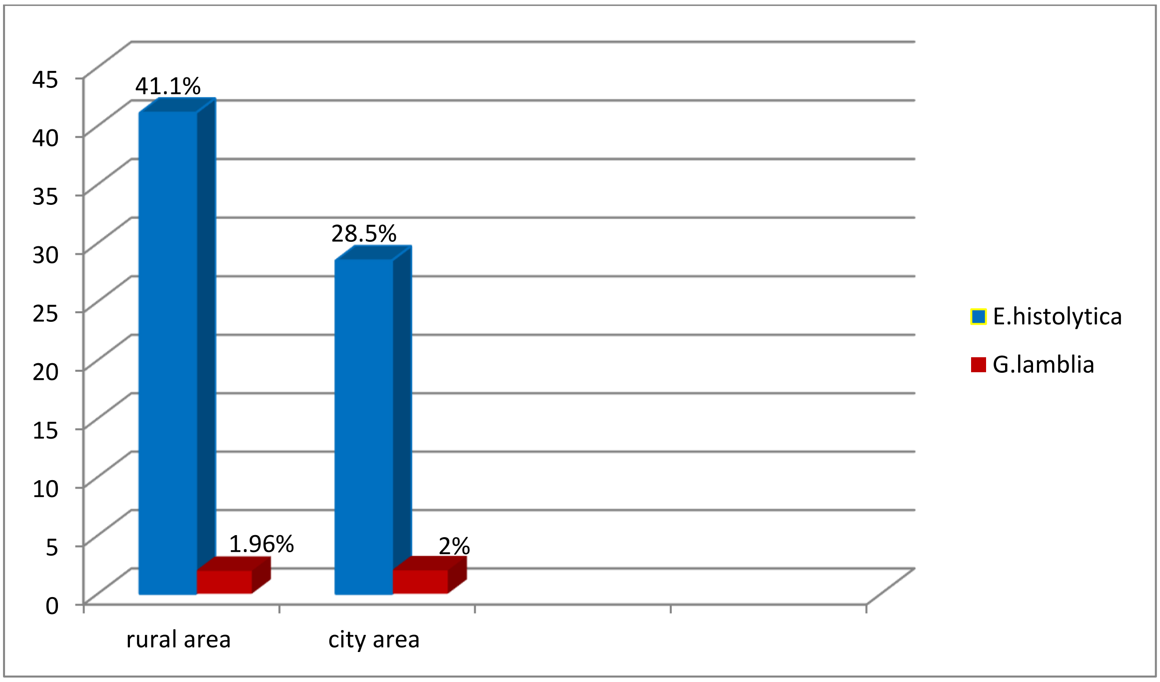
Appendix (1): Total percentage of infection for parasites and bacteria that cause diarrhea in the present study



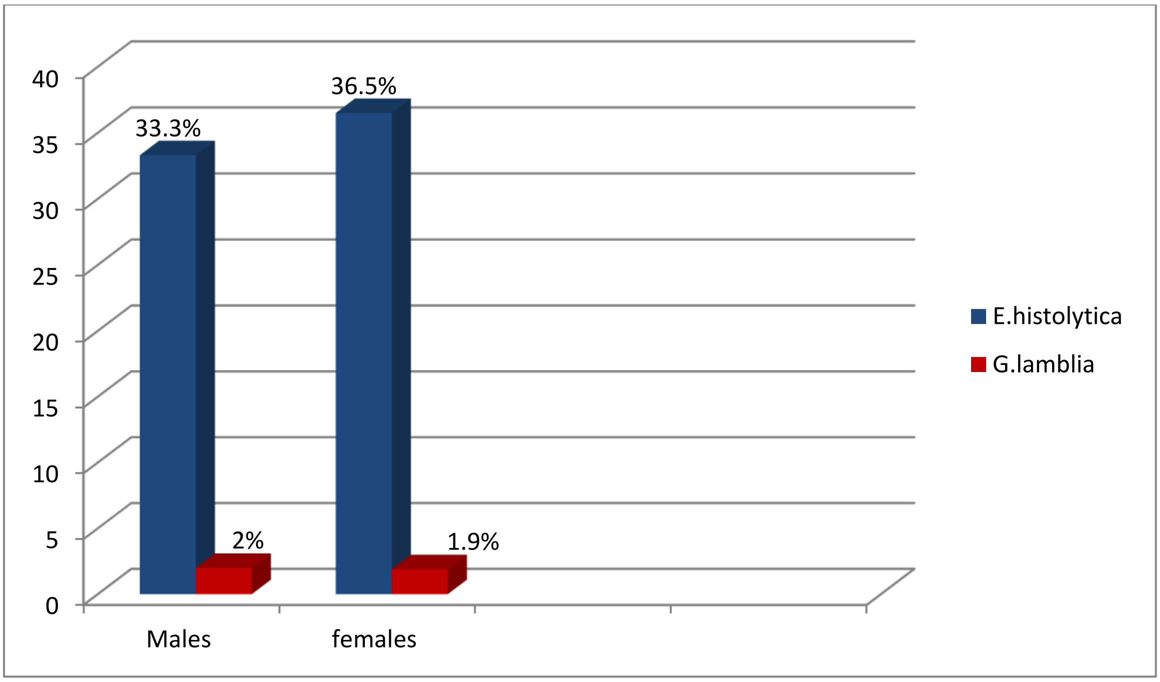
Appendix (2): Frequency of E. *coli* and other bacteria in clinical samples.



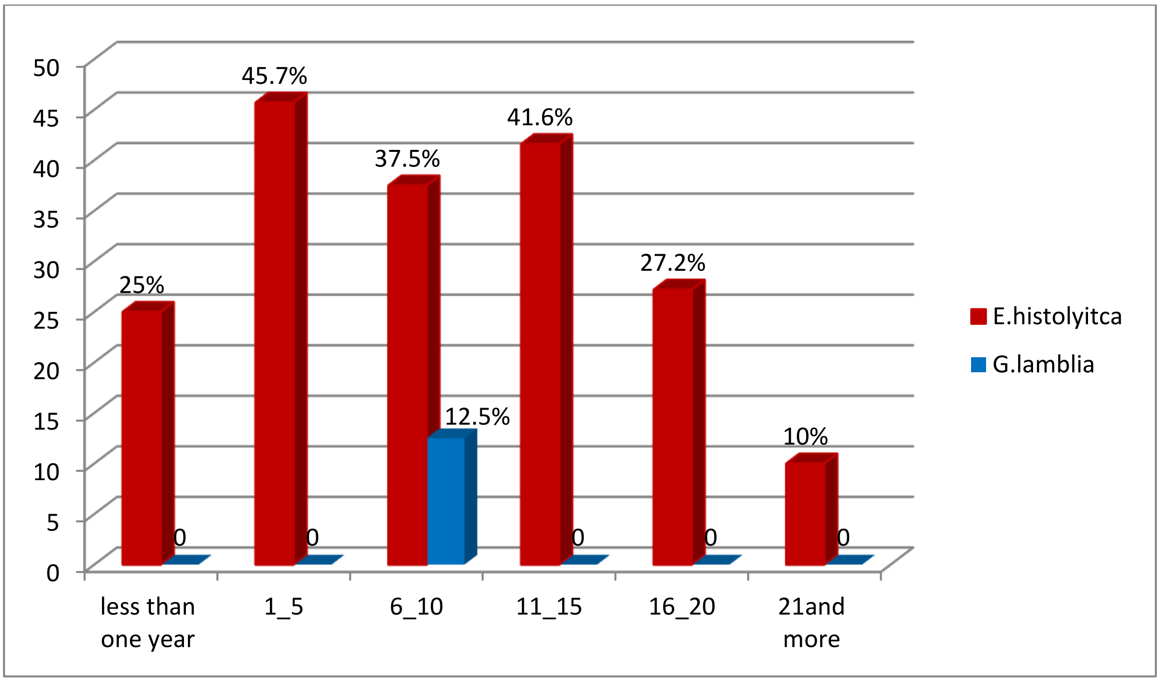
Appendix (3): Percentage of Infection for parasites that cause diarrhea according to residence area by using direct smear method.



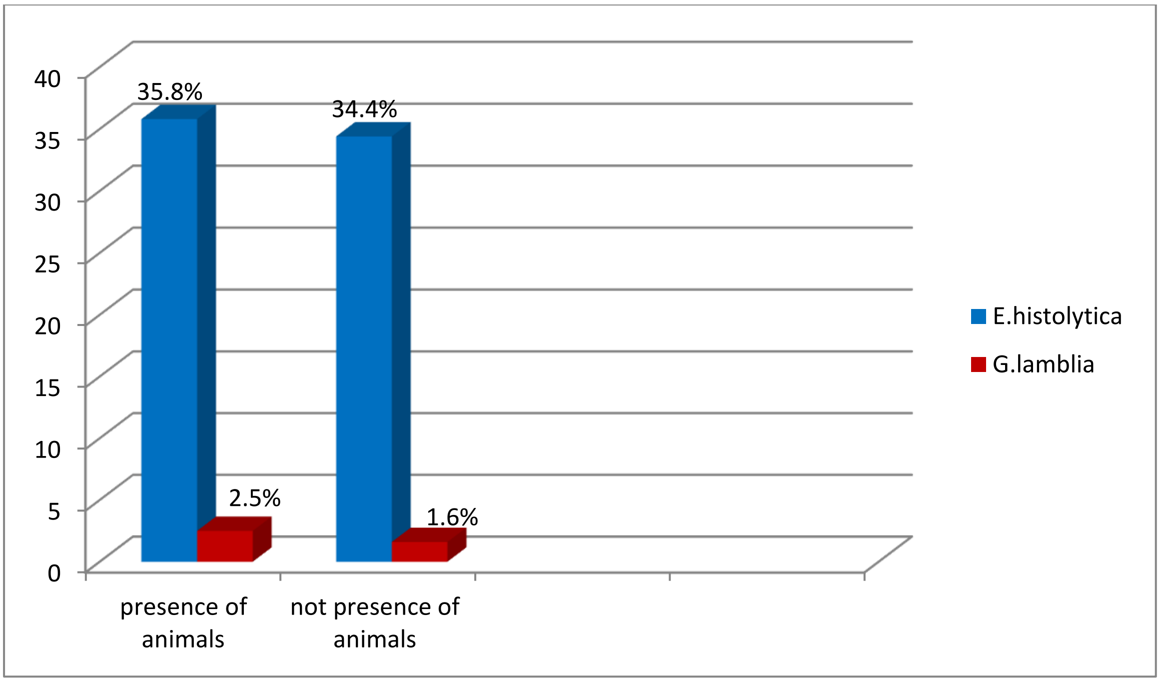
Appendix (4): Percentage of Infection for parasites that cause diarrhea according to the sex of the patient by using direct smear method



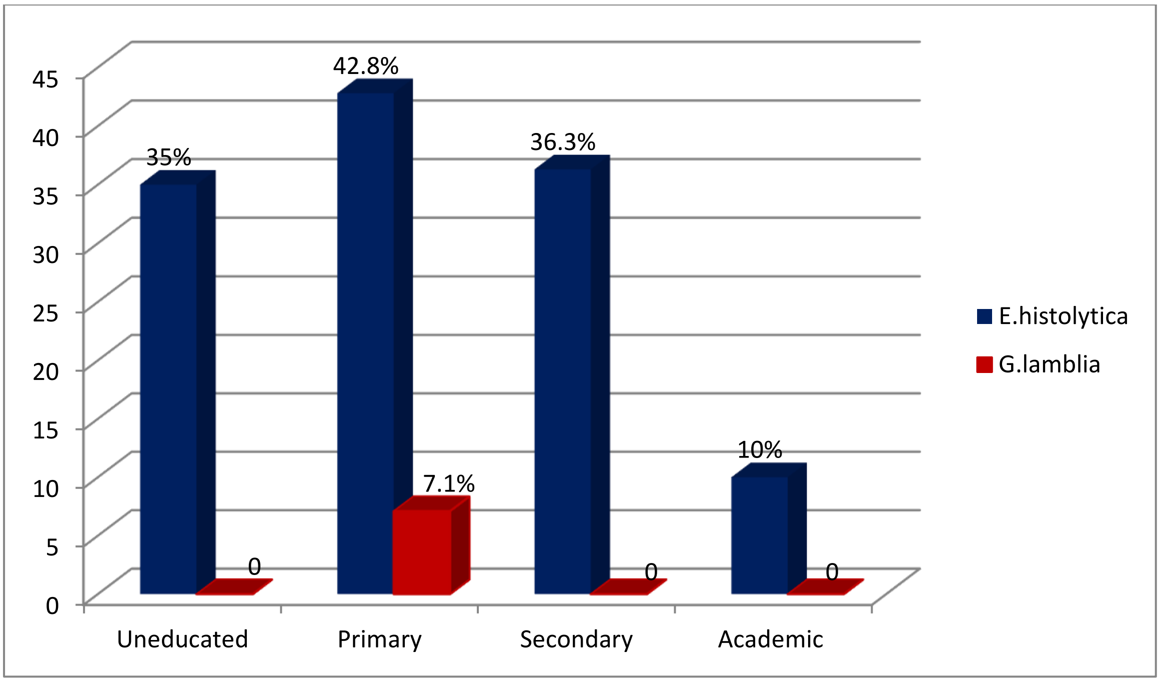
Appendix (5): Percentage of Infection for parasites that cause diarrhea according to age groups of patients by using direct smear method.



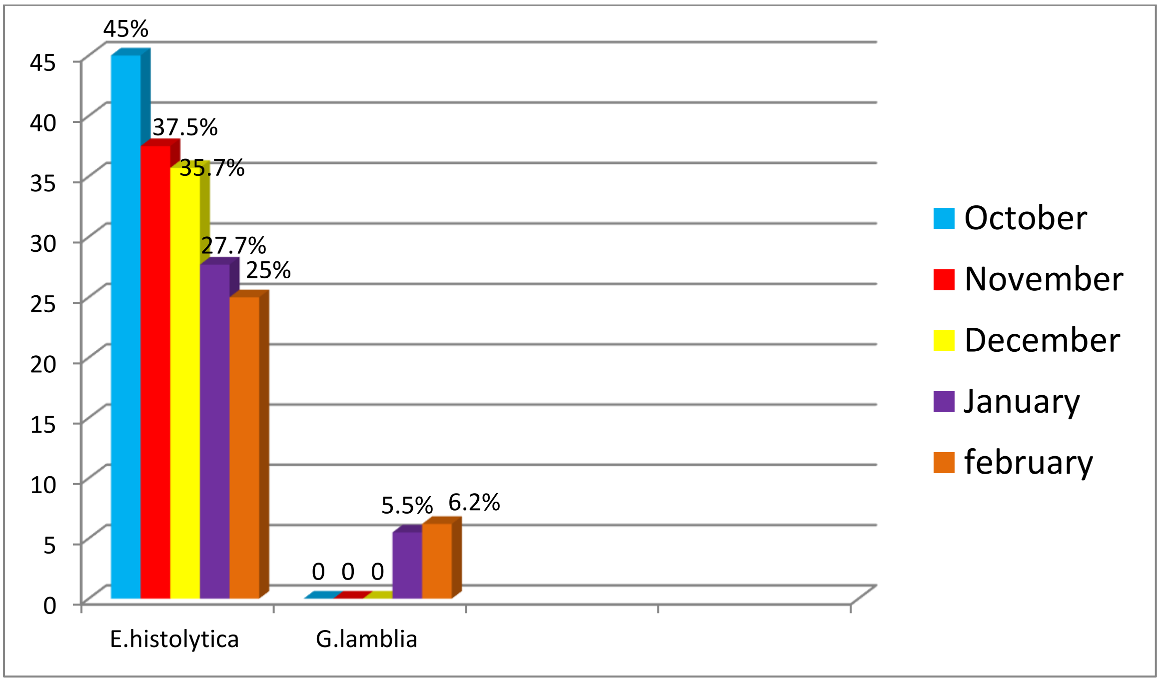
Appendix (6): Percentage of Infection for parasites that cause diarrhea according to the presence of animals in the houses or not by using direct smear method.



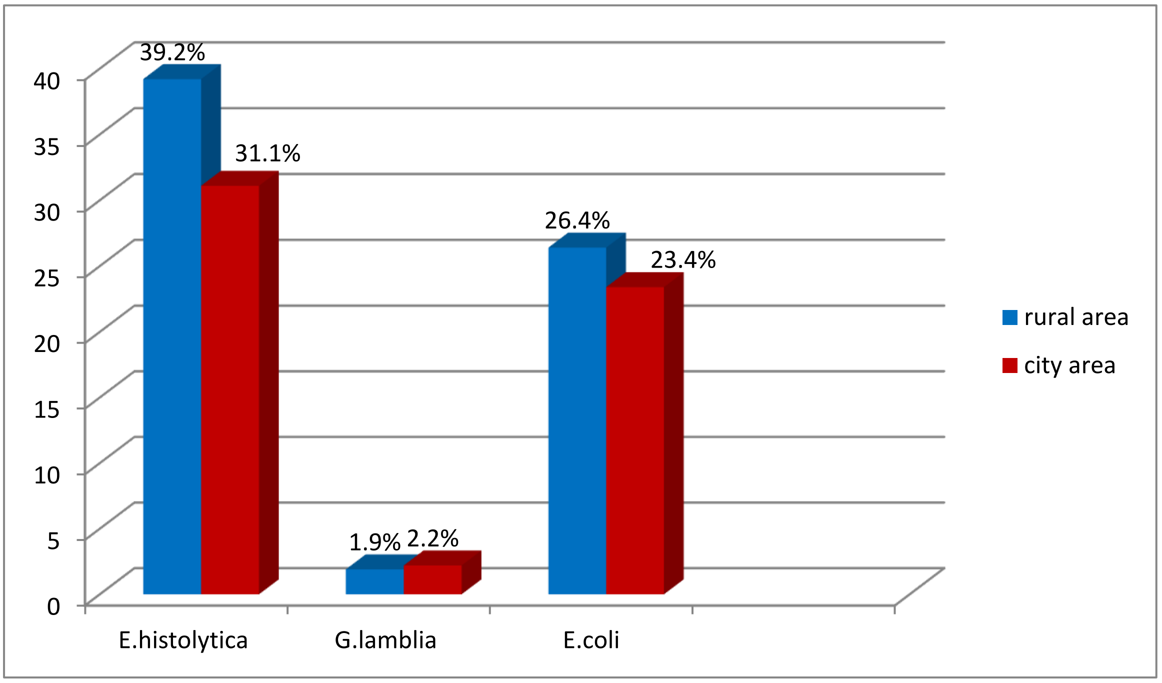
Appendix (7): Percentage of Infection for parasites that cause diarrhea according to educational level of patient by using direct smear method.



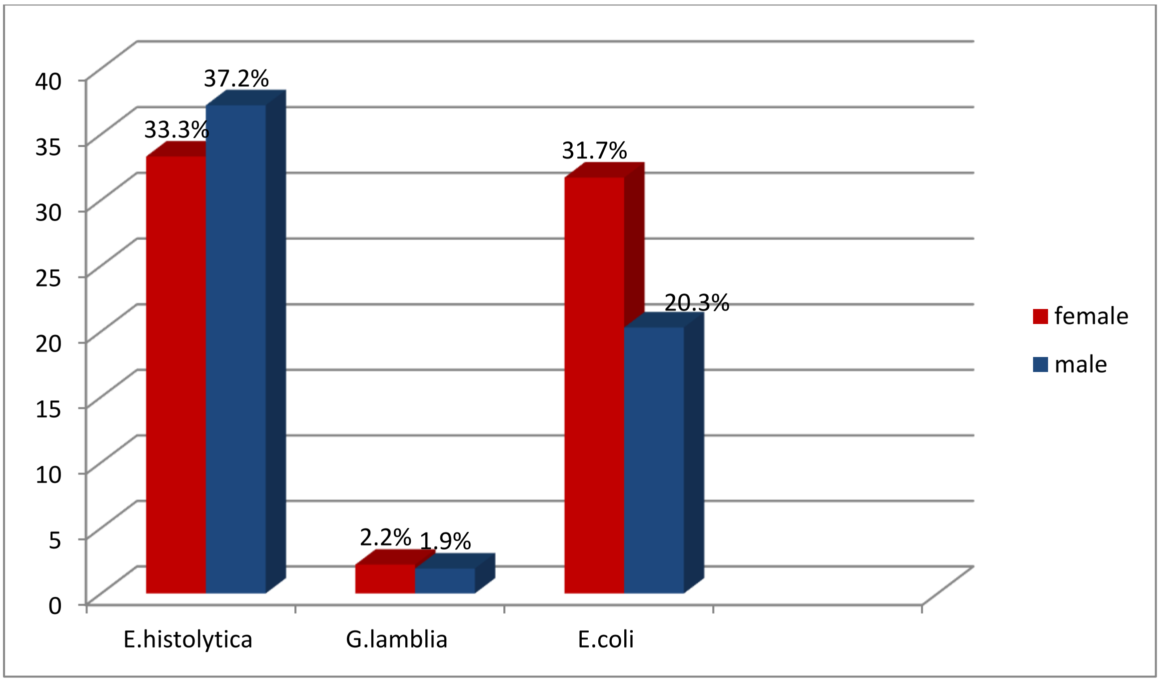
Appendix (8): Percentage of Infection for parasites that cause diarrhea according to the collecting sample months by using direct smear method .



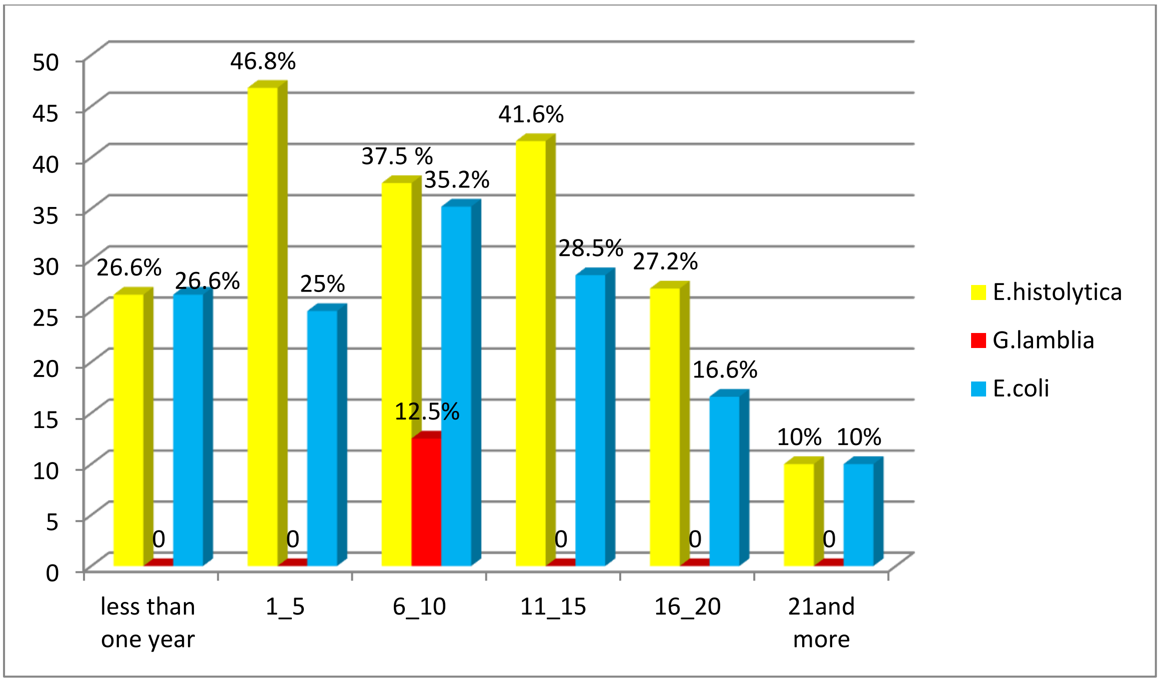
Appendix (9): Percentage of Infection for parasites and bacteria that cause diarrhea according to the residence area by using pcr technique.



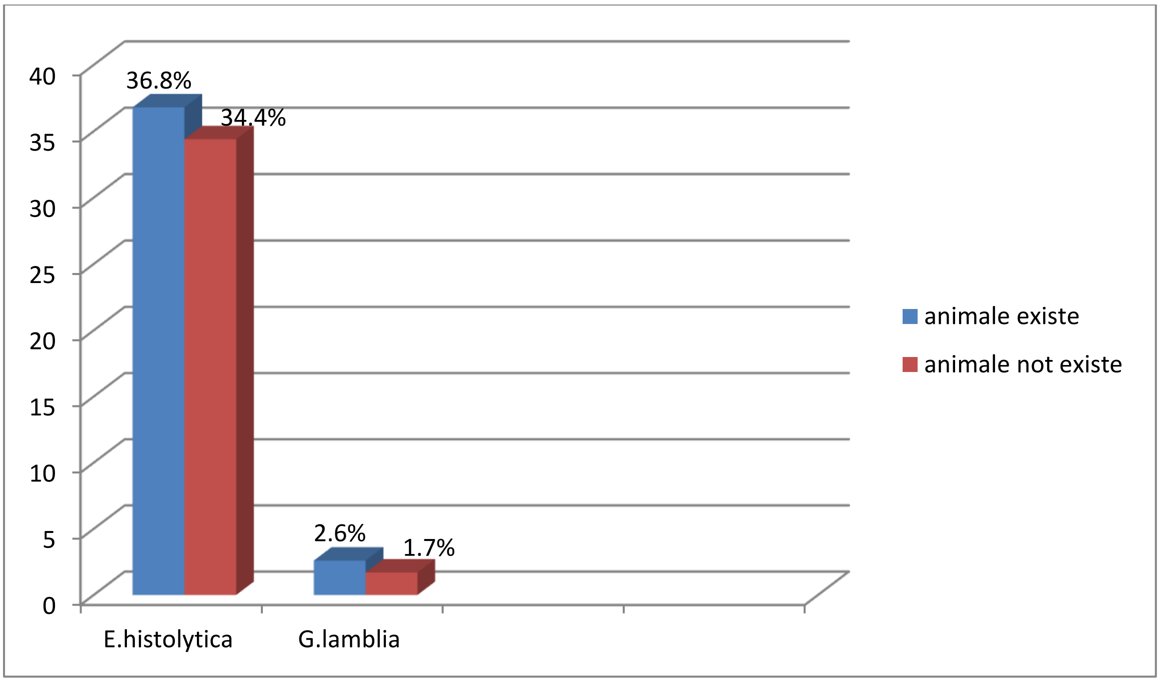
Appendix (10): Percentage of Infection for parasites and bacteria that cause diarrhea according to gender of the patient by using pcr technique.



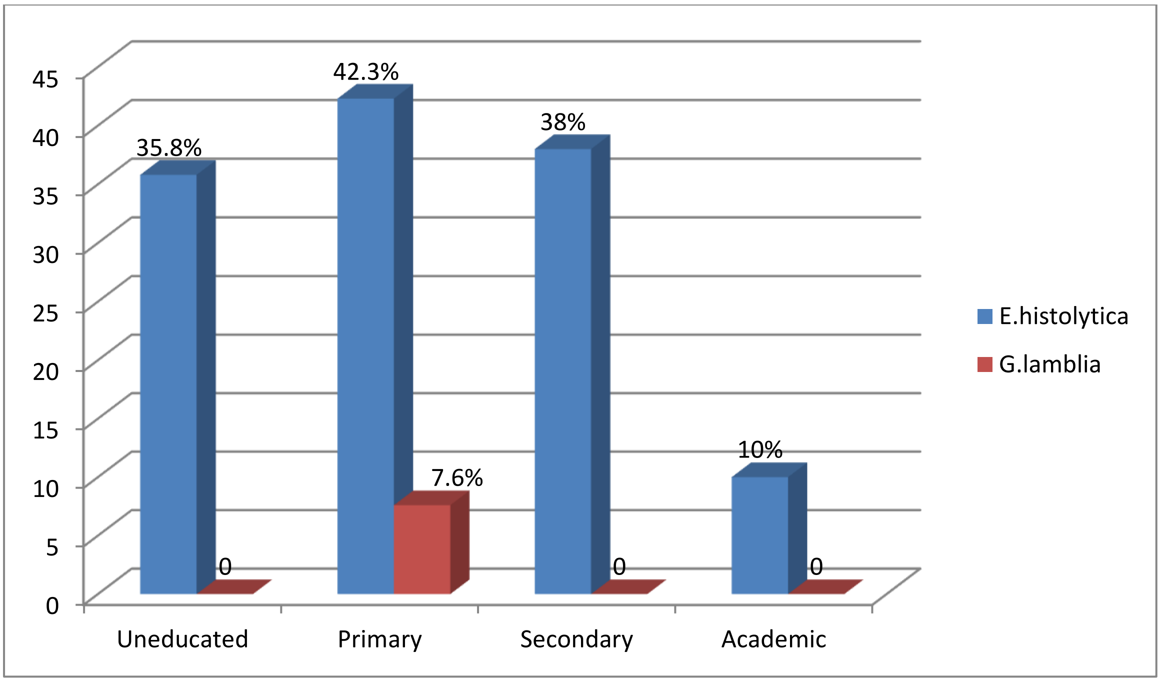
Appendix (11): Percentage of Infection for parasites and bacteria that cause diarrhea according to age groups of patients by using pcr technique.



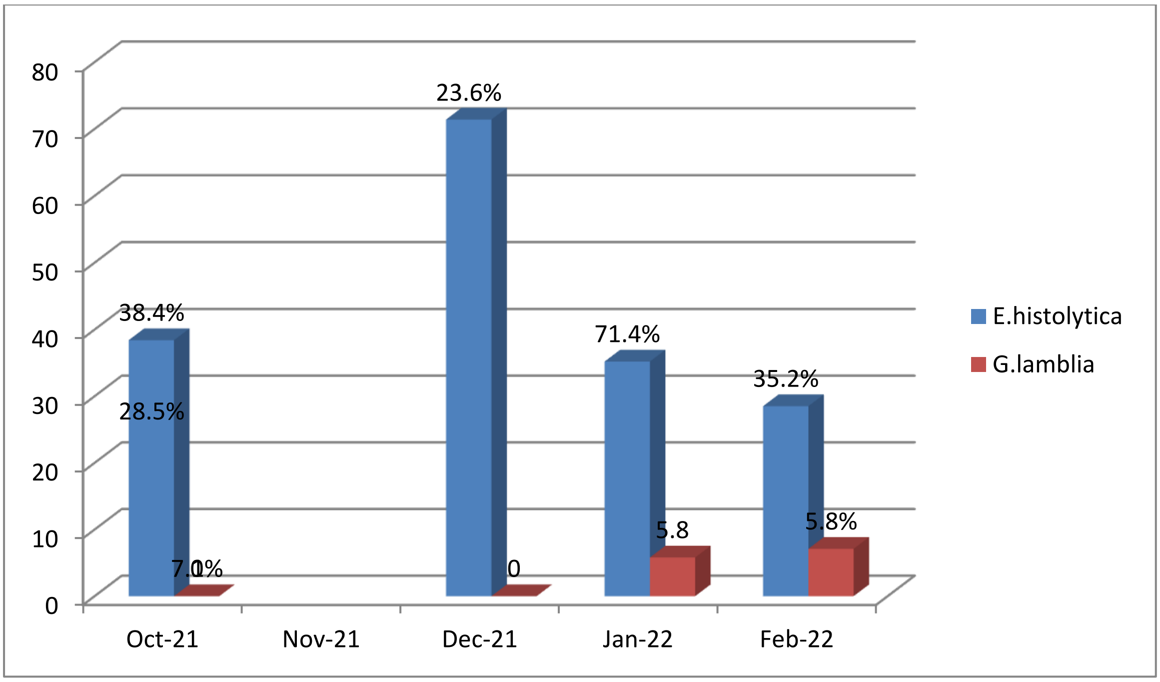
Appendix (12): Percentage of Infection for parasites that cause diarrhea according to the presence of animals in the houses or not by using pcr technique.



Appendix (13): Percentage of Infection for parasites that cause diarrhea according to educational level of patient by using pcr technique.



Appendix (14): Percentage of Infection for parasites that cause diarrhea according to collecting sample months by using pcr technique.



Appendix (15): Gathering information form.

1 – Residence area

2 – Sex

3 – Age groups

4 – The presence of animals in the houses or not

5 – Educational level

6 – Months

7 – Type of infection