T.R
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
BIOLOGY DEPARTMENT

PREVALENCE STUDY OF *ENTAMOEBA HISTOLYTICA*/ *DISPAR* BY MICROSCOPIC AND SEROLOGICAL DIAGNOSIS (ELISA) USING SPECIES VERIFICATION BY NESTED PCR IN NORTH IRAQ REGION

Ph.D. THESIS

PREPARED BY: Arshad Mohammad ABDULLAH
SUPERVISOR: Prof. Dr. Hasan YILMAZ

VAN-2018

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ACCEPTANCE and APPROVAL PAGE

This thesis entitled "**Prevalence study of *Entamoeba histolytica/ dispar*, by microscopic and serological diagnosis (ELISA) using species verification by Nested PCR in North Iraq region**" presented by Arshad Mohammad ABDULLAH under supervision of Prof. Dr. Hasan YILMAZ in the department of biology has been accepted as a Ph.D. thesis according to Legislations of Graduate Higher Education on 13/04/2018 with unanimity / majority of votes members of jury.

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Director of Institute

THESIS STATEMENT

I declare that all information presented in this thesis were obtained within the framework of ethical behavior and academic regulations. Moreover, I inform that I cited properly all expressions and information that does not belong to me in this thesis, which has been written according the rules given in thesis writing guide.

Arshad Mohammad ABDULLAH

ABSTRACT

PREVALENCE STUDY OF *ENTAMOEBA HISTOLYTICA*/ *DISPAR*, BY MICROSCOPIC AND SEROLOGICAL DIAGNOSIS (ELISA) USING SPECIES VERIFICATION BY NESTED PCR IN NORTH IRAQ REGION

Arshad Mohammad ABDULLAH
Ph.D. Thesis, Department of Biology
Supervisor: Prof. Dr. Hasan YILMAZ
Van 2018, 106 Pages

Amebiasis is one of the important diseases caused by *Entamoeba histolytica*, a protozoan parasite, affecting the human intestinal mucosa and other organs. The aim of this study was to determine the prevalence of *E. histolytica* and *Entamoeba dispar* in patients hospitalized in Duhok, Erbil and Sulaymaniyah by microscopy (by native-Lugol, trichrome staining), ELISA and Nested-PCR methods. It was examined stool samples from 162 hospitalized persons in this study. Firstly, the samples were examined under light microscopy using native-Lugol and trichrome staining methods. In the study, ELISA and PCR methods were used to distinguish *E. histolytica* and *E. dispar* from each other. *Entamoeba spp*. were detected in 58 (35.8%) of the 162 stool samples examined. *Entamoeba spp*. were found in 22 (33.8%), 17 (34%) and 19 (40.4%) persons in Duhok, Erbil and Süleymaniye cities, respectively. In the second stage, all stool samples were examined by ELISA, and *E. histolytica* was detected in eight (4.9%) of the stool samples. *E. histolytica* was determined in 4 of 65 persons (6.1%) in Duhok city, 2 of 50 persons (4%) in Erbil city, and 2 of 47 persons (4.2%) in Sulaimaniyah city. Then, all stool specimens were examined by Nested PCR, and *E. histolytica* in nine of the samples (5.5%), *E. dispar* in 37 (22.8%) and mixed infection in three (1,8%) (*E. histolytica* and *E. dispar* together) were detected. In Duhok City, *E. histolyca* was found in five patients (7.6%), *E. dispar* in 14 patients (21.5%), and mix infection (*E. histolyca* and *E. dispar*) in two patients (3%) by PCR. In Erbil City, *E. histolica* was found in two patients (4%), *E. dispar* in 10 patients (20%), and mix infection

(*E. histolyca* and *E. dispar*) in one patient (2%) by PCR. In Sulaimaniyah City, *E. histolyca* was found in two patients (4.2%), and *E. dispar* in 13 patients (27.6%) by PCR. In this study, it was found that the PCR method is much more sensitive than the other diagnostic methods in the detection and differentiation of *E. histolytica* and *E. dispar*, and it is also a very useful and easy method of ELISA for the detection of *E. histolytica* in stool.

Keywords: *E. histolytica*, *E. dispar*, ELISA, Microscopy, North Iraq, PCR

ÖZET

KUZEY IRAK BÖLGESİNDE *ENTAMOEBA HISTOLYTICA* / *DISPAR* YAYGINLIĞININ MİKROSKOPİ VE ELISA İLE ARAŞTIRILMASI VE BU TÜRLERİN NESTED PCR İLE DOĞRULANMASI

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Amebiasis, protozoon bir parazit olan *Entamoeba histolytica*'nın neden olduğu ve insanın bağırsak mukozası ve diğer organlarını etkileyen önemli hastalıklardan biridir. Bu çalışmanın amacı, Duhok, Erbil ve Süleymaniye'de hastaneye yatırılan hastalarda *E. histolytica* ve *Entamoeba dispar'ın* mikroskopi (nativ-Lugol ve trikrom boyama ile), ELISA ve Nested-PCR yöntemleri kullanılarak prevalansının belirlenmesidir. Bu çalışmada, hastanelerde yatan 162 hastanın dışkı örnekleri incelendi. İlk olarak, örnekler ışık mikroskobu altında nativ-Lugol ve tricrom yöntemleri kullanılarak incelendi. *E. histolytica* ve *E. dispar'ı* birbirinden ayırt etmek için ELISA ve PCR yöntemleri kullanıldı. Dışkı örnekleri incelenen 162 hastanın 58'inde (%35.8) *Entamoeba spp*. saptandı. *Entamoeba spp*., Duhok, Erbil ve Süleymaniye şehirlerinde sırasıyla 22 (%33.8), 17 (%34) ve 19 (%40.4) kişide saptandı. İkinci aşamada bütün dışkı örnekleri ELISA ile incelendi ve toplam olarak sekiz (% 4.9) dışkı örneğinde *E. histolytica* saptandı. Duhok'ta 65 kişinin dördünde (6,15), Erbil'de 50 kişinin ikisinde (%4) ve Süleymaniye'de 47 kişinin ikisinde (%4.2) *E. histolytica* belirlendi. Daha sonra, tüm dışkı örnekleri Nested PCR ile incelendi ve örneklerin dokuzunda (%5.5) *E. histolytica*, 37'sinde (%22.8) *E. dispar* ve üçünde (%1.8) miks enfeksiyon (*E. histolytica* ve *E. dispar* birlikte) saptandı. Duhok şehrinde Nested PCR yöntemi ile beş hastada (%7.6) *E. histolyca*, 14 hastada (%21.5) *E. dispar* ve iki hastada (%3) miks enfeksiyon (*E. histolytica* ve *E. dispar* birlikte) saptandı. Erbil şehrinde Nested PCR yöntemi ile iki hastada (%4) *E. histolytica*, 10 hastada (%20) *E. dispar* ve bir hastada

(%2) miks enfeksiyon (*E. histolyca* ve *E. dispar*) saptandı. Süleymaniye şehrinde Nested PCR yöntemi ile, iki hastada (%4.2) *E. histolyca* ve 13 hastada (%27.6) *E. dispar* bulundu. Çalışmada, Nested PCR yönteminin *E. histolytica* ve *E. dispar'ın* saptanması ve birbirinden ayırt edilmesinde diğer tanı yöntemlerinden çok daha duyarlı olduğu ve ayrıca ELISA yönteminin *E. histolytica'nın* saptanması için çok kullanışlı ve kolay uygulanabilir bir yöntem olduğu gözlendi.

Anahtar Kelimeler: *Entamoeba histolytica / dispar*, ELISA, Kuzey Irak, Mikroskopi, Nested PCR.

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TABLE OF CONTENTS

Pages

ABSTRACT.. i

ÖZET... iii

ACKNOWLEDGMENT v

CONTENTS vii

LIST of TABLES xi

LIST of FIGURES x

SYMBOLS AND ABBREVIATIONS xiii

LIST OF APPENDIX.. xvii

1. INTRODUCTION 1

2. LITERATURE REVIEWS 3

2.1. History 3

2.1.1. *Entamoeba dispar* 4

2.1.2. *Entamoeba moshkovskii* 4

2.2. Taxonomy of Parasite 5

2.3. Mophology of Parasite 5

2.3.1. Trophozoite 5

2.3.2. Precyst 6

2.3.3. Cyst 7

2.4. Life Cycle 8

2.5. *E.histolytica* Pathogenesis 10

2.5.1. Pathology 10

2.5.2. Pathogenesis 12

2.5.3. Trophozoites interaction with patient intestinal flora 13

2.5.4. Trophozoite and target host cell contact and adhesion 13

2.5.5. Target host cells lysis by toxins of trophozoites 14

2.5.6. Phagocytosis process by *Entamoeba* trophozoites 15

2.6. Host Immune Response of Human to *E. histolytica* 15

Pages

2.6.1. Innate immunity ... 16
2.6.2. Adaptive immunity ... 17
2.7. Global Epidemiology of Amoebiasis ... 17
2.7.1. Iraq ... 17
2.7.2. Turkey ... 18
2.7.3. Iran ... 18
2.7.4. United States ... 19
2.7.5. Central and South America ... 19
2.7.6. England ... 19
2.7.7. Asia ... 20
2.7.8. Africa ... 21
2.8. Symptoms of Parasite ... 21
2.8.1. Asymptomatic colonization of *Entamoeba* parasite ... 22
2.8.2. Dysentery and amebic colitis ... 23
2.8.3. Extraintestinal amebiasis in human ... 24
2.9. Diagnosis Methods ... 26
2.9.1. Microscopic diagnosis ... 27
2.9.2. Culture diagnosis method ... 28
2.9.3. Isoenzyme analysis method ... 29
2.9.4. Antibody detection tests ... 29
2.9.5. Antigen detection tests ... 30
2.9.6. Immunochromatographic assays ... 31
2.9.7. Conventional PCR ... 32
2.9.8. Real-time PCR ... 33
2.10. Treatment ... 34
2.11. Prevention ... 34
3. MATERIALS AND METHODS ... 35
3.1. Materials ... 36
3.1.1. Instruments ... 36

Pages

3.1.2. Chemicals 36

3.1.3. Sample collection 38

3.2. Methods 39

3.2.1. Microscopic examination 39

3.2.2. Serologic examination 39

3.2.3. Stock solutions and buffers preparation 40

3.2.3.1. M Tris –HCl pH 8.0 40

3.2.3.2. M EDTA (0.5) pH 8.0 40

3.2.3.3. Loading buffer 40

3.2.3.4. Sodium chloride 40

3.2.3.5. Tris-EDTA buffer (TE buffer) 40

3.2.3.6. Lysis buffer 41

3.2.3.7. Tris-borate-EDTA buffer (10XTBE) 41

3.2.4. DNA extraction 41

3.2.4.1. QIAamp DNA stool kit procedure 42

3.2.4.2. Manual DNA extraction procedure 43

3.2.4.3 DNA Purity and concentration determination 44

3.2.4.4. Detection of *E. histolytica* SSUrDNA gene by PCR 45

3.2.4.5. Agarose gel electrophoresis 47

4. RESULTS 49

4.1. Microscopic Examination 50

4.2. Serological Examination 51

4.3. Molecular Examination (Nested PCR Technique) 52

5. DISCUSSION 57

6. CONCLUSIONS 63

RECOMMENDATION 65

REFERENCES 67

APPENDIX (EXTENDED TURKISH SUMMARY)........ 81

CURRICULUM VITAE 87

LIST OF TABLES

Table **Pages**

Table 2.1. Prevalence of ALA in some countries.. 21

Table 2.2. Prevalence of *Entamoeba histolytica* in some countries............................. 22

Table 3.1. QIAamp DNA stool kit contents.. 40

Table 3.2. Primers of *Entameba genus, E. histolytica* and *E. dispar*.............................44

Table 3.3. Master reaction mixture (25 ml) for PCR.. 45

Table 3.4. Nested PCR program.. 47

Table 4.1. Prevalence of *Entamoeba* in diarrheal patient according to microscopic, Serologic and Nested PCR, in Duhok, Erbil and Sulaimaniyah....................49

Table 4.2. Prevalence of *E. spp*.. infection in diarrheal patient according to age and sex in Duhok City...50

Table 4.3. Prevalence of *E. spp*. infection in diarrheal patient according to age and sex in Erbil City..52

Table 4.4. Prevalence of *E. spp*. infection in diarrheal patient according to age and sex in Sulaimaniyah City. 53

LIST OF FIGURES

Figure **Pages**

Figure 2.1. *Entamoeba histolytica* trophozoites... 6

Figure 2.2. Cyst stage of *Entamoeba histolytica*... 7

Figure 2.3. *Entamoeba histolytica* cysts.. 8

Figure 2.4. Excystation of *Entamoeba histolytica*.. 9

Figure 2.5. Life cycle of *Entamoeba histolytica*.. 10

Figure 2.6. intestinal ulcer (Flask-shaped) by *Entamoeba histolytica*..................... 12

Figure 2.7. Host Immune Response to Intestinal Amebiasis................................. 16

Figure 2.8. United Kingdom.. 20

Figure 2.9. Human amebiasis: intestinal ulcers... 24

Figure 2.10. Human amebic liver abscess... 26

Figure 3.1. General scheme of genomic Stool DNA extraction............................ 43

Figure 3.2. PCR conventional thermocycler.. 46

Figure 3.3. Agarose gel electrophoresis... 48

Figure 4.1. ELISA test for diagnosis of *Entamoeba histolytica*............................. 50

Figure 4.2. Stool samples eppendorf preparing 52

Figure 4.3. *Entamoeba histolytica* (PCR amplification in 439 bp).................. 55

Figure 4.4. *Entamoeba histolytica* (PCR amplification in 439bp.)................. 56

Figure 4.5. *Entamoeba dispar* (PCR amplification in 174 bp)......................... 56

Figure 4.6. *Entamoeba dispar* (PCR amplification in 174 bp)................................ 56

Figure 4.7. Prevalence of *Entamoeba* infections in Duhok city................................56

Figure 4.8. Prevalence of *Entamoeba* infections in Erbil city....................................57

Figure 4.9. Prevalence of *Entamoeba* infections in Sulaimaniyah city......................57

SYMBOLS AND ABBREVIATIONS

Some symbols and abbreviations used in this study are presented below, along with descriptions.

Symbols	Explanation
WHO	World Health Organization
ALA	Amoebic liver abscess
ELISA	Enzyme-linked immunosorbent assay
IHA	Indirect hemagglutination
LAT	Latex agglutination
IE	Immunoelectrophoresis,
CIE	Counterimmunoelectrophoresis
IFA	Immunofluorescence assay
PCR	Polymerase chain reaction
RBC	Red blood cell
CHO	Chinese hamster ovary
GalNAc	Galactose or N-acetyl-D-galactosamine
GlcNAc	N-acetyl-D-glucosamine
IECs	Intestinal epithelial cells
TNF-a	Tumor necrosis factor-a
IFN-c	Interferon-c
LPS	Lipopolysaccharides
ROS	Reactive oxygen species
CRD	Carbohydrate recognition domain
IgA	Immunoglobulin A
RG	Resident group
FDA	Food and Drug Administration
CDC	Centers for Disease Control

PMNs	Polymorphonuclear cells
ALP	Alkaline phosphatase
SAF	Sodium acetate- acetic acid-formalin
PVA	Polyvinyl alcohol
TPP	Triage parasite panel
DNA	Deoxyribonucleic acid
SrDNA	Small-subunit rDNA gene
SREPH	Serine-rich *E. histolytica* protein
IDN	Integrated DNA Technologies
bp.	Base pair

LIST OF APPENDIX

Pages

APPENDIX 1 EXTENDED TURKISH SUMMARY..81

APPENDIX 2. Patient Information Form……………………………………....85

APPENDIX 3. DNA sequence specification sheets of *E. histolytica* and
E. dispar (IDT) ………………………………………………..... 86

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1. INTRODUCTION

Entamoeba histolytica is an intestinal protozoan parasite, which causes human amebiasis, the disease amebiasis considered as fourth death leading causes and its very dangerous in chronical case and it is one of the important worldwide distributed protozoan parasite. This infection listed in category B priority biodefense pathogen as National Institute of Health in the United States (WHO, 1998). Reports show the infection by*E. histolytica* occur in one-tenth population of the world (Walsh, 1986), and a large number of infected persons (100,000 deaths worldwide) die each year (Diamond and Clark, 1993; Anon, 1997; Petri et al., 2000; Haque et al., 2003). This parasite is the fourth mortality causes after malaria, African trypanosomiasis and leishmaniasis and third morbidity causes after malaria and trichomoniasis (Anon, 1998). The infection of this parasite in developing countries depends on person age, sanitation, economic and cultural level (Petri, 1996). *Entamoeba* genus contains several species which are: *E. histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni*, *E. gingivalis, E. polecki* and *E. chattoni* (Kuroki et al., 1989; Chacin-Bonilla, 1992; Sargeaunt et al., 1992; Verweij et al., 2001; Clark et al., 2006). Some species of this parasite morphologically are identical under normal light microscopy, for example *E. histolytica* and *E. dispar* are morphologically similar, but *E. dispar* is not pathogen specie like as *E. histolytica* (Diamond and Clark, 1993). The parasite of *E. histolytica* lives in human intestines and the multiplication of this parasite takes place in the human gut and produce cysts, which these cysts are exit through feces and can infect new persons by contaminated water or food (Bray, 1996). This parasite develops by simple life cycle, which the cyst stage (10 to 15 µm in diameter) of this parasite are infective stage, also there is another stage which called trophozoite stage (10 to 60 µm in diameter) that sometimes seen in infected human feces. The symptoms of amoebiasis occur in form of colonization to amoebic colitis (dysentery or diarrhea) and to form of extraintestinal amoebiasis such as liver abscesses (Fotedar et al., 2007). World Health Organization reports show that there are about 40-50 million cases of amoebic colitis and amoebic liver abscess (ALA) (WHO, 1997; Stanley, 2003; Ravdin, 2005;). Most researchers report that 90% of global prevalence of *E. histolytica* infection are asymptomatic while the 10% of cases are clinically symptomatic (Jackson *et al.,* 1985 and

Haque et al., 1999). The diagnosis of *E. histolytica* is occurring by detection of cysts or trophozoites stage in stool. The simple diagnosis does by stool examination under light microscope in forms of direct smear examination either as a wet mount or fixed by stain. Also the serological examination of this parasite is useful for diagnosis in developed countries, and in these countries individuals are constantly exposed to *E. histolytica* which used serological tests for diagnosis of this parasite (Caballero *et al*., 1994 and Ohnishi et al., 1997). Serological test in amebic infection specific in amoebic liver abscess (ALA) is very important (Zengzhu et al., 1999). The main assays for the detection of *E. histolytica* infections include: indirect hemagglutination (IHA), latex agglutination, immunoelectrophoresis, counterimmunoelectrophoresis (CIE), the amebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA) (Fotedar et al., 2007). ELISA is one of the best methods for the diagnosis of amoebiasis infections especially in epidemiologically studies in developed countries in both cases of intestinal and extraintestinal amoebiasis, ELISA is very sensitive in diagnosis of this parasite which shown about 97.9% sensitivity and 94.8% sensitive in amoebic liver abscess (ALA) patients (Hira et al., 2001). There is another technique for diagnosis of amoebiasis by using of PCR-based techniques, this technique is useful for clinical and epidemiological studies (Acuna-Soto et al., 1993; Katzwinkel et al., 1994; Calderaro et al., 2006 and Hamzah et al., 2006). Molecular technique (PCR-based methods) for diagnosis of amoebiasis is a high sensitive method and can be used to detect *E. histolytica* in stool, tissues, and liver lesion aspirates (Mirelman et al., 1997).

The aims of this study:

1. Determination of the prevalence of *E. histolytica* in Duhok, Erbil and Sulaimaniyah city,

2. Determine the best diagnostic method for detection of *Entamoeba spp*. among the microscopy, serological and molecular techniques and also determine the differentiation of pathogenic *Entamoeba spp*. from the non-pathogenic.

2. LITERATURE REVIEW

2.1. History

Entamoeba histolytica is a protozoan parasite that causes amoebic dysentery and liver abscesses. The disease is common in tropical regions of the world, where hygiene and sanitation are often substandard. The *Entamoeba* parasite has various species, the most notables are *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli* and *E. hartmanni*, which live in the human intestinal lumen. *E. histolytica* is the only species of this parasite which considered as the pathogenic parasite and infects human hosts, whil other species of this parasite are non-pathogenic in the human body (Clark and Diamond, 1991; Garcia and Bruckner, 1997). Friedrich Lösch (Russian physician) reported a case of dysentery caused by a protozoan parasite of an amoeba in 1875. His description of this parasite was as a result of the motility of amoeba, the nucleus, and ingested RBC, this amoeba now is called *Entamoeba histolytica*. Firstly, Lösch named this organism *Amoeba coli*, because this organism was found in the colon (Lesh, 1975). In 1903, the name of this organism changed to *Entamoeba histolytica* by Fritz Schaudinn, as he noted this organism had the ability to cause tissue lysis (Clark, 1998). In that years many species of this parasite that was similar to *E. histolytica*, was named in other names. In 1919, Clifford Dobell reported various species of amoeba which were similar to *E. histolytica* and that produced cysts with four nuclei (Marianne Lebbad, 2010). In 1925, Emile Brumpt reported another amoeba parasite with quadrinucleate cysts, which he named *Entamoeba dispar*. Brumpt later described this parasite as being identical to *E. histolytica*, but that it did not cause disease in humans. Brumpt detected this parasite in one of the epidemiological study of amoeba parasite in European countries, he reported his findings and hypothesis in the London Royal Society of Tropical Medicine and Hygiene meeting in 1928 (Marianne Lebbad, 2010). Walker and Sellard (in 1913), in the Philippines, reported that the amoeba was asymptomatic in some individuals but caused dysentery in others (Clark, 1998). Sargeaunt and Williams reported (in 1978) research undertaken into the field of isoenzyme electrophoresis

performed on cultured parasites, and suggested that the *E. histolytica* parasites have two groups, one group are invasive and can be considered as pathogenic and cause infection, whilst the other group is non-invasive and considered as non-pathogenic as they do not cause infection. These researchers isolated about 6000 samples of *Entamoeba* parasites which they saw that all samples were in two groups of pathogenic and non-pathogenic (Sargeaunt et al., 1978). The extensive biochemical, immunological, and genetic research undertaken in 1993 supported the existence of two morphological species of *Entamoeba*, one of which was pathogenic, and the other a non-pathogenic species of *Entamoeba* (Strachan et al., 1988; Petri et al., 1990; Tannich et al., 1991). The pathogenic parasite was found to cause dysentery, and represented an invasive form of *Entamoeba* was subsequently named *E. histolytica*, whilst the non-pathogenic *Entamoeba* was named *E. dispar* (Diamond and Clark, 1993). In 1997, four years later, the World Health Organization (WHO) accepted this classification of *Entameba*, and concluded that when diagnosis is via normal light microscopy of *Entamoeba* cysts, the cyst should be reported as *E. histolytica*/ *E. dispar* (WHO, 1997).

2.1.1. *Entamoeba dispar*

Entamoeba dispar is one of the non-pathogenic *Entamoeba spp*. that does not cause disease in humans, this species of parasite is identical to *E. histolytica* morphologically, but is not pathogenic. This difference was first described by Brumpt in 1925 though this species was later distinguished by immunological, biochemical and molecular analyses (Diamond and Clark, 1993; Stauffer and Ravdin, 2003; Tanyuksel and Petri, 2003).

2.1.2. *Entamoeba moshkovskii*

Entamoeba moshkovskii is identical to *E. histolytica* and *E. dispar* morphologically, and was first described by Tshalaia in (1941) from samples taken from sewage in Moscow (Tshalaia, 1941). This species was subsequently reported in various countries (Scaglia *et al.*, 1983;Clark and Diamond, 1991).

2.2. Taxonomy of Parasite

Classification of *Entamoeba histolytica* according to Beaver and Jung (1985):
Sub-kingdom: Protozoa
Phylum: Sarcomastigophora
Sub-phylum: Sarcodina
Super class: Rhizopoda
Class: Lobosa
Sub-class: Gymnamoebia
Order: Amoebida
Sub-order: Tubulina
Genus: *Entamoeba*
Species: *Entamoeba histolytica*

Entamoeba histolytica is an anaerobic protozoan parasite which is nonflagellate and uses pseudopodia for movement and obtain food. It is classified in the phylum Sarcomastigophora, Class: Lobosa, Order: Amoebida, Genus *Entamoeba*, where the *Entamoeba genus* contains several species but only *Entamoeba histolytica* is pathogenic and is thus considered a medically important parasite (Cavalier, 2004).

2.3. Morphology of Parasite

E. histolytica parasite has in three stages: trophozoite, precyst and cyst.

2.3.1. Trophozoite

The trophozoite form is the invasive and motile form of the parasite, is pleomorphic in shape and measures about 15-40 µm (Avila and Calderon, 1993). This form of parasite is found in the lumen and large intestine wall and is invasive form of parasite, and can be seen in fresh stools. It contains short, blunt pseudopodia, and contains a thin ectoplasm that is distanced from granular endoplasm. It is very difficult to see its nucleus in the live trophozoit form, and it can usually only be seen after

fixation and staining with iron hematoxlin. The size of nucleus is about 1/6 to 1/5 of the cell diameter and is spherical in shape. In the centre of nucleus, the karyosome and the achromatic fibrils can be seen to radiate within the inner space of the nucleus membrane, the inner surface of which contains a chromatin material in granular form which can be seen as a dark circle. Further, there are food vacuoles, and some time there are RBC in the trophozoite form of the parasite. (Avila and Calderon, 1993; Lee et al., 2000).

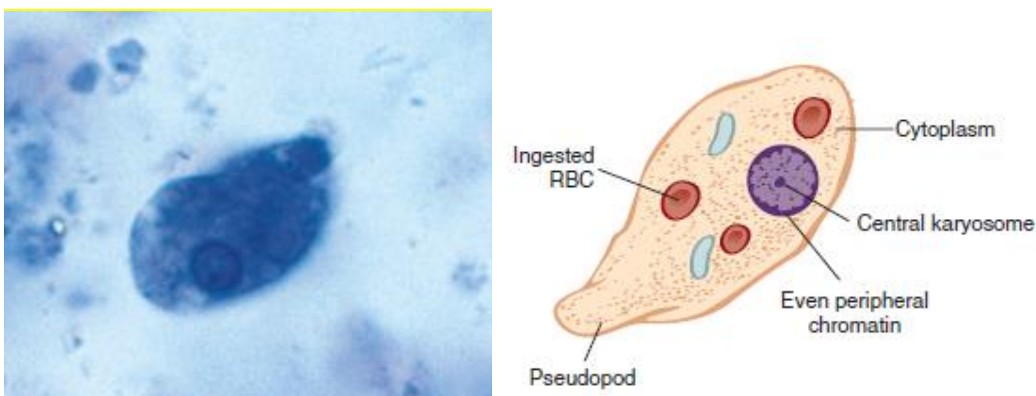

Figure 2.1. *Entamoeba histolytica* trophozoites (Mahon et al., 2011).

2.3.2. Precyst

When stool is passed from an infected individual, the stool matter dehydrates and the parasite encysts. In the stool, the trophozoite form of parasite is called precyst when it condenses to a sphere. There are a large number of glycogen vacuoles in the precyst, and the chromatoid bars are an irregular, spherical and curved shape, whereas the shape of the parasite is round, thick and short shape. The precyst forms of the parasite secretes a thin, tough hyaline cyst wall to form cysts that usually are in round shape and about 10 µm to 20 µm wide (McLaughlin, 1985).

2.3.3. Cyst

The cyst form of the parasite is the infective stage and have one to four-nuclei. The younger stage has only one nucleus, but this nucleus multiple to two and four-nuclei. Cysts become matures through a nuclear multiplication process then chromatoidal bodies and the glycogen vacuoles disappear. Some times in semi formed stools we can see the precyst and cyst forms of the parasite, and cysts might have one to four nuclei. In formed stools, we can see quadrinucleate cysts or metacysts, which is the stage that can live in outside of the human body and can infect healthy individuals. The excysting process takes place in the small intestine, where the nuclei and cytoplasm divide to form eight small metacystic trophozoites. These are identical to mature trophozoite form but are smaller in size than mature trophozoites. Cysts of the *E. histolytica* parasite can survive in a cool, moist environment for about 12 days, but can remain viable in water for about 30 days. The parasite cyst cannot survive temperatures below 5°C and above 40°C and is rapidly killed by such extremes. The cysts are resistant to chlorinated water and can still cause infection in such an environment. In the stomach, the cysts are inactive in acidic environment but when they reach the small intestine with its alkaline medium the metacyst form and move to their cyst walls and the division of quadrinucleate amoebas take place, and divide into amebulas that are swept downward into the cecum. This form of parasite firstly colonizes in host and then contact with the mucosa (McLaughlin, 1985).

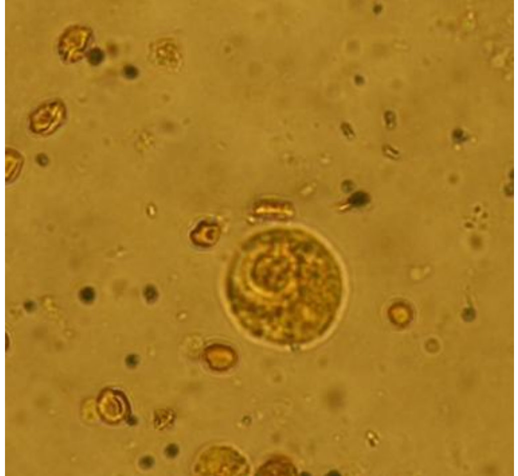

Figure 2.2. Cyst stage of *Entamoeba histolytica* (Koçman, 2012).

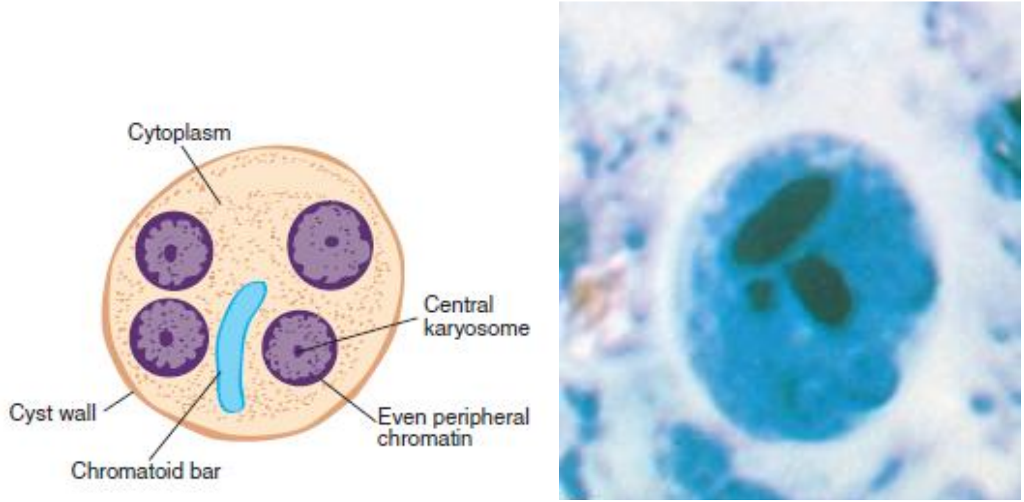

Figure 2.3. *Entamoeba histolytica* cysts (Forbes *et al.*, 2007).

2.4. Life Cycle

The life cycle of *E. histolytica* contain a motile and non-motile stage, the motile stage is trophozoit stage and non-motile stage is cyst stage (Stanley, 1996). The cyst stage of *Entamoeba* is an infective stage of this parasite; after the host has ingested the cyst and become infected with the *Entamoeba* parasite, and in this time the parasite in host body colonize in the gastrointestinal tract. The cysts size is about 9 to 25 mm, and sometimes accidently infect from infected asymptomatic individuals stool or infect from infected stool of patients with clinical signs of amoebic diarrhoea. Contaminated food and water with infected stool acts as an excellent medium for infection and transmission of the cyst from one infected host to another healthy host (Stanley, 1996). The cyst walls of *Entamoeba* parasite can resist the low pH of the host stomach and gastrointestinal enzymes (Kimura, 1997). First, in the infected host gastrointestinal tract, the cyst undergoes a number of changes, and once the tetranucleated cyst has begun to nuclear and cytoplasmic division, the excystation of the parasite occurs in the small intestine to trophozoites form. The trophozoite stage of the *Entamoeba* parasite is the motile and feeding form, which transmits to the large intestine and feeds on cellular debris and sometimes invades the colonic mucosa of the host (Ravdin, 1995). Colonic amoebic lesions are characterized by a minimal inflammatory response and extensive cellular destruction. Research studies using experimental animals infected with

Entamoeba parasite show the tissue destruction in host is not directly caused by the *Entamoeba* parasite, host tissues are destroyed by lysosomal enzymes after lysis of the tissue leukocytes and monocytes (Kimura, 1997). Trophozoites of *E. histolytica* can attach to intestinal epithelia via lectins and then release extracellular proteinase and invade the colon (Reed, 1995). Further, the trophozoite stages of *E. histolytica* can pass through bowel wall and move to another extraintestinal organs such as liver via the portal circulation and cause liver abscesses, or can infect other extraintestinal organs including the lungs and brain and the genitourinary system (Reed, 1995). The life cycle of the *E. histolytica* has two stages (cyst and trophozoit) that have an important role in the development and transmission of amoebiasis, where these stages are connected together by encystation and excystation process which these two processes are fundamental factors in differentiation of *Entamoeba* parasite (Aguilar et al., 2011). Large number of mature cysts of *Entamoeba* parasite in the large intestine of the infected host leave the host and remain viable and infective in a moist, cool environment for at least 12 days and can infect other man via contaminated food and water, and the life cycle of *Entamoeba* parasite will continue as follow (McLaughlin and Aley, 1985).

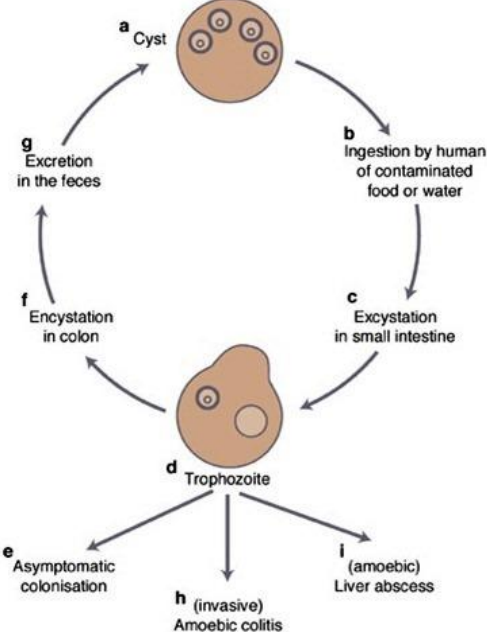

Figure 2.4. Excystation of *Entamoeba histolytica* (Huston *et al.*, 1999).

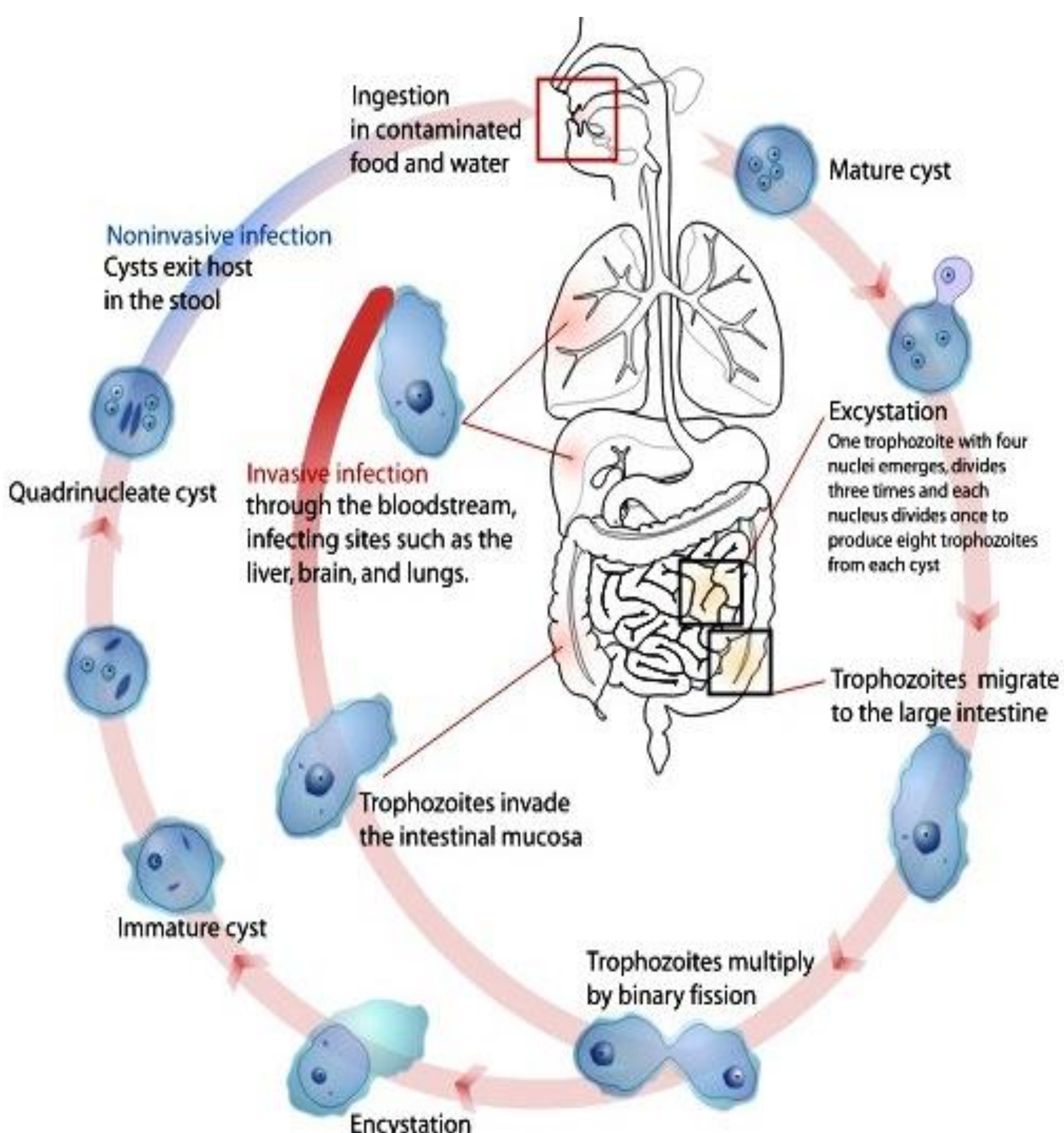

Figure 2.5. Life cycle of *Entamoeba histolytica* (Wikimedia, 2009).

2.5. Pathogenesis of *E. histolytica*

2.5.1. Pathology

Infection of human intestine by *E. histolytica* begins with luminal colonization and continues to invasion of mucosal membrane (Joyce and Ravdin 1988). Pittman and Henniger (1974) reported that the trophozoite stage of *Entamoeba* parasite is initially present in the mucus of intestinal lumen, after which the trophozoites attach to the

interglandular epithelium and are associated with mucosal microulcerations. The authors also reported non-specific colitis, with haemorrhage and oedematous mucosa are symptoms of this stage of infection. This is followed by amoeba attachment, where the epithelial cell layer disintegrates after invasion of the submucosa by *Entamoeba* trophozoites. The human inflammatory response to the *Entamoeba* parasite is poor, which may be as a result of *E. histolytica* lysing inflammatory cells (Guerrant et al., 1981; Salata et al., 1985). Following ulcer extends into the lamina propria and further into muscularis mucosa, after which perforation stops and necrotic debris begins to accumulate in the centre of the ulcerated area. At the base of the ulceration in the leading edge we can see the trophozoite stage of the *Entamoeba* (Brandt and Perez-Tamayo, 1970; Prathap and Gilman, 1970). Brandt and Perez-Tamayo (1970) discovered that the ulcers of intestine which produce by *E. histolytica* are typically "flask-shaped". Some researchers discovered that in the edges of the ulcers there are inflammatory response which involves mononuclear, neutrophils and giant cells (Brandt and Perez-Tamayo, 1970; Pittman et al., 1973). Patient mucosal ulceration is considered as invasive disease hallmark which produce by *E. histolytica*. In parts of the large intestine (ascending colon and caecum), ulceration can be extensive. In human acute colitis cases by *Entamoeba* parasite, sometimes perforations occur (about 20%) as a result of peritonitis (Brandt and Perez-Tamayo 1970). Brandt and Perez-Tamayo (1970) and Prathap and Gilman (1970) have reported that in the chronic ulceration by *E. histolytica*, the formation of a proliferative tuft can occasionally be seen, which its forms by remaining mucosa as a mass in the lumen. Further, the trophozoite of *E. histolytica* can infect the liver, causing liver abscesses as trophozoites find their way to the liver portal venules; liver abscesses can be 10 cm in diameter and are mostly produced in its right lobe. Dead cells are mostly found in the centre of the abscesses, the trophozoites can be found on their periphery of abscesses. Bacteria are conspicuous in liver abscesses because of bacterial absence, and most research was shown that 95% of patients with liver abscesses die (Brandt and Perez, 1970).

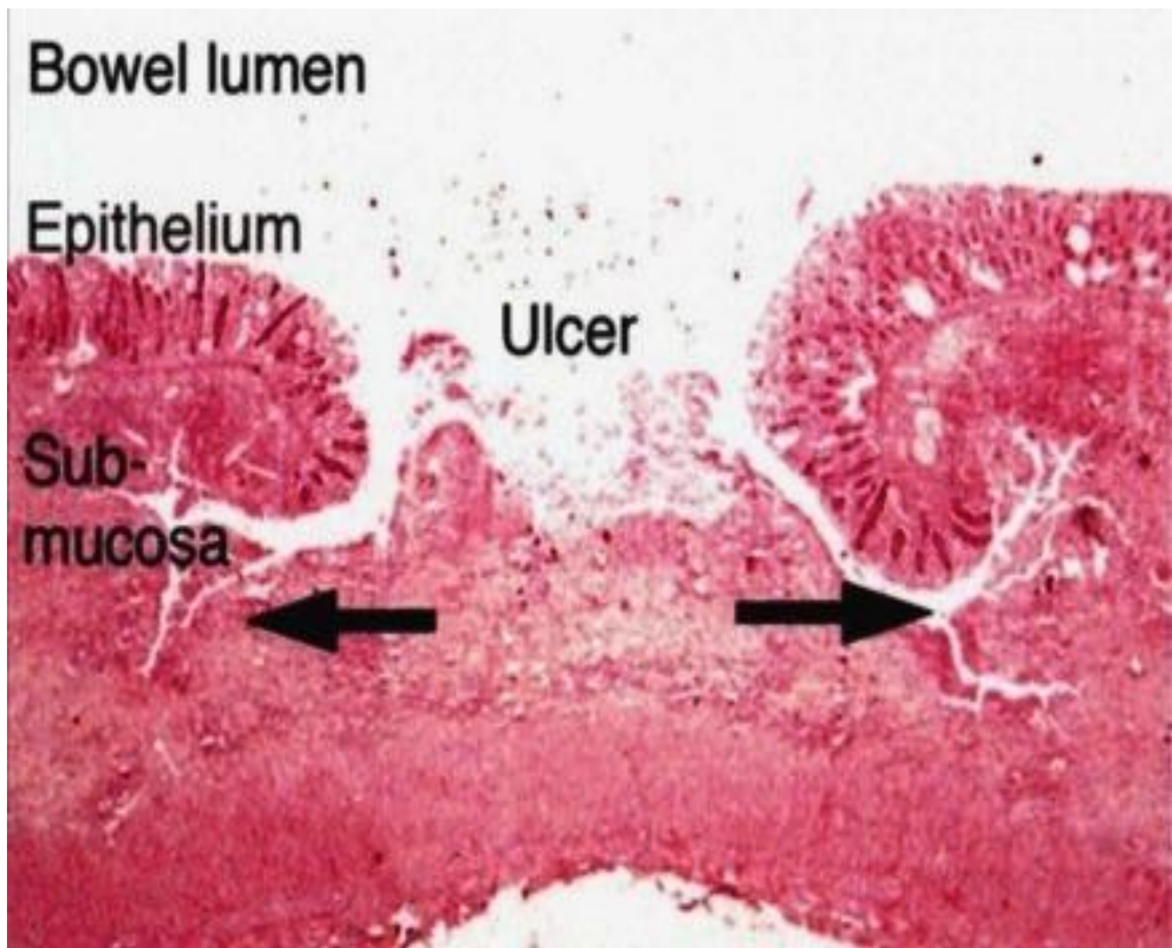

Figure 2.6. Intestinal ulcer (Flask-shaped) by *E. histolytica* (Pritt and Clark, 2008).

2.5.2. Pathogenesis

Liver lesions produce after penetration of the trophozoites stage of *E. histolytica* into liver tissue. Various steps of the pathogenesis can be show using in vitro models of pathogenesis studying (Petri and Ravdin, 1988). We can score parasite adherence by means of Chinese hamster ovary (CHO) cells, erythrocytes or bacteria. We can show the lysis by disruption percent of cell culture monolayers. We can use the ingested number of erythrocytes by trophozoite as particular measure of phagocytosis. For killing of target cells and organs by trophozoites stage of *E. histolytica* several experimental approaches can use (Petri and Ravdin, 1988). Pathogenesis is described below.

2.5.3. Trophozoites interaction with patient intestinal flora

Trophozoites stages of *E. histolytica* continually interact with intestinal flora, whereby the trophozoites undergo several changes on interacting with bacteria, as has been shown in a several research studies. Axenic *E. histolytica*, which is a non-virulent type, sometimes regain it when linked and associated with some bacteria such as *Salmonella typhosa*, *Escherichia coli* or *Salmonella paratyphi*. Non-attached and non-ingested strains of bacteria by trophozoites stages of *E. histolytica* do not affect virulence (Bracha et al., 1982). Trophozoite stages of *E. histolytica* strain 200: NIH have various levels of virulence depending on culture associates. When the trophozoites stage of *Entamoeba* is cultured with intestinal flora of rabbits or NRS bacteria, it causes acute disease in animals. Wittner and Rosenbaum (1970), in research about pathogenesis, described that an association of viable bacteria with *E. histolytica* was required to observe such virulence. Trophozoites of *E. histolytica* that connect to certain forms of live bacteria (Adhere amoeba) for 30 min and increase the virulence of parasite which shown in vivo but there is no association with *Entamoeba* invasion measurements (Bracha and Mirelman, 1984). When trophozoites were associated with a specific bacterium, this caused changes to the architecture and surface of the cells, and further changes to the properties of the cell itself (Bhattacharya et al., 1992).

2.5.4. Trophozoite and target host cell contact and adhesion

Adherence and association of trophozoite stages of *E. histolytica* to target cells is very important and necessary for cytotoxicity (Ravdin and Guerrant, 1981). The following observations demonstrate this process. It has been shown that when the trophozoites of *E. histolytica* associate and interact with target cells on a glass coverslip, the trophozoites and target cells in direct contact with the cell membrane release from the coverslip, but the cells that do not have direct contact with trophozoites are unaffected and remain viable. Ravdin and Guerrant (1981), described that when the trophozoites stages of *Entamoeba* mix with target cells in high molecular weight dextran (10%) and are incubated, the lysis process does not occur because dextran does

not allow the adherence of trophozoites and target cells. They showed that there are two cytochalasins, B and D, which inhibit the adherence of the trophozoites and target cells at 37°C as a result of amoebic microfilament function being implicated in this process. Ravdin et al. (1985) also observed that the adherence of trophozoites and target cells can be inhibited by a Ca^{2+} channel blocker, because intracellular Ca^{2+} flux is of particular importance to microfilament function. There are certain surface molecules that are responsible for adherence of trophozoites and target cells, and these molecules are inhibited by galactose or N-acetyl-D-galactosamine (GalNAc) (Bracha and Mirelman 1983; Petri et al., 1987; Ravdin and Guerrant 1981; Ravdin et al., 1985) and the other molecule is inhibited by N-acetyl-D-glucosamine (GlcNAc) polymers (Kobiler and Mirelman,1981). The adherence property of trophozoites can be inhibited by pretreatment with galactose or GalNAc, while pretreatment of trophozoites with neuraminic acid, mannose, maltose and GlcNAc does not have any apparent effect (McCoy, 1994). This molecule plays an important role in the adherence of trophozoites to target cells, which are shown in the following data: (a) association and adherence of trophozoites to target cells are inhibited by 90-95% by 50 mM galactose and GalNAc, whilst other sugars do not affect the binding process (Ravdin and Guerrant 1981; Ravdin et al., 1985; Salata et al., 1985; Salata and Ravdin 1986; Chadee, 1987) (b) defects in the production of N- and O-linked galactose-terminal oligosaccharides can be associated with the amount and number of target cells which are resistant in process of adherence; (c) Petri *et al*. (1987) have described that the complex molecules of polysaccharides which containing galactose groups at their termini were 1,000-fold more effective by weight than galactose in inhibiting adherence to CHO cells.

2.5.5. Target host cells lysis by toxins of trophozoites

The invasion of mucus membrane by trophozoit stage of *E. histolytica*, starts with the depletion and disruption of the mucous epithelial barrier. The function of microfilament amoebic trophozoit is to cytolysis the target cells, microfilament function is very important and necessary as a result of inhibition of the lysis process in cells at 25°C (37°C is the optimal temperature for cytolysis) (Pollard, 1976). Bos (1979)

proposed that there are two ways to cells being killed by the *E. histolytica* trophozoite: the first way is rapid and begins at close contact and attachment, whilst the second is slow and operates through soluble substances. Lushbaugh et al. (1978) observed that in the absence of serum, the extraction of new-grown trophozoites has a cytopathic effect on other cultured cells. Bos (1979) and Lushbaugh et al. (1979) discovered that the cytotoxic substance which extracted from trophozoit stage of *E. histolytica* caused to cell rounding. Salata and Ravdin (1986) discovered that when the trophozoit stage of *E. histolytica* contacts a cell layer, the neutrophils are lysed by trophozoits toxic products. Guerrant et al. (1981) showed that virulent amoeba has a cytolytic function on human leukocytes. Salata and Ravdin (1985) reported that in *vitro* culture, *E. histolytica* can kill the T lymphocytes and macrophages, but macrophages can be activated by concanavalin A and can kill the trophozoite stage of the amoeba parasite; also, the T lymphocytes of a strong immune system can kill *E. histolytica* trophozoites when incubated with antigen of trophozoites.

2.5.6. Phagocytosis process by *Entamoeba* trophozoites

Some particles, cells, bacteria, protozoa and erythrocytes can phagocytose trophozoites of *E. histolytica* (Trissl et al., 1978). McCaul (1977) reported that patients infected by trophozoites of *E. histolytica* contain a large number of ingested erythrocytes and reported a high rate of erythrophagocytosis as compared with healthy carriers. Phagocytosis of erythrocytes or human erythrophagocytosis by *E. histolytica* was by electron microscopy in tissue culture (McCaul, 1977).

2.6. Host Immune Response of Human to *E. histolytica*

Human contain several immune ways by which to destroy the *E. histolytica* parasite as they invade the body. The interaction of the host's immunity mechanisms and *E. histolytica* parasite can be described in the following ways (Shannon et al., 2013):

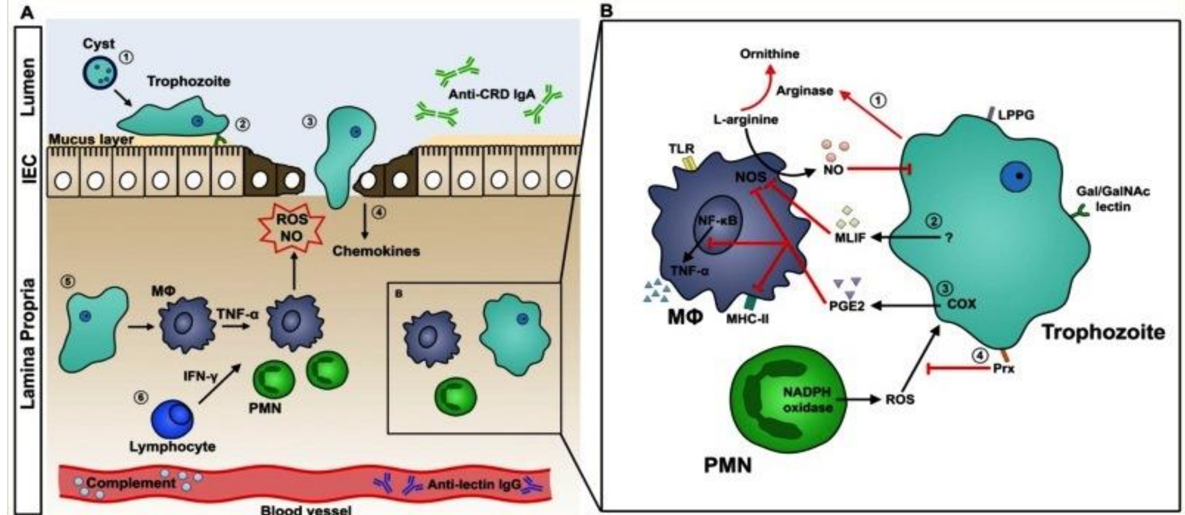

Figure 2.7. Host immune response to intestinal amebiasis (Shannon *et al.*, 2013).

2.6.1. Innate immunity

Innate immunity is the first line of defence against amoebiasis, whereas acid in the stomach is the first lien of defence against parasites; and can kill several parasites, infection with *Entamoeba* cyst is different because *Entamoeba* cyst stage has high resistance to the acidic environment in the host stomach. The mucus layer in the wall of the host intestine is the other defence in terms of the host's innate immunity against *E. histolytica* parasite and acts as a protective mechanism to prevent the invasion of intestinal epithelial cells (IECs) by *E. histolytica* parasite. Mucin is a glycoprotein produced by the submucosal glands and goblet cells which binds to the parasite and caused inhibition of Gal/GalNAc adherence lectin of the *E. histolytica* (Chadee et al., 1987). Secretion of cysteine proteases (CPs) and glycosidases by trophozoites of *Entamoeba* parasite is very important and these secretions can disrupt the intestinal mucus layer and allow the penetration of trophozoites into the colonic mucosa (Lidell et al., 2006). The first immune response cells are neutrophils, which have a special role in amoebic invasion, and become active by tumor necrosis factor-a (TNF-a), interferon-c (IFN-c) and lipopolysaccharides (LPS) which act against the amoeba parasite by producing reactive oxygen species (ROS) (Guerrant et al., 1981; Denis and Chadee, 1989). Host macrophage cells also play an important role against intestinal infection by

the *Entamoeba* parasite, these cells stimulate by tumor necrosis factor-a (TNF-a) or interferon-c (IFN-c) (Ghadirian and Denis, 1992; Lin et al., 1994).

2.6.2. Adaptive immunity

The *Entamoeba* trophozoites surface adhesion molecule (Gal/GalNAc lectin) bind to the mucosal layer of the intestine; this molecule has heavy chains and contains the carbohydrate recognition domain (CRD) that is important to the binding process (Houpt et al., 2004; Abdalla et al., 2012). There are several studies about vaccine production against amoebiasis which using IgA antibodies against Gal/GalNAc lectin is associated with protection against infection by *E. histolytica* (Houpt et al., 2004; Abd Alla et al., 2012). Haque et al (2001), in Bangladesh, observed that when mucosal IgA is directed by the carbohydrate recognition domain (CRD), the host body is protected against *E. histolytica* infection (Haque et al., 2001). Interferon gamma (IFN- y) play an important role in protection against amoeba parasites by ability to activate the macrophages and neutrophils which allow cells to kill the *Entamoeba* parasite (Haque et al., 2007).

2.7. Global Epidemiology of Amoebiasis

Amoebic dysentery is worldwide distribution but is most prevalent in tropical, temperate and developing countries where sanitation facilities are poor. Most WHO reports show that there are high percentages of infection by *E. histolytica* parasite in such countries. We can list some reports about epidemiology of this parasite as follows:

2.7.1. Iraq

Amoebic infection in Erbil city, Iraq, was reported about 18.6% (Molan and Faraj, 1989). The prevalence of amoebic dysentery among children in Pediatric Hospital of Sulaimaniyah, Iraq, was 38.13% (Ali and Mohammed, 2010). Al-Dawdi, (1998) reported the rate of infection by *E. histolytica* 10.4%. Al-Ganabi (2002) in his research

showed that the infection percentage with amoebic dysentery was 44.4% in Baghdad city of Iraq, whilst in another report, Al-Ganabi (2002) recorded 23.3% amoebiasis infection in Al-Karkh Hospital. In Duhok City, Hussein (2010) recorded about 10.15% (115 cases) of amoebiasis infection among 1132 collected stool samples of children in primary schools.

2.7.2. Turkey

Yilmaz *et al*. (1999) in Van City, Turkey, obtained different infection rates for *E. histolytica*, where their study considered two different societies with different socio-economic statuses. They found that the rate of *E. histolytica* infection was 11.6% in an immigrant group (IG) and 5.9% in the resident group (RG). Taş et al. (2005) reported the infection rate of amoebiasis through the examination of 2975 primary school children, and they were found that 1.1% of children were infected with *E. histolytica* / *E. dispar* parasites in Van City, Turkey. In Diyarbakır City, Turkey, the rate of infection with *E. histolytica* was 1.5% through the examination of 800 stool specimens, as reported by Tuba Dal and Sinan Dal (2009). Yılmaz *et al*. (2009) reported the infection rate of *E. histolytica* / *E. dispar* in Van City from the examination of 6267 samples (13 years old and under), in this research they were found 0.1% infection rate among the examined stool samples. In other research Alver *et al*. (2011) reported a prevalence rate of 29.3% with *E. histolytica* parasite which diagnosed by the serological method in Turkey.

2.7.3. Iran

In epidemiological research, Hoshyar et al. (2004) randomly examined 16592 stool samples via the molecular technique in northern, central, and southern Iran, they showed that 7.9% of stool samples were positive for *E. histolytica*. Kia et al. (2007) found that the infection by the *E. histolytica* / E. *dispar* parasite in Mazandaran Province, Iran, was 1.2%. Also, Tappeh, (2008) reported the prevalence of infection by *E. histolytica*/ *E. dispar* was 0.4% in the West Azerbaijan Province of Iran.

2.7.4. United States

Amoebic infection in United States is not endemic, and the number of infections by this parasite decreased from 4433 to 2983 cases between 1985 to 1994, and was removed from the list of notifiable diseases in 1995 (FDA, 2013). Centres for Disease Control and Protection (CDC) reported amoebic infection in US mostly found in immigrant persons and infect from travel to endemic countries, especially immigrants people from poor sanitation condition (CDC, 2013; CDC, 1994). Amoebic dysentery infection was reported in Chicago in 1933, which a large number of people were infected with *Entamoeba* parasite through contaminated drinking water, in this year was shown about 58 cases of death (FDA, 2013).

2.7.5. Central and South America

Infection with *E. histolytica* in Mexico was particularly high, with incidences of this disease at about 543.37 cases per 100,000 persons in 2007, this represented one of the major causes of disease in Mexico, and serological studies showed that up to 9% of people in Mexico City were infected with amoebic dysentery, especially children between 5-9 years of age (Caballero et al., 1994; Ximenez et al., 2009). In Brazil, the rate of infection with *Entamoeba* parasite varied according to region, from between 2.5 to 11% in the south and southeast, and 10 to 19% in the north and northeast (Benetton, 2005).

2.7.6. England

Amoebic dysentery in the England shows a relatively low infection rate, and this disease is not included among notifiable diseases; and in recent years the infection rate become decreased. In 2001, the infection number was 214 cases, and decreased to 68 cases in 2008; also, the researcher further reported that the cause of infection was by contaminated food and water from endemic countries (Nichols, 2000). Amoebic dysentery infection by the *E. histolytica* parasite in Twycross Zoo, England was 16.2%, as reported by Regan (2014).

Figure 2.8. United Kingdom (Carl *et al.*, 2014).

2.7.7. Asia

In Malaysia, the rate of infection by *E. histolytica* was reported about 21%, especially in mountainous and tropical areas and 18.5% was shown in population of aborigines, but the infection rate by this parasite was very low in Kuala Lumpur which it was about 0.4%. This variation between infection rate was shown to be due to the different levels of personal hygiene and socio-economic circumstances (Tengku and Norhayati, 2011). Ngui et al. (2012), in their research, recorded infection rate of 75% with *E. histolytica* by using molecular techniq in Peninsular Malaysia. Amoeba infection in Japan is included in the notifiable diseases list; there is a high prevalence of amoebiasis in this country, which reported increasing percentage from 377 to 747 cases between 2002 to 2006. A large number of infected people were considered to be local cases, with sexual contact being one of the modes of transmission. 90% of infections occurred in individuals between 30-60 years old from 2003 to 2006, and infection rate with this parasite was increased by 1.7 times as compared with infection rate of 1999 to 2002 (IDSC, 2007).

2.7.8. Africa

Gatharim et al. (1987) showed that 90% of individuals infected by the *Entamoeba* parasite with non-pathogenic *Entamoeba* (*E. dispar*), and only 10% of infections was by *E. histolytica*. Saeed *et al. (*2011) in their epidemiological study in Sudan, found 196 positive samples of *E. histolytica* out of 246 stools samples by using the molecular diagnostic method. During 2011- 2012 in Zawia City, Libya the infection with *E. histolytica* parasite was 3.1% (Elsaid et al., 2012).

There are large number of studies about epidemiology of amebiasis, we list some epidemiological studies bellow, in Table 2.1. and Table 2.2.

Table 2.1. Prevalence of ALA in some countries

Country	Period	No. of ALA cases	References
Vietnam	1990–1998	2031	Blessmann et al. (2002)
USA	1979–1994	56	Seeto and Rockey (1999)
Thailand	1992–2001	62	Wiwanitkit (2002)
France	1995–1999	20	Djossou et al. (2003)
Spain	1991–2001	7	Ruiz de Gopegui et al. (2004)
South Korea	1990–2005	31	Park et al. (2007)
Mexico	2000–2005	319	Valenzuela et al. (2007)
Taiwan	1994–2005	40	Hung et al. (2008)

Table 2.2. Prevalence of *Entamoeba histolytica* in some countries

Method of diagnosis	Country	Prevalence (%)	Reference
Microscopy	Korea	1.8	Lee *et al.* (2000)
	Thailand	7.1	Sirivichayakul et al. (2003)
	Lebanon	2.3	Saab *et al.* (2004)
	Iran	1.4	Hooshyar *et al*. (2004)
	Mexico	12.8	Ramos *et al.* (2005)
	Brazil	5.8	Pinheiro *et al*. (2005)
	Australia	2.9	Fotedar *et al*. (2007)
ELISA	Mexico	8.4	Salcedo *et al.* (1994)
	Bangladesh	76.0	Haque *et al.* (2006)
	Tanzania	0.8	Nesbitt *et al*. (2004)
	South Africa	18.8	Samie *et al*. (2006)
	Egypt	9	El-Kadi *et al*. (2006)
	Saudi Arabia	2.7	Barnawi *et al*. (2007)
PCR	Mexico	5.4	Ramos *et al*. (2005)
	Italy	5.6	Calderaro *et al*. (2006)
	Australia	5.6	Fotedar *et al*. (2007)
	India	3.5	Khairnar *et al*. (2007)
	Brazil	0.8	Santos *et al*. (2007)
	Iran	11.7	Hooshyar *et al*. (2004)
	Vietnam	11.2	Blessmann *et al*. (2003)

2.8. Symptoms of Parasite

2.8.1. Asymptomatic colonization of *Entamoeba* parasite

Asymptomatic infections show that the *Entamoeba* parasite lives in perfect conditions within the host (Kammanadiminti and Chadee, 2006). About 90% of individuals infected with *E. histolytica* can be considered as asymptomatic or mildly symptomatic, more studies about this parasite have been based on fecal examination by light microscope (Walsh, 1986; WHO, 1997). Some infected individuals have cysts in their feces, but there are no any symptoms, stool of these patients can contain cysts without trophozoites or ingested red blood cells (RBCs) trophozoites. These patients contain *E. histolytica* parasite and can produce antibody titers in their body without symptoms or invasive disease (Jackson et al., 1985; Gathiram and Jackson, 1987; Ravdin et al., 1990). In asymptomatic patients, with *E. histolytica* colonization without

treatment can lead to amoebic diarrhoea with highly invasive infection, but in most cases the infection resolves without the disease developing (Gathiram et al., 1987; Haque et al., 2001; Blessmann, 2002; Blessmann, 2006).

2.8.2. Dysentery and amoebic colitis

When an individual infected with *E. histolytica* parasite and after an incubation period, the parasite maybe invades the mucosa membrane of the host's colon and cause ulcerative lesion and bloody diarrhoea (Boettner et al., 2002; Mortimer and Chadee, 2010). In asymptomatic infective individuals with *E. histolytica* parasite, after one year about 4% to 10% of these individuals develop amoebic colitis or other extraintestinal disease occurs, so treatment of asymptomatic cyst carriers is necessary and important (Haque et al., 2001; Blessmann et al., 2006). Infection symptoms in patients include abdominal pain, tenderness and watery, bloody or mucous dysentery. Most individuals infected (80%) with amoebic colitis have abdominal pains and some patients only have intermittent diarrhoea. In some patients' stained stools or submucosal tissue we can see trophozoites stage. When the parasite invades and penetrates colonic mucosa, in this time feces are shown in bloody form, and further we can see Charcot-Leyden crystals and blood acute stage of amoebic infection and in amoebic dysentery, sometimes macrophages, polymorphonuclear cells (PMNs) and red blood cells can be seen microscopically and fever can be seen in 40% of symptomatic patients (Adams and MacLeod, 1977). Other symptoms in patients with amoebic colitis are bloody diarrhoea, fever, abdominal pain, peritoneal signs, extensive involvement of the colon, ameboma, toxic megacolon, rectovaginal fistulae and cutaneous amebiasis (Takahashi et al., 1997; Adams and MacLeod, 1977; Lysy et al., 1991; Mhlanga et al., 1992).

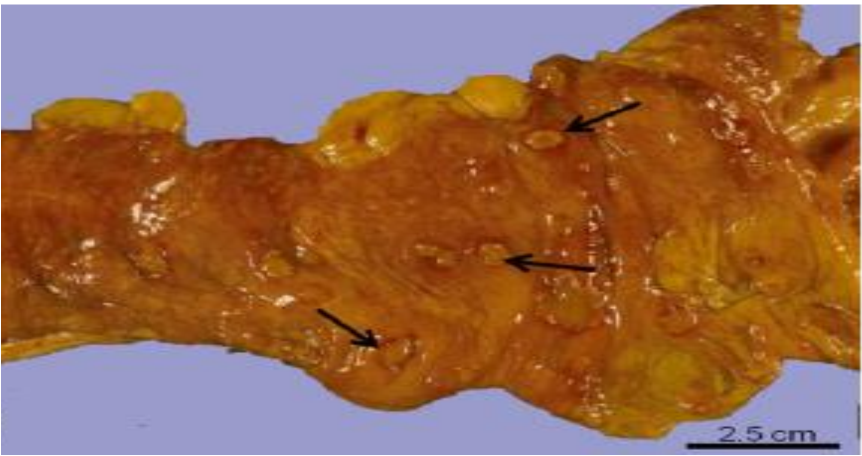

Figure 2.9. Human amebiasis: intestinal ulcers (Shibayama *et al.,*1997).

2.8.3. Extraintestinal amebiasis in human

Amoebic liver abscess (ALA) is one of the common cases of extraintestinal amebiasis in patients with high percentage of morbidity and mortality in humans extraintestinal amebiasis. This type of infection was one of the important and dangerous fatal disease during the last century, but with the development of medical science and good diagnostic methods and treatments, the mortality rate in this disease decreased to between 1 to 3% (Boonyapisit et al., 1993; Shandera et al., 1998). Amebic liver abscess (ALA) occurs in the case of trophozoite invasion from the colon to the liver by means of the hepatic portal vein, this process of infection by trophozoites frequently take place in the right hepatic lobe, which drains the most amount of blood draining the cecum and ascending colon (Rustgi and Richter, 1989). In some individuals infected with ALA there is amoebic colitis, but these persons haven't any bowel symptoms, and in these patients the fecal examination are negative for detection of cysts and trophozoites stage of *E. histolytica* parasite (Adams and MacLeod, 1977; Thompson et al., 1985; Rosenblatt et al., 1995;). ALA infection can remain in individuals more than months after travel in the endemicity area, so it is very important to carefully travel to endemic areas of this parasite (Barnes et al., 1987; Knobloch and Mannweiler, 1983; Shandera et al., 1998). ALA maybe suspected in individuals with a history of travel in endemic areas with symptoms such as fever, right upper quadrant pain and cough (Adams and MacLeod, 1977; Thompson et al., 1985; Barnes et al., 1987; Shandera et al., 1998).

ALA symptoms in the first 10 days of infection are usually acute, but this infection can be chronic which anorexia with weight loss are most occurrence symptoms. Anemia, leukocytosis, increasing in the sedimentation rate of erythrocytes and high concentration of ALP (alkaline phosphatase) are the most occurrence blood parameters in individuals infected with ALA (Adams and MacLeod, 1977; Thompson et al., 1985; Barnes et al., 1987; Shandera et al., 1998). The most significant and dangerous symptoms of ALA are rupture and bacterial superinfection. Rupture of the pleura is rare, and early diagnosis of the infection and treatment can be cause to decrease in mortality rate of infected individuals to 1% (Adams and MacLeod, 1977). In patients with extraintestinal amebiasis, there are several complications which we can named as brain abscess, pleuroplumonary amebiasis secondary to rupture of amoebic liver abscess through the diaphragm urogenital amebiasis. Brain abscess in patients can be diagnosed via microscope to find the amoeba parasite in brain biopsy, but in new researches and studies, diagnosis can be completed via PCR techniques which is most recommended (Solaymani et al., 2007). In liver abscess the main and important diagnostic method which strongly recommended include serological test which these tests are high sensitive (94%) and highly specific (95%). Sometimes within the first 10 days of infection a false-negative serological test may occur, but a repeat test will usually give a positive result. In ALA cases the abdominal ultrasound or tomography scan do not give good results and not specificity for these cases of infection. The PCR test is an important and sensitive diagnostic method for diagnosis but it is not routinely available, so a positive serological test with an abdominal X-ray and imaging is useful for diagnosis. Most recently researches show that the treatment of ALA patients gives good results (Blessmann et al., 2006).

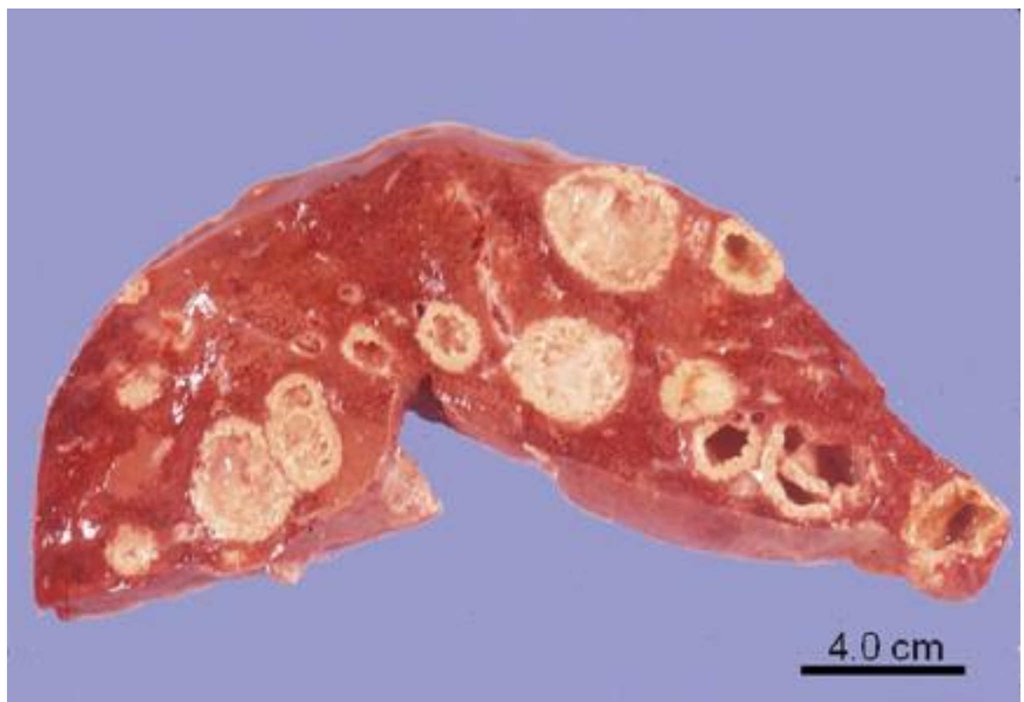

Figure 2.10. Human amoebic liver abscess (Shibayama *et al.*, 1997).

2.9. Diagnosis Methods

We cannot differentiate the pathogenic form of *Entamoeba* parasite (*E. histolytica*) from non-pathogenic *Entamoeba* by microscopic examination, for example identification and differentiation of *E. histolytica* from *E. dispar* in stool samples and liver abscess is impossible because the trophozite form of *E. histolytica* is similar to *E. dispar* with ingested erythrocytes under the microscope (Strachan et al., 1988; Gonzalez-Ruiz et al., 1994). Microscopic identification and differentiation between the pathogenic and non-pathogenic species of *Entamoeba* parasite (*E. histolytica* and *E. dispar*) is also impossible because of the misidentification of macrophages with *E. dispar* and *E. moshkovskii*, which are considered as non-pathogenic parasites (Krogstad et al., 1978; Gonzalez-Ruiz et al., 1994).

2.9.1. Microscopic diagnosis

The wet preparation and concentration method with several smears staining methods in fecal examination used as microscopically techniques in diagnosis of *E. histolytica* / *E. dispar* in clinical laboratory. There is a high insensitivity (10%) in microscopic stool examination using the wet mount or direct saline method on fresh stool specimens in the laboratory (Huston et al., 1999). Stool samples in clinical laboratory should be tested within the first hour after collection to see motile form of trophozoites with ingested erythrocytes. However, there is no trophozoite with ingested RBCs in non-acute dysentery patients. In stool of asymptomatic patients there is only the cyst stage of *Entamoeba* parasite and there is no trophozoite stage. In fecal concentration method, we can see the cyst stage of *Entameba*; also the staining method for example trichrome or iron hematoxylin, is very important in identification of *Entamoeba spp*. but these microscopic techniques are less reliable methods for identification of *Entameba spp*. as compared with the culture method or serological tests (Krogstad et al., 1978; Haque et al., 1995). The poor sensitivity of microscopic examination is more than 60%, special in misidentification of macrophages as the trophozoites stage of *Entamoeba* (Gonzalez-Ruiz et al., 1994; Haque et al., 1995; Haque et al., 1997; Haque et al., 1998; Tanyuksel and Petri, 2003). Unfixed and refrigerated stool samples not recommended, because these may effect on trophozoites form of *Entamoeba*, so collected stool samples should be preserved to prevent deformation of parasite stages form, concentration and staining method of stool samples is very important and fixation materials are include merthiolate iodine formalin, Schaudinn's fluid, sodium acetate-acetic acid-formalin (SAF), or 5% or 10% formalin (Proctor, 1991). In permanently stained samples, fixatives materials include Ziehl-Neelsen stains, modified polyvinyl alcohol (PVA) and iron hematoxylin (Li and Stanley, 1996). Sometime we can differentiate between *E. histolytica* and *E. dispar* by microscopic fecal examination in patients with dysentery when tropozoites contain ingested RBCs, however there is no trophozoites containing erythrocyte in all patients (Gonzalez-Ruiz et al., 1994; Strachan et al., 1988). Recent research has shown that the specificity and value of microscopic findings is very low when they find the ingested RBCs in *E.*

dispar, because vitro researches shown the ingesting ability of RBCs by *E. dispar* (Trissl et al., 1978; Haque et al., 1995). In one research the microscopic fecal examination specificity for detection of *E. histolytica* / *E. dispar* was only 9.5% when compared with other serological diagnostic methods (Pillai et al., 1999).

2.9.2. Culture diagnosis method

The culture method in stool for *Entamoeba* parasite isolation was used in many years ago, in this method xenic was used (diphasic and monophasic). In xenic cultivation, the increasing and growth of *Entamoeba* parasite occur in an undefined flora (Clark and Diamond, 2002). In 1925 Boeck and Drbohlav discovered the xenic culture of *E. histolytica* (Clark and Diamond, 2002). Now there are many various media which used in culture methods, we can list several of these media for example Jones's medium (Jones, 1946), TYSGM-9 (Diamond, 1982) and egg yolk infusion medium (Balamuth, 1946). In cultivation of *E. histolytica* more common media which are used include Robinson's medium, monophasic TYSGM-9 and diphasic Locke-egg (Robinson, 1968; Diamond, 1982). The culture method of *E. histolytica* can be used in fecal samples, specimens from rectal biopsy or aspirates from liver abscess (Blessmann et al., 2002). Some researchers have reported that the success rate of culture method for *E. histolytica* is about 50 to 70% (Clark and Diamond, 2002). Cultivation method in stools samples and liver abscesses patients are not used in routine clinical tests, and in any case, it is a difficult method. In xenic culture method *E. dispar* can be grown and isolate, but the growth of *E. histolytica* is more than of *E. dispar*, and the viability rate of some *Entamoeba* strain in a xenic culture is low (Clark, 1995; Kobayashi, 1998). Several studies have reported that the YI-S medium for *E. dispar* culture is not a suitable medium (Kobayashi, 1998; Clark and Diamond, 2002). The culture method for the isolation of *E. histolytica* is not recommended for diagnosis in laboratory routine examination because excessive growth of other protozoa, bacteria and fungi make more problems in diagnosis procedure during culture (Clark and Diamond, 2002).

2.9.3. Isoenzyme analysis method

Sargeaunt et al. (1978) reported that zymodemes analysis of *Entamoeba* culture can be used for *Entamoeba* differentiation. Isoenzyme or zymodeme are some *Entamoeba* strains that have ability to share the similar electrophoretic pattern of enzymes, these enzymes include hexokinase, malic enzyme, phosphoglucomutase isoenzyme and glucose phosphate isomerase (Sargeaunt et al., 1987). About 24 different isoenzymes have been discovered, among these isoenzymes there are 21 isoenzymes which isolated from humans, which are nine strains of *E. histolytica* and 12 strains of *E. dispar* (Blanc and Sargeaunt, 1991). The technique of isoenzymes or zymodeme analysis of amoeba culture can be used in distinguish and differentiation of non-pathogenic *Entamoeba* (*E. dispar*) from pathogenic *Entamoeba* (*E. histolytica*). This method was considered as gold standard for diagnosis of *Entamoeba* infection prior to the discovery and development in molecular diagnostic method, but there are several problems and disadvantages in isoenzyme analysis of amoeba culture technique, for example this method takes more time, it is difficult to perform and this technique is not always successful and sometimes the results of this method are negative in some microscopic-positive samples (Strachan et al., 1988; Gonzalez et al., 1994; Haque et al., 1997).

2.9.4. Antibody detection tests

Serological examination in detection, identification and differentiation of *Entamoeba spp*. is very useful especially in countries where *E. histolytica* is not a more common species of amoebiasis infection (Ohnishi and Murata, 1997; Weinke et al., 1990). Antibodies detection is a very important and useful method in amoebic liver abscess (ALA) patients, because in some ALA patients cannot detect *Entamoeba* parasite in their stool. Antibodies detection sensitivity in serum of ALA patients is about 100% which has been reported by several researchers (Zengzhu et al., 1999). There are many different assays which can be used for the serological examination for detection of antibodies in infected individuals with *Entamoeba* parasite, several

serological antibody tests are latex agglutination test, indirect hemagglutination test (IHA), immunoelectrophoresis, complement fixation test, indirect immunofluorescence assay (IFA), counterimmunoelectrophoresis (CIE) and enzyme-linked immunosorbent assay (ELISA). The above serological tests have different sensitivity, complement fixation tests are less sensitive as compared with other serological tests and don't used in most medical laboratories, indirect hemagglutination test (IHA) is very easy in performing and has a high sensitivity (99.1%) in *Entamoeba* diagnosis (Hung et al., 1999). There are commercial kits for *Entamoeba* diagnosis which are available and these kits can give a result within 10 minutes but these kits are nonspecific reactions kits (Sanchez et al., 2000). Sheehan et al. (1979) observed that the diagnosis and detection of *E. histolytica* antibodies in extraintestinal amebiasis in patients by counterimmunoelectrophoresis (CIE) is high sensitivity (100%), but takes more time. Antibody detection in amebiasis patients by indirect immunofluorescence assay (IFA) is reliable and rapid, especially in ALA patients is very useful test and differentiation of amebiasis infection between patients (past disease and present disease) can be down undertaken by indirect immunofluorescence assay (IFA) (Garcia et al., 1982). The most popular serological test that used in most area of world in epidemiological study of *Entamoeba* is ELISA. This test is used especially in individuals with ALA infection and it is a simple test with easy procedure in clinical laboratories (Gonzalez et al., 1995). ELISA is a useful test, especially in extraintestinal amoebiasis in which fecal examination result are negative and *Entamoeba* parasite cannot be detected in the stool (Rosenblatt et al., 1995). The sensitivity of the ELISA test is 97.9% and 94.8% specific for antibodies detection and diagnosis of *E. histolytica* in individuals with amoebic liver abscess (ALA) infection (Hira et al., 2001).

2.9.5. Antigen detection tests

Serologists developed ELISA test as an antigens detection in stool of infected patients with *E. histolytica*. The ELISA test for antigen detection is highly sensitive as compared with culture method of stool and its easy and rapid test in laboratories. ELISA Kits of TechLab in 1993 were designed for antigen detection of *E. histolytica* parasite in

individual stool and by this test the Gal/GalNAc lectin of *Entamoeba* parasite is detected in infected individuals' feces (Haque et al., 1997; Haque et al., 1998). These antigen-based ELISA kits are used for the detection and identification of *E. histolytica* and *E. dispar* in stool samples because there are differences in antigens of lectins in both *E. histolytica* and *E. dispar* and the antigen detection rate in this method is very high (Haque et al., 1993; Mirelman et al., 1997). There is problem in this test that is the denaturation of antigen in fixed feces samples, but this test is highly sensitive for antigen detection and identification of *E. histolytica* in asymptomatic and amoebic colitis patients (Haque et al., 1997; Haque et al., 1998). There is a good correlation between nested PCR and TechLab ELISA, in antigen detection of *E. histolytica* parasite in stools of patients (Haque et al., 1998). Kits of *E. histolytica* (TechLab) are very sensitive and very useful for diagnosis and detection of lectin antigen in pus of liver abscess patients and serum lectin antigen (Haque et al., 2000). Haque et al. (2000) reported a 96% antigen detection rate in serum and 100% antigen detection in liver abscess pus in patients with ALA diseases. Stool-specific antigen detection of *E. histolytica*/ *E. dispar* via ELISA test is an excellent method and mostly used in epidemiological researches that the PCR method not be used (Haque et al., 1997). Antigen based ELISA test is easy and more effective method for antigen detection of *E. histolytica* parasite as compared with other diagnostic methods such as microscopic examination, antibody detection test, isoenzyme analysis and culture method (Mirelman et al., 1997).

2.9.6. Immunochromatographic assays

The first immunochromatographic assay which was used for the detection of specific antigen in *E. histolytica* / *E. dispar*, *Giardia lamblia* and *Cryptosporidium parvum*, is called Triage parasite panel (TPP). The immunochromatographic strip covered with monoclonal antibodies specific for *E. histolytica*/*E. dispar* antigen surface (29-kDa). There is a high specificity (99.1% - 100%) and a high sensitivity (96-100%) in Triage parasite panel (TPP) kits in antigen detection of *E. histolytica*/*E. dispar* parasites as compared with microscopic fecal examination. Triage parasite panel (TPP)

kits are useful and require about 15 minutes for to perform and can use in all unfixed, fresh and frozen stool samples forms (Garcia et al., 2000; Sharp et al., 2001).

2.9.7. Conventional PCR

PCR-based techniques are a diagnostic method that used in many developed countries by researchers in epidemiologically studies and used in clinical laboratories as a method for differentiation between *Entamoeba spp*., this method endorsed strongly by World Health Organization (WHO) (Calderaro et al., 2006; Hamzah et al., 2006; Haque and Petri, 2006). The *Entamoeba histolytica* parasite can be detected and diagnosed in many type of clinically samples, for example can be detect in stool, tissues and infected liver abscess (Tanyuksel and Petri, 2003). Molecular diagnosis or PCR of 18S rDNA (small-subunit rDNA gene) is very sensitive and its sensitivity is 100 times more than the ELISA technique and other diagnostic methods (Mirelman et al., 1997; Troll et al., 1997). Some researchers in their study has been applied PCR method in amplification of special gene which encodes 125-kDa antigen surface and then adapted subsequently to differentiation and distinguishing between *Entamoeba spp*. via restriction digestion (Edman et al., 1990; Tannich et al., 1991; Burchard, 1991). The first study of PCR techniques in *Entamoeba spp*. were undertaken by Edman et al. (1990) and Tannich and Burchard (1991) which they extracted DNA from control positive samples of isolated *Entamoeba spp*. in laboratory (Edman et al., 1990; Tannich and Burchard, 1991). PCR technique in diagnosis and differentiation of *Entamoeba spp*. by using specific primers with highly repetitive sequences present in *Entamoeba spp*. (pathogenic and non-pathogenic *Entamoeba*) (Garfinkel et al., 1989; Romero et al., 1992). Detection of 18S rDNAs gene of *Entamoeba spp*. is very important and is target way for distinguish and differentiation among *Entamoeba spp*. (Clark and Diamond, 1991; Que and Reed, 1991; Clark and Diamond, 1992; Clark and Diamond, 2002). In research laboratory all microscopically positive stool samples and culture samples of *Entamoeba spp*. were prepared and DNA extraction in these samples were done by automated and manual methods and then these extracted DNA were performed in PCR machine for diagnosis and differentiation of *Entamoeba spp*., and researchers reported the high sensitivity in

detection and differentiation of *Entamoeba* parasite (Clark and Diamond, 1993; Clark and Diamond, 1997; Heckendorn et al., 2002; Moran et al., 2005). Detection of 18S rDNA gene by PCR technique in researches used widely for diagnosis and detection of *Entamoeba spp*. (Bhattacharya et al., 1989). Acuna-Soto et al. (1993) were the first research team group which they reported the epidemiologically study of *Entamoeba* infection by using PCR technique, firstly they isolated DNA from stool samples and then added a specific primer to amplification of extrachromosomal DNA. In more molecular studies and conventional PCR about *Entamoeba spp*., the QIAGEN kits for isolation of DNA and also primers for antigen gen of 29-kDa/30-kDa have been more used for detection and differentiation of pathogenic and non-pathogenic *Entamoeba spp*. (Aguirre et al., 1995; Verweij et al., 2002). In PCR technique there are two other genes which are also widely used in *Entamoeba spp*. distinguish and differentiation which these genes are the chitinase gene and serine-rich *E. histolytica* protein (SREPH) gene (Stanley et al., 1990; De la Vega et al., 1997). SREPH gene was shown in DNA amplification of *Entamoeba* positive fecal samples (Ramos et al., 2005). A nested PCR of target SREPH gene in stool extracted DNA was used to detection and investigation of *Entamoeba* parasite in individuals (Ayeh kumi et al., 2001). Actin gene and cysteine proteinase gene were used as target genes in PCR method in extracted DNA from stool in *Entamoeba* epidemiological studies (Freitas et al., 2004). More researchers used a multiplex nested PCR technique, which its developed method that by this method two pathogenic and non-pathogenic *Entamoeba* (*E. histolytica* and *E. dispar*) can be detect from extracted DNA from positive sample of *Entamoeba spp*. (Evangelopoulos et al., 2000; Hung et al., 2005). Detection of *E. histolytica* in amoebic liver abscess patients by PCR method was achieved by using the gene encoding the 30 kDa antigen which the sensitivity of this method was 100% (Tachibana et al., 1992).

2.9.8. Real-time PCR

One of the new and highly attractive forms of PCR is real-time PCR, which is used for detection and diagnosis of *Entamoeba* parasite in infected patients with amoebiasis and require shorter time for performing than conventional PCR and

characterized by a low contamination risk (Klein, 2002). This technique is performed by amplicon (PCR product) detection during polymerase chain reaction via binding to one or two fluorescence-labelled probes. The sensitivity of real-time PCR is more than conventional PCR which can detect parasite in small amount of stool (Blessmann et al., 2002). Real-time PCR is very sensitive and quantitative technique and can show the number of infected parasites in stool (Blessmann et al., 2002; Calderaro et al., 2006). Diagnosis of *E. histolytica* in stool and amoebic liver abscess samples by real-time PCR technique work as by targeting the 18S rDNA gene (Roy et al., 2005).

2.9.9. Treatment

For treatment of amoebiasis, in the first should be differentiate *E. histolytica* than *E. dispar* or other non-pathogenic *Entamoeba*, the treatment should not be undertaken according to microscopic diagnosis, because there are morphological similarities between pathogenic *E. histolytica* and non-pathogenic *E. dispar*, where non-pathogenic *E. dispar* does not require any treatment (WHO, 1997). Drug for treatment of amoebiasis depend on the type of infection which are:

A) Luminal amoebicides: in this case the trophozoite stage of *Entamoeba* parasite present in the intestinal lumen and should be treated with paromomycin, diiodohydroxyquin or diloxanide furoate.

B) Tissue amoebicides: infection of tissues by *Entamoeba* parasite which treated with tinidazole, metronidazole, 2-dehydroemetin and emetine hydrochloride (WHO, 1997).

2.11. Prevention

E. histolytica infect human by cyst stage, so prevention of fecal-oral route is very important to prevention of this disease, good cooking of food, good washing of vegetables and treat with vinegar (for 15 min) and drinking water boiling method in developing countries and good personal hygiene is the best methods to prevent and control of this parasite. (Petri et al., 1991; Zhang and Stanley., 1994).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments

The current study includes microscopy, serology and molecular diagnosis to differentiate between *E. histolytica* and other non-pathogenic *Entamoeba*, we used various equipment and materials in this study as follows:

Instruments

Light Microscope

ELISA plate reader

PCR thermocycler

Gel electrophoresis

Deep Freeze

Bench Centrifuge

Micro centrifuge

Magnetic Stirrers

Incubator

Ice maker

Micro plate shaker

Micro pipettes

Nano drop

pH Meter

Refrigerator

Sensitive microbalance

Water bath Water

distillatory

3.1.2. Chemicals

In this study, it was used various chemical materials in the preparation of the required buffers and the solution used for detection and differentiation of *Entamoeba spp*.

Chemicals and Kits

Iodine solution

Normal saline

Ladder

Agarose

Ethidium bromide solution

Bromophenol blue

Glycerol

Boric acid

Hydrochloric acid (HCl)

Sodium hydroxide (NaOH)

Sodium chloride (NaCl)

Ammonium acetate

Absolute ethanol

Phenol chloroform isoamyl

Isopropanol

Sodium dodecyl sulphate (SDS)

Sodium chloride (NaCl)

Tris-base

Agarose

ELISA Kit of *E. histolytica*

QIAamp stool DNA extraction kit

Master Mix kit

3.1.3. Sample collection

In the study, 162 stool samples were collected from diarrheal patients in three cities in northern Iraq: Duhok (65 samples), Erbil (50 samples) and Sulaimaniyah (47 samples). All of these samples were collected randomly from diarrheal patients during the period between January 2016 to May 2016. All data and information about the collected samples such as number collected, sex and age were tabulated. Then, the collected stool samples were stored in a special container in a laboratory deep freezer.

3.2. Methods

3.2.1. Microscopic examination

In this stage of work I used direct stool examination, or the ‘wet smear’ method, which does not require complex or specific equipment, I used only slides, cover slips and a drop of normal saline for each stool sample. This method is generally used for the diagnosis of intestinal parasites and the *Entamoeba* parasite, but without differentiation between *Entamoeba spp.*. In this method, I placed a small amount of the patient’s stool on a glass slide using a wooden stick and mixed it with drop of normal saline then covered with cover slip and examined under a light microscope. I also added a drop of Iodine solution and a small amount of stool on a second glass slide and mixed well for the detection of *Entamoeba spp.* under the microscope.

3.2.2. Serologic examination

It was used a serological test (ELISA) for all stool samples stored in the laboratory deep freezer for the detection of *E. histolytica* using *E. histolytica* II kits according to the kits manufacturer’s instructions (TechLab made in USA).

Kit components

1. Microwells containing anti-*E. histolytica* antibodies.
2. Reagent 1: Anti-*E. histolytica* antibodies bottle (blue dye).
3. Reagent 2: Antibodies conjugated to horseradish peroxidase bottle (red dye).
4. Positive Control: *E. histolytica* antigen vial in buffer.
5. Negative Control: buffer vial.
6. Substrate.
7. Wash Concentrate (20X).
8. Stop Solution.

E. histolytica ELISA kit procedure

1. Fecal specimen dilution: It was added a small amount of stool to 400 µL diluent.
2. One drop (50 µL) of conjugate was added to each well.
3. 200 µL of diluted stool or control specimen was added to wells containing conjugate and then was incubated for 2 hours at normal room temperature.
4. After two hours, the wells were washed with dilute wash solution five times, after which the well was inverted and slapped hard onto the paper between each wash.
5. Two drops (100 µL) of substrate were added to the wells and then incubated at room temperature for 10 min.
6. Then, one drop (50 µL) of stop solution was added to the wells and mixed, and then wait for two min.

7. Sample result readings were made available within 10 min of adding the stop solution at 450/620-650 nm. in the ELISA plate reader, where samples testing positive for *E. histolytica* are considered to be those with absorbance readings of 0.15 or above OD units**.**

3.2.3. Stock solutions and buffer preparation

3.2.3.1. 1M Tris–HCl pH 8.0

To prepare this buffer, we dissolved Tris-base (121.1g) in distilled water (800 ml) and added a few drops of NaCl to raise the pH to 8, after which the total volume of the solution was increased to 1000 ml by addition of distilled water.

3.2.3.2. 1M EDTA (0.5) pH 8.0

This solution was prepared by dissolving EDTA (186.1g) in distilled water (800 ml) and adding a few drops of NaOH to raise the pH to 8, the solution was then made up to 1000 ml using distilled water.

3.2.3.3. Loading buffer

This buffer was prepared by the addition of glycerol (30 ml) to bromophenol blue (0.25 g), which was then made up with distilled water (50 ml) and a few drops of 10M NaOH to adjust the pH to 8. Finally, the solution volume completed to 100 ml by adding distilled water.

3.2.3.4. Sodium Chloride

This solution was prepared by adding distilled water (400 ml) to sodium chloride (146.1 g) and completed solution volume up to 500 ml.

3.2.3.5. Tris-EDTA buffer (TE buffer)

This buffer prepared by adding Tris-base (1 M, 5 ml) with 1ml of EDTA (5M, 1 ml) and total volume of the solution was completed to 500 ml by adding distilled water.

3.2.3.5. Lysis buffer

Preparation of this buffer: 0.5 M of Tris-HCl and 20 mM of Ethylene diamine tetraacetic acid (EDTA) were mixed with 10 mM of sodium chloride (NaCl) and finally adding 0.1% sodium dodecyl sulphate (SDS) at pH 9.0.

3.2.3.7. Tris-Borate-EDTA buffer (10XTBE)

This buffer prepared by dissolving boric acid (55 g) and Tris-base (108 g) to in 40 ml of EDTA at pH 8.0 (0.5 M) and then adding distilled water (800 ml). The pH was then adjusted to 7.8 by adding a few drops of HCl, after which the total volume completed to 1000 ml using distilled water.

3.2.4. DNA extraction

For DNA extraction from stool samples, we used two methods of DNA purification. The first used the QIAamp DNA stool kit (QIAgen, Germany) according to the instructions of the DNA kit manufacturer (Table 3.2.) and the second one by manual DNA extraction method, where we prepared the solution, buffers and required chemicals.

Table 3.1. QIAamp DNA stool Kit Contents

QIAamp Stool DNA extraction mini kit	
Mini Spin QIAamp columns	50
Tubes for collection (2 ml)	200
Tablets of Inhibit	50
ASL buffer	140 ml
AL buffer	33 ml
AW1 buffer	19 ml
AW2 buffer	13 ml
AE buffer	15 ml

3.2.4.1. QIAamp DNA stool kit procedure

1. It was added about 200 mg stool sample to a microcentrifuge tube (2 ml) then was placed this tube on ice.

2- Then it was added ASL Buffer (1.4 ml) to each stool sample and was vortexed for about 1 min to homogenize the sample.

3- It was heated the sample at 70°C for about 5 min.

4- Again it was vortexed the sample for about 15 second and was placed the sample in high speed centrifuge for 1 min.

5- It was taken 1.2 ml of the sample supernatant in a small microcentrifuge tube.

6- Then it was added one inhibit EX tablet to the sample and immediately vortexed for about 1 min and then incubated it at room temperature for 1 min.

7- It was centrifuged the sample solution in a high-speed centrifuge for 3 min.

8- It was placed the solution supernatant in a new microcentrifuge tube (1.5 ml) and centrifuged again in a high-speed centrifuge for 3 min.

9- It was added 15 µl proteinase K into sample tube.

10- It was taken 200 µl of the supernatant from step 8 and added it to the 1.5 ml tube containing proteinase K.

11- It was added 200 µl of AL buffer to sample and vortexed for 15 seconds.

12- It was incubated sample for 10 min at 70°C.

13- It was placed 200 µl of ethanol (96–100%) in sample and vortexed it to mix sample solution.

14- It was taken new QIAamp spin column (label the lid) and placed in a 2 ml collection sample tube which is then sealed and centrifuged at high speed for about 1 min then added spin column in a new tube (2 ml) and then discarded containing filtrate tube.

15- Then a QIAamp spin column was opened and Buffer AW1 (500 µl) was added carefully. Then the spin column was sealed and speed centrifuged for 1 min. Later the QIAamp spin column was placed in a new tube (2 ml collection tube) and the filtrate collection tube was discarded.

16- The QIAamp spin column was then opened and Buffer AW2 (500 µl) added. The cap was then sealed and placed in a high-speed centrifuge for 3 min, after which the filtrate collection tube was discarded.

17- Also the QIAamp spin column was added to a new tube (2 ml) and the old filtrate collection tube discarded. The new tube was centrifuged in a high-speed centrifuge for about 1 min.

18- The QIAamp spin column was then placed in a new microcentrifuge tube (1.5 ml) and carefully open a QIAamp spin column and place AE Buffer (200 µl) in the QIAamp membrane then close the tube cap. The sample tube was incubated at room temperature for 1 min and then was centrifuged in a high-speed centrifuge for 1 min to elute the stool DNA.

3.2.4.2. Manual DNA extraction procedure

It was used a manual DNA extraction method for several of the stool samples in which the DNA purity and concentration were shown in low percentage in kit method of QIAamp stool DNA extraction. In this method, it was used the following procedure according to Machiels *et al*. (2000).

1. It was added 0.25 gr stool sample to each of two tubes: tube A and tube B.
2. It was added 2.5 ml of lysis buffer to each tube (A & B).
3. It was vortexed for 10 minutes and shaked each tube for 5 minutes (A & B).
4. It was added 2.5 ml of lysis buffer to each tube (A & B) again.
5. It was shaked each tube for 5 min (A & B).
6. It was centrifuged at 400 rpm for 12 min.
7. It was taken supernatant + half the volume (1/2) of Ammonium acetate + two volumes of absolute ethanol (cool ethanol).
8. It was incubated each tube (A & B) at -20ºC for 25 min.
9. It was centrifuged each tube (A & B) at 400 rpm for 15 min.
10. It was taken the supernatant of each tube (A & B) and added 200 µl of TE buffer to each tube.
11. It was mixed the contents of the two tubes together (tube A+ tube B).

12. It was incubated the tube in water bath 65°C for 15 min.

13. It was added an equal volume of phenol chloroform isoamyl to the tube.

14. It was centrifuged the tube at 1000 rpm for 10 min in a microcentrifuge.

15. At this stage, two layers appear in the tube, of which the upper layer was taken.

16. Then, 0.6 volumes of isopropanol was added. For exam. 300 µl of tube solution × 0.6= 180 (0.6 volume of isopropanol).

17. It was centrifuged the tube at 1000 rpm for 10 minutes in a microcentrifuge.

18. It was removed supernatant (sometimes all the solution in the tube).

19. It was added 150 µl of TE buffer to the tube. The current solution is stool DNA.

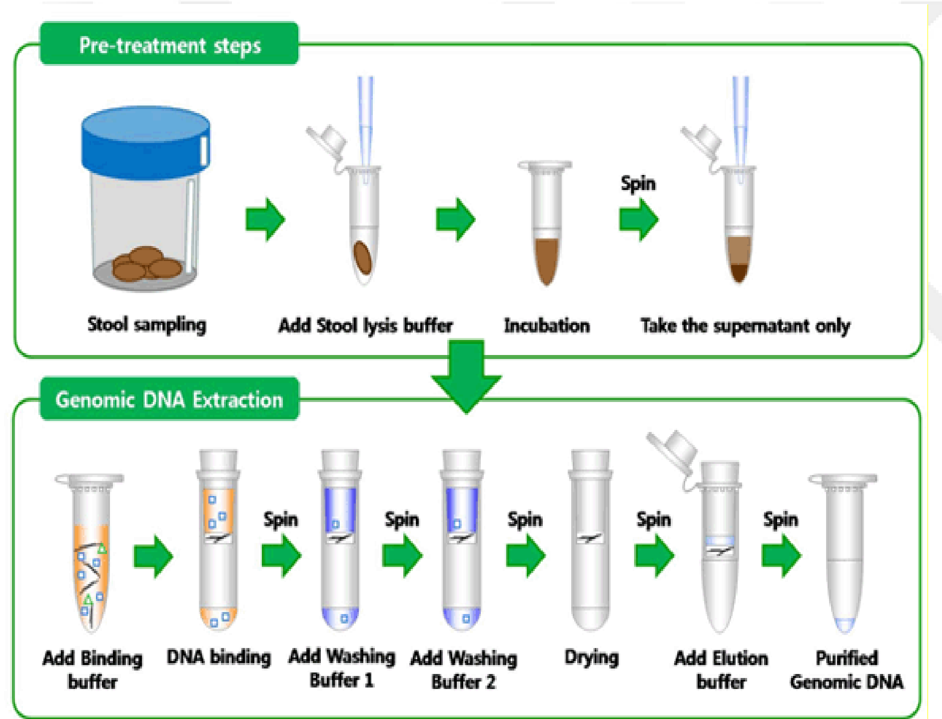

Figure 3.1. General scheme of genomic Stool DNA extraction (QIAamp, 2012).

3.2.4.3. DNA purity and concentration determination

After DNA extraction from all stool samples using either the QIAamp Stool DNA extraction kit or the manual DNA extraction method, it was used a NanoDrop spectrophotometer (Thermo Scientific, USA) to determine the concentration and purity of the stool DNA obtained from the samples.

3.2.4.4. Detection of *E. histolytica* SSUrDNA gene by PCR

To detect and amplify the SSUrDNA gene fragment of *Entamoeba spp*. it was used the polymerase chain reaction method (Nested PCR) as a special protocol by using special primers of *Entameba spp*., *E. histolytica* and *E. dispar* (Khairnar and Parija, 2007). These primers are used to obtain SSUrDNA fragments from the *Entamoeba* parasite. These primer sets are prepared by integrated DNA technologies (IDN), as shown in Table 3.4.

Table 3.2. Primers of *Entameba spp*., *Entameba histolytica* and *Entameba dispar*

Name of primer species	Nucleotide sequence of primers
Entameba spp.	F. (5'-TAA GAT GCA GAG CGA AA-3') R. (5'-GTA CAA AGG GCA GGG ACG TA-3')
Entameba histolytica (439 bp.)	F. (5'-AAG CAT TGT TTC TAG ATC TGA G-3') R. (5'-AAG AGG TCT AAC CGA AAT TAG-3')
Entameba dispar (174 bp.)	F. (5'-TCT AAT TTC GAT TAG AAC TCT-3') R. (5'-TCC CTA CCTATT AGA CAT AGC-3')

In this study we used the Nested PCR technique for the detection of the ribosomal gene which characterize the *Entamoeba spp*., *E. histolytica* and *E. dispar* (Que and Reed,1991). In this technique, we used the samples from the first PCR step to obtain the primary PCR product, which we then subjected to secondary PCR to produce the secondary PCR product used to characterize *E. histolytica* (439 bp.) and *E. dispar* (174 bp.), according to method described by Khairnar and Parija (2007). According to this technique, we prepared a master reaction mixture (25 ml) for Nested PCR. In this stage, it was used 162 special PCR tubes, adding 12.5 µl of master mix to each tube (GeneDirex, USA) with 2.5 µl of both the forward and reverse primers, further adding 4 µl of genomic DNA with 3.5 µl distilled water, as shown in Table 3.4. We then placed tubes containing the master reaction mixture in the thermal cycler machine and

programmed the PCR machine according to the protocol described by Que and Reed (1991) to start amplification of the special *Entamoeba spp*. gene fragments to allow detection and differentiation of the *Entamoeba* parasite.

Table 3.3. Master reaction mixture (25 ml) for PCR

Component	Volume (µl)
Master mix	12.5
Forward primer, 10 mm	2.5
Reveres primer, 10 mm	2.5
Genomic DNA	4.0
DH2O	3.5
Total	25

According to this method, to obtain the primary PCR product the samples in the PCR machine were heated (96°C) for 2 min, this step represents the initial denaturation which is followed by 30 further cycles. Then, in the denaturing step, the samples are heated to 92°C for 1 min and heated to 56°C (1 min) in the annealing step. Finally, the sample is heated at 72°C for 7 min (extension step).

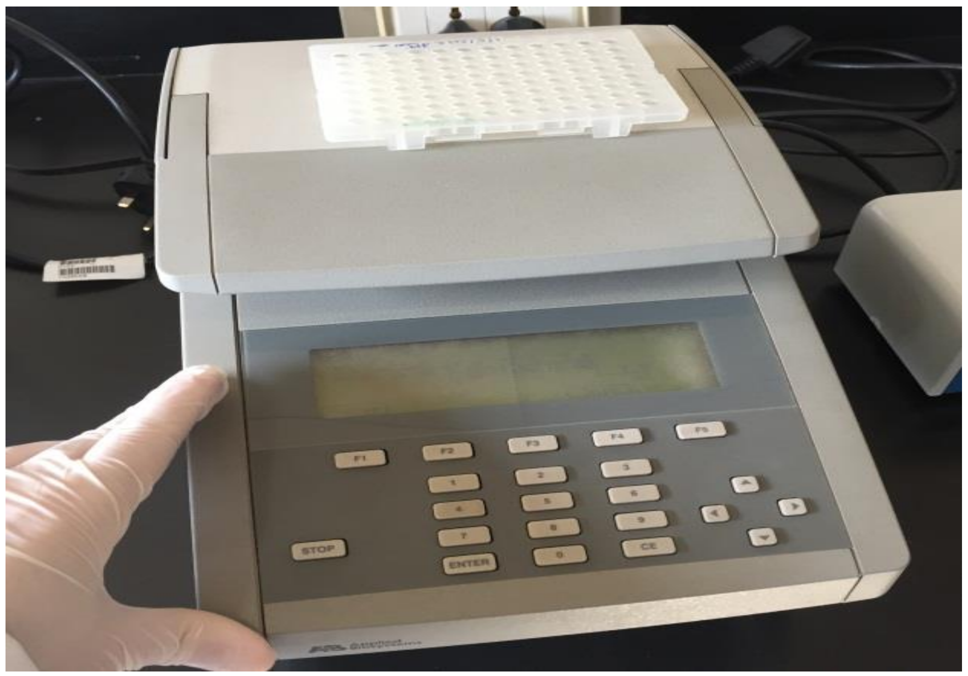

Figure 3.2. PCR conventional thermocycler.

In second stage, the primary PCR product is subjected to secondary PCR product to detect of *Entamoeba spp*. (*E. histolytica* and *E. dispar*). In secondary stage of the Nested PCR process, to produce the secondary PCR product we used the same heating program on the PCR machine except for changing the temperature of the annealing step to 48°C, and the duration of the extension step was changed to 1 min, as per the following Table (Table 3.3). For the gel agarose run, we used the secondary PCR product in the electrophoresis (100 V for 35 min) in gel agarose (2%), staining with ethidium bromide to visualize bands under UV light, of which photos were taken.

Table 3.4. Nested PCR program

Step	Temperature	Duration	No. of cycles
Initial denaturation	96°C	2 min	
Denaturation	92°C	1 min	30 cycles
Annealing	Primary PCR product: 56°C Secondary PCR product: 48°C	1 min 1 min	
Extension	72°C	Primary PCR product: 1 min 30 sec. Secondary PCR product: 1 min	
Final extension	72°C	7 min	

3.2.4.5. Agarose gel electrophoresis

The preparation of Gel Agarose is performed according to the type of molecular technique and is generally in different concentrations, for example, we used Gel Agarose 1% (w/v) in TBE for the detection of extracted DNA, in this type of Gel Agarose preparation we added Agarose (1 g) in 1X TBE buffer (100 ml) which we then dissolved by heating at high temperature (boiling temp.), and then left the product to cool (55°C) before adding it to the casting plate and placing the comb at edge of the gel and leaving it to cast. Then, it was added 1X TBE to a gel tank and placed the gel plate

in the horizontal position in the electrophoresis tank. Then, it was mixed each sample of DNA (5 µl) with loading buffer (1 µl) and then mixed the DNA with a loading buffer which was then carefully added to each well. A voltage of 45 V was then applied for 15 min and then 85 V for about 1 to 2 hours, in this time, the DNA in the wells started to run and moved through the Gel agarose which was then stained with ethidium bromide dye and placed in distilled water with dye at a concentration of 0.5 µg/ml for 15-30 min. It was then quickly washed with distilled water and illuminated with U.V. to visualize the DNA bands. A photograph was taken of the illuminated bands (Maniatis et al., 1982).

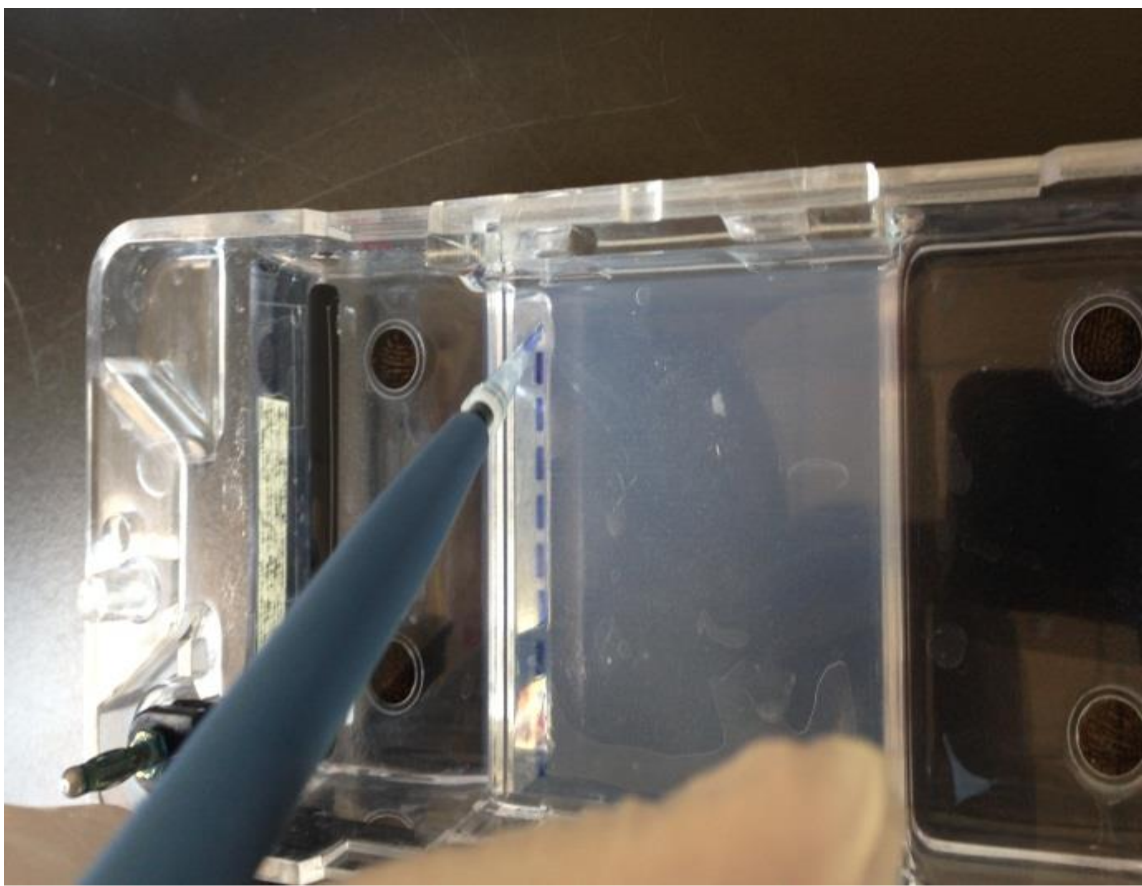

Figure 3.3. Gel Agarose electrophoresis.

4. RESULTS

4.1. Microscopic Examination

In the current study, all of these samples were examined microscopically, the results of which are recorded in this study (Table 4.1.). The prevalence of *Entamoeba* infections was found to be 58 (35.8%) of diarrheal patients in all three cities which microscopically were examined and found to be positive for the *Entameba spp*. parasite. In Duhok city, a total of 65 samples were collected from hospitalized diarrheal patients, of whom the number infected with *Entameba spp*. was 22 (33.8%);10 of the infected persons were between 1-6 years old, eight were between 7-15 years old, and four were more than 15 years old, as shown in Table 4.2.

Table 4.1. Prevalence of *Entamoeba* infection according to Microscopic, Serologic and Nested PCR studies in Duhok, Erbil and Sulaimaniyah

City	Microscopy *E. spp.*	Serology *E. histolytica*	PCR Diag. *E. histolytica*	PCR Diag. *E. dispar*	PCR Diag. *E. histolytica + E. dispar*
Duhok (N=65)	22 (33.8%)	4 (6.1%)	5 (7.6%)	14 (21.5%)	2 (3%)
Erbil (N=50)	17 (34%)	2 (4%)	2 (4%)	10 (20%)	1 (2%)
Sulaimaniyah (N=47)	19 (40.4%)	2 (4.2%)	2 (4.2%)	13 (27.6%)	0
Total N= 162	58 (35.8%)	8 (4.9%)	9 (5.5%)	37 (22.8%)	3 (1.8%)

In Duhok city, 12 infected persons were female and 10 infected persons were male. In Erbil city stool samples were collected from 50 diarrheal patients, 17 (34%) were found microscopically positive for *Entameba spp*. (Table 4.3.). According to age, seven infected persons were between 1-6 years old, six were between 6-15 years old and four patients were more than 15 years old. According to sex, 10 infected persons were male and seven were female (Table 4.3). In Sulaimaniyah, a total of 47 stools samples were collected, as shown in Table 4.4., according to microscopic examination 19 persons (40.4%) were infected with *Entameba spp*., which 10 infected patients were between 1-6 years old, four were between 7-15 years old and five were more than 15 years old.

(Table 4.4.). In Sulaimaniyah city, 11 infected persons with *Entameba spp.* were male and eight were female.

Table 4.2. Prevalence of *Entamoeba spp*. infection in diarrheal patient according to age and sex in Duhok City

Age/No.	Microscopy *E. spp.*	Serology *E. histolytica*	PCR Diag. *E. histolytica*	PCR Diag. *E. dispar*
(1-6)/ N=33	10 (30.3%)	2 (6%)	3 (9%)	6 (18.1%)
(7-15) /N=18	8 (44.4%)	2 (11.11%)	2 (11.1%)	5 (27.7%)
(15 >)/ N=14	4 (28.5%)	0	0	3 (21.4%)
Total/ N= 65 28♂/ 37♀	22 (12♂, 10♀)	4 (3♂, 1♀)	5 (3♂, 2♀)	14 (8♂, 6♀)

4.2. Serological Examination

According to serological examinations of stool samples of the patients, the total positive samples from all collected samples from patients from Duhok, Erbil and Sulaimaniyah were eight (4.9%) persons diagnosed, as shown in Table 4.1. In Duhok city, 65 stool samples were collected for which the ELISA test showed that there were four infected people (6.1%). In Erbil city, there were two infected persons (4%) among the 50 collected stool samples. In Sulaimaniyah city, 47 stool samples were collected from hospitalized patients of which two (4.2%) persons were infected with the *E. histolytica* parasite according to the serological test (ELISA).

Table 4.3. Prevalence of *Entamoeba spp*. infection in diarrheal patients according to age and sex in Erbil city

Age/No.	Microscopy *E. spp.*	Serology *E. histolytica*	PCR Diag. *E. histolytica*	PCR Diag. *E. dispar*
(1-6)/ N=22	7 (31.8%)	1 (4.5%)	1 (4.5%)	4 (18.1%)
(7-15)/N= 19	6 (31.5%)	1 (5.2%)	1 (5.2%)	4 (21%)
(15 >)/ N= 9	4 (44.4%)	0	0	2 (22.2%)
Total/ N=50 32 ♂/ 18♀	17 (10 ♂, 7♀)	2 (1 ♂, 1♀)	2 (1 ♂, 1♀)	10 (6 ♂, 4♀)

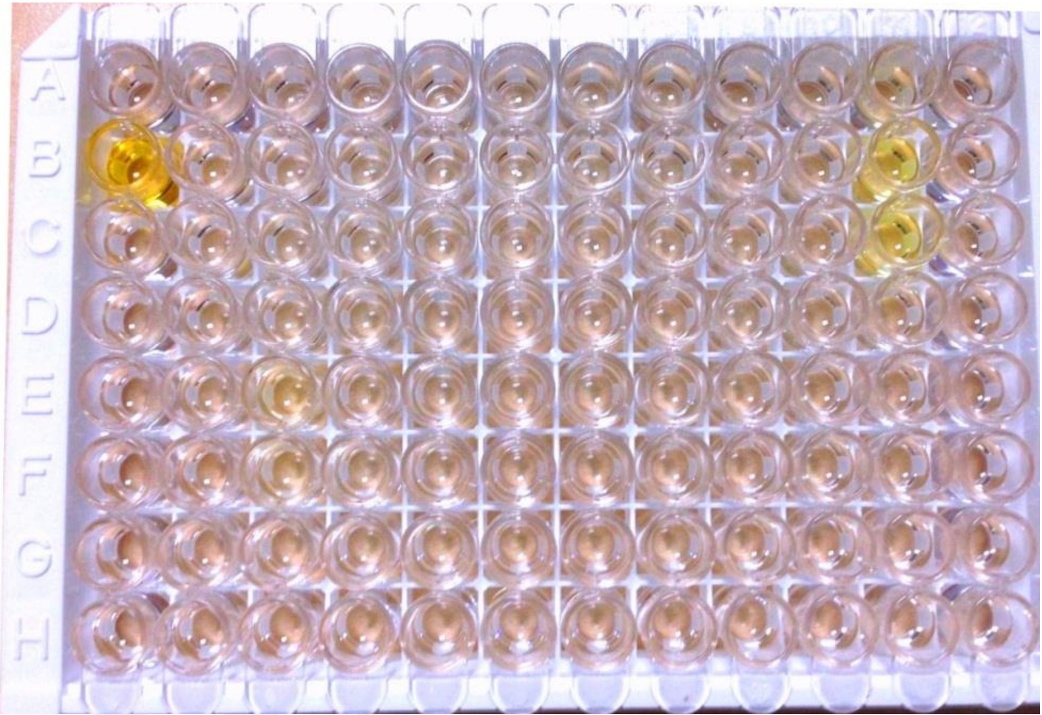

Figure 4.1. ELISA test for diagnosis of *E. histolytica.*

4.3. Molecular Examination (Nested PCR Technique)

In this part of research, I used the Nested PCR technique to amplify and characterize *Entamoeba spp*. parasite to detection *E. histolytica* and *E. dispar*. In this study, I examined 160 stool samples and I detected both bands associated with *E. histolytica* with 439 bp. (Figures 4.3. and 4.4.) and *E. dispar* with 174 bp. (Figures 4.5. and 4.6.).

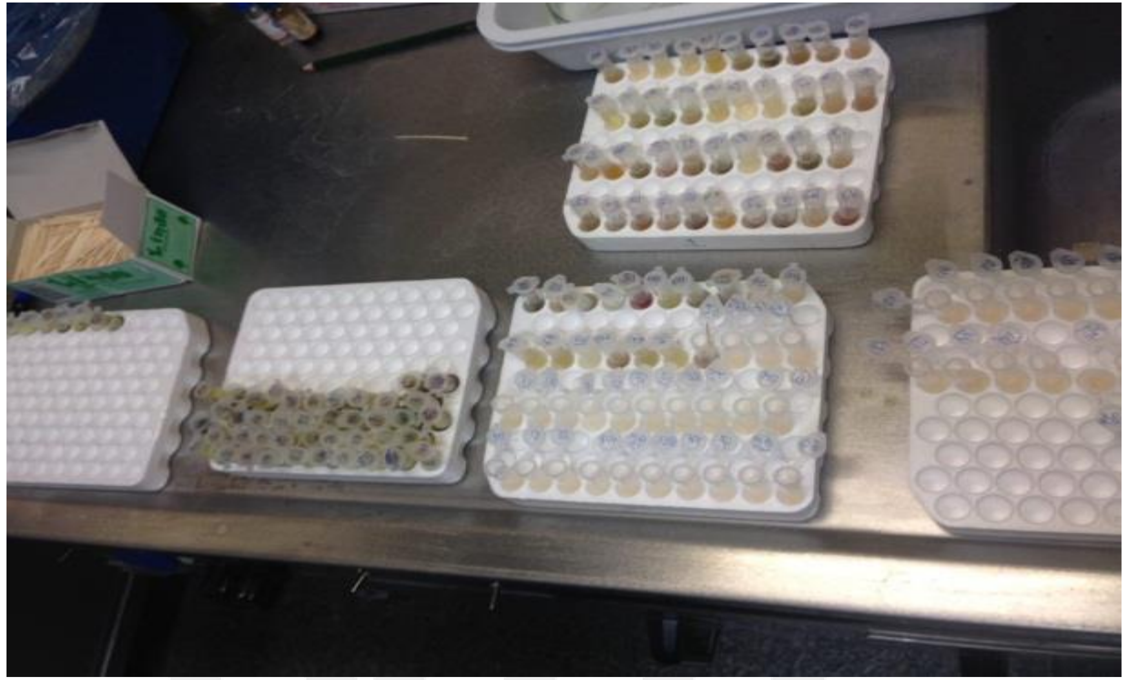

Figure 4.2. Stool samples eppendorf preparation.

After gel running via this technique, which are shown in Table 4.1. the number of stool samples testing positive for *E. histolytica* was five (7.6%) in Duhok City, two (4%) in Erbil City and two (4.2%) in Sulaimaniyah city, but the numbers of positive stool samples of *E. dispar* were 14 (21.5%) in Duhok City, 10 (20%) in Erbil City and 13 (27.6%) in Sulaimaniyah City. Total stool samples infected by *E. histolytica* (Duhok City, Erbil City and Sulaimaniyah City) were nine (5.5%) and the total number of stool samples infected by *E. dispar* were 37 (22.8%). There were three (1.8%) stool samples which were infected with both *E. histolytica* and *E. dispar* (mixed infection), two (3%) of these samples were detected in Duhok city and one (2%) in Erbil City (Table 4.1.).

Table 4.4. Prevalence of *Entamoeba spp*. infection in diarrheal patient according to age and sex in Sulaimaniyah City

Age/No.	Microscopy *E. spp.*	Serology *E. histolytica*	PCR Diag. *E. histolytica*	PCR Diag. *E. dispar*
(1-6)/ N= 21	10 (47.6%)	2 (9.5%)	2 (9.5%)	7 (33.3%)
(7-15)/N=14	4 (28.5%)	0	0	3 (21.4%)
(15 >)/N=12	5 (41.6%)	0	0	3 (25%)
Total/ N= 47 29 ♂/ 18♀	19 (11 ♂, 8♀)	2 (2 ♂, 0♀)	2 (2 ♂, 0♀)	13 (6 ♂, 7♀)

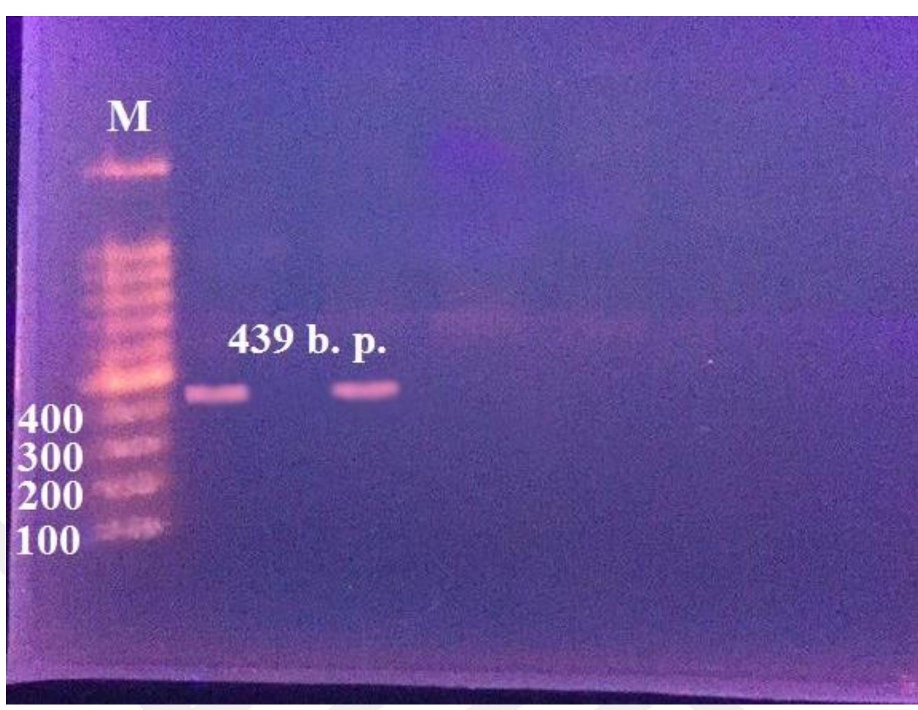

Figure 4.3. *Entamoeba histolytica* (PCR amplification in 439 bp).

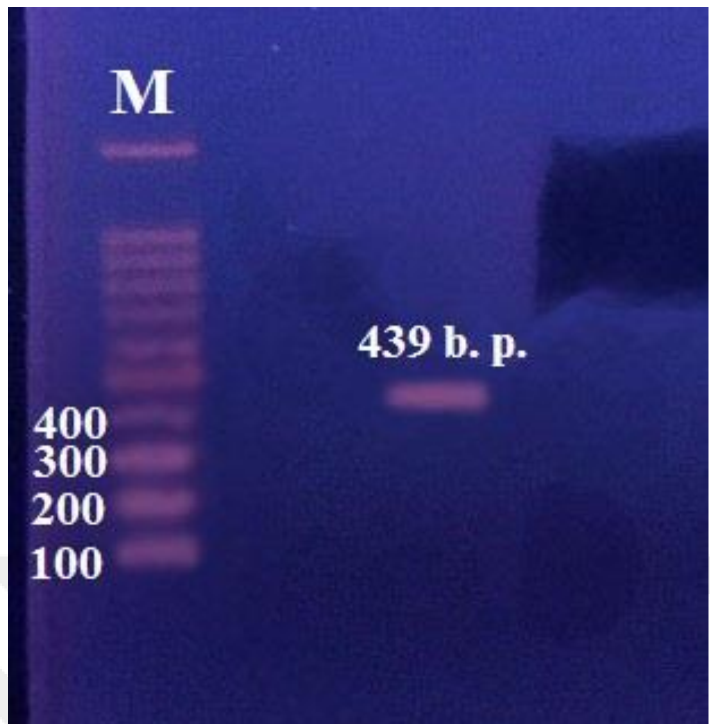

Figure 4.4. *Entamoeba histolytica* (PCR amplification in 439 bp.).

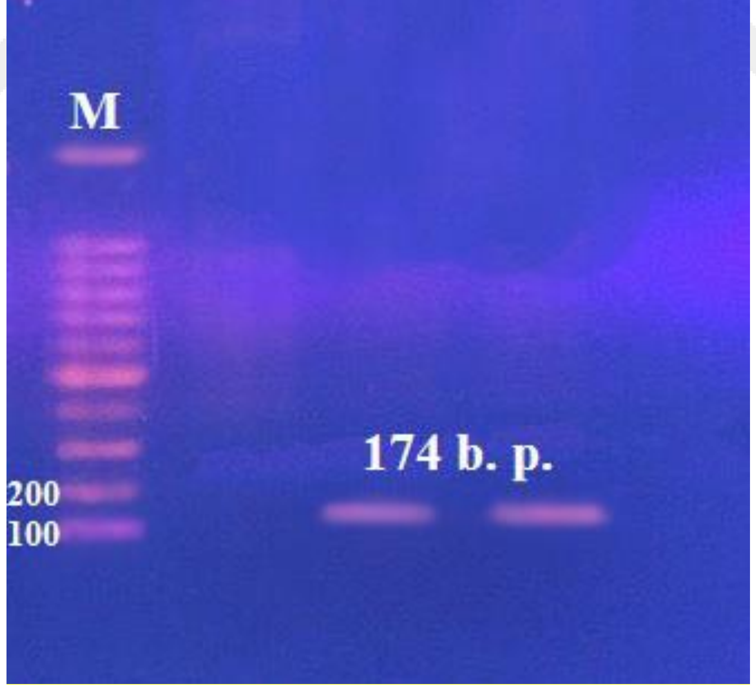

Figure 4.5. *Entamoeba dispar* (PCR amplification in 174 bp).

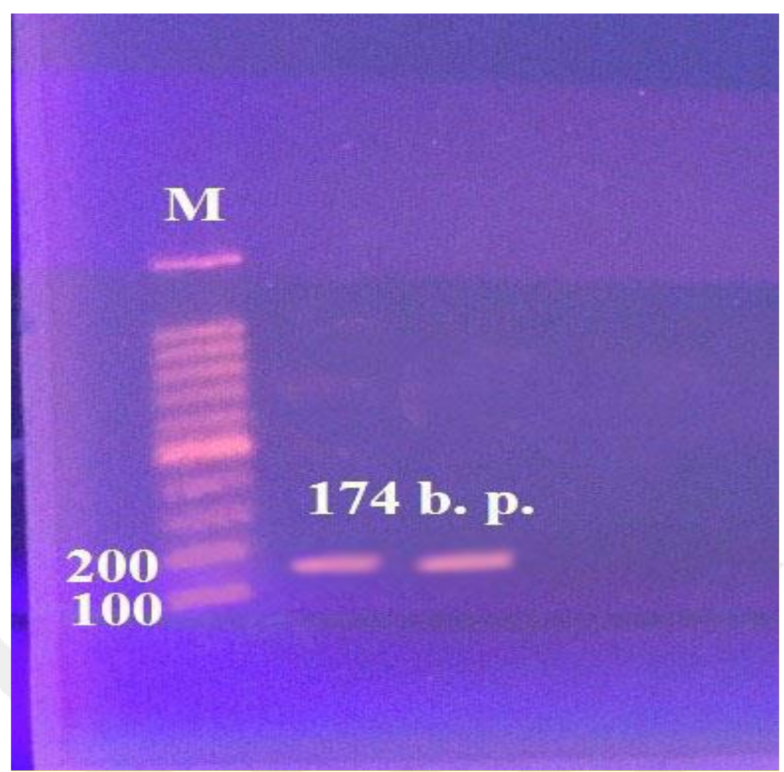

Figure 4.6. *Entamoeba dispar* (PCR amplification in 174 bp).

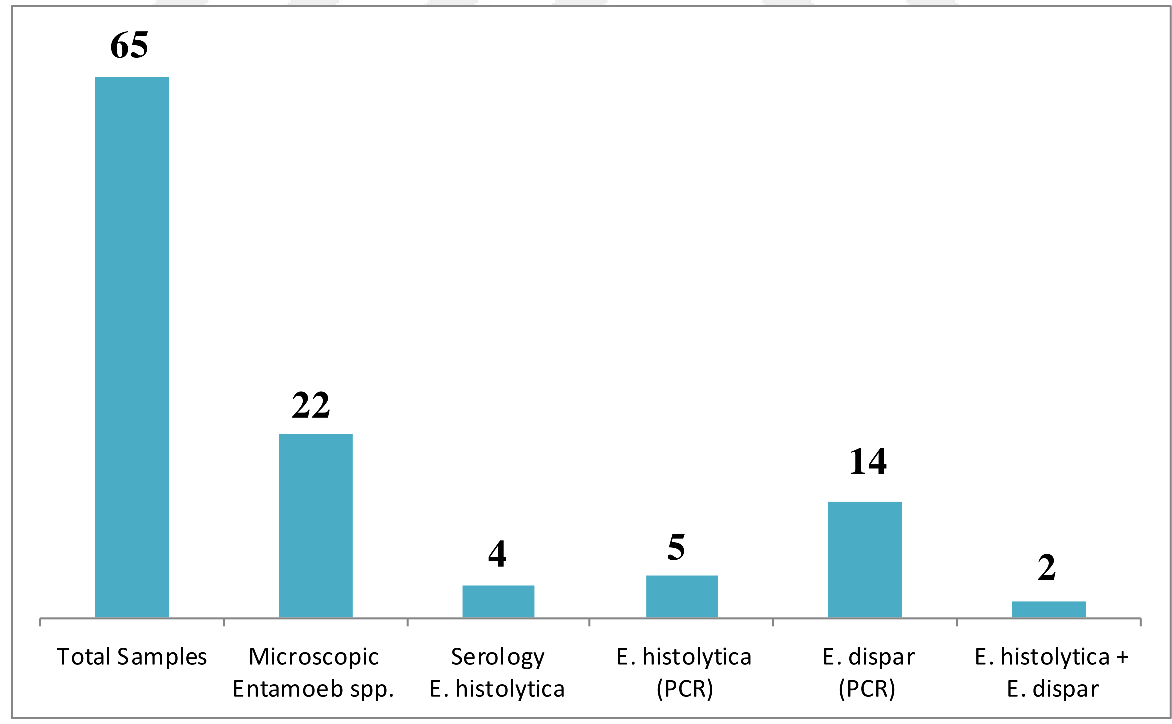

Figure 4.7. Prevalence of *Entamoeba* infections in Duhok city.

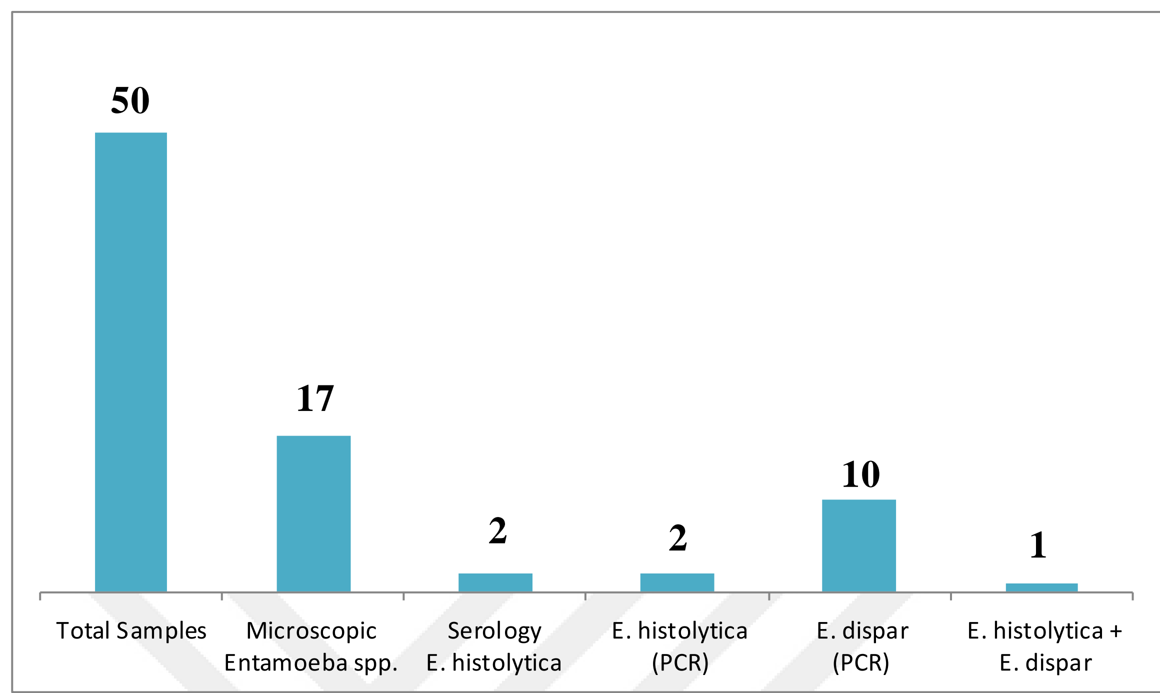

Figure 4.8. Prevalence of *Entamoeba* infections in Erbil city.

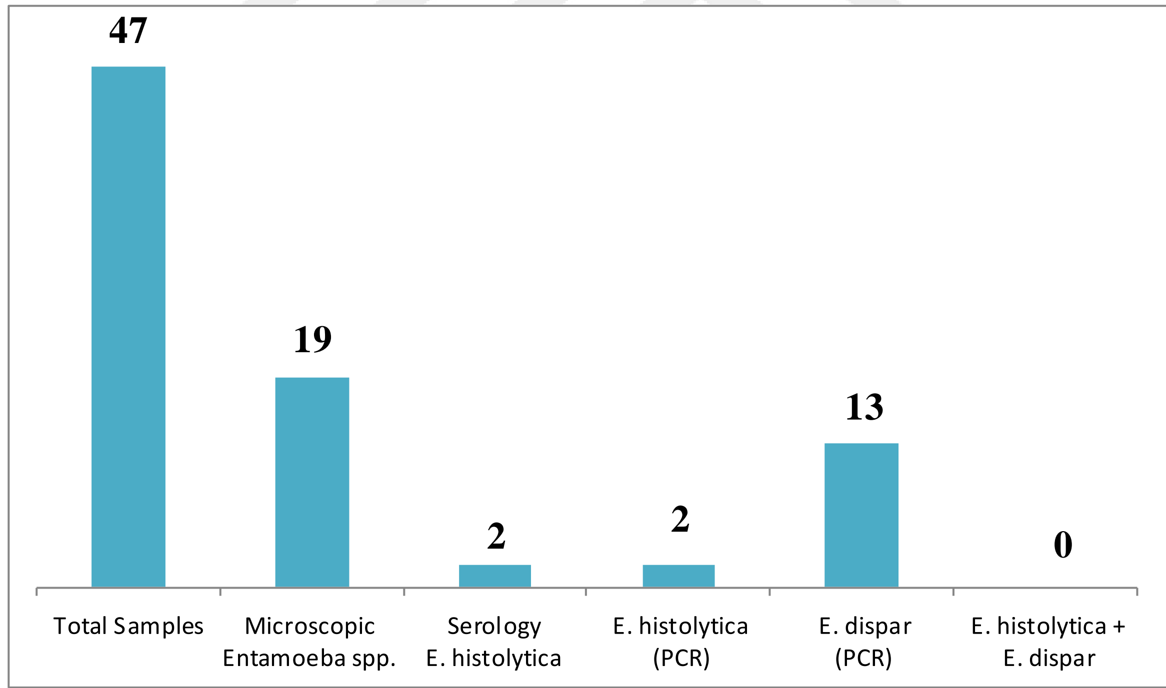

Figure 4.9. Prevalence of *Entamoeba* infections in Sulaimaniyah city.

5. DISCUSSION

Amoebiasis is one of the important protozoan parasite which causes health problems in Iraq, and is more prevalence in developing countries. A large number of researches has been undertaken before differentiation of these two species of *Entamoeba* (*E. histolytica* and *E. dispar*), which morphologically are similar. It is very important to use new techniques and new diagnostic methods in epidemiological studies of amoebiasis to distinguish and differentiate between two species of *Entamoeba*. Microscopic examination is not a sufficiently sensitive method of diagnosis to differentiate between *E. histolytica* and *E. dispar*, so there are other important diagnostic techniques such as serological examination (ELISA test) and the PCR test to distinguish and differentiate between pathogenic *Entamoeba* (*E. histolytica*) and non-pathogenic *Entamoeba* (*E. dispar*) (Zaki and Clark, 2001). There were used microscopical, serological and molecular (PCR) methods for diagnosis and differentiation between *E. histolytica* and *E. dispar* in this study. In the present study, the microscopic diagnosis of *Entameba spp*. showed that the prevalence of *Entamoeba* infection was higher in Sulaimaniyah City (40.4 %) than both Duhok City (33.8 %) and Erbil City (34 %). The total number of *Entamoeba* infections was recorded as 58 positive cases among 162 hospitalized individuals, or about 35.8 % in Duhok City, Erbil City and Sulaimaniyah City. Results from the same diagnostic method (microscopic diagnosis) have been reported by other researchers in the above cities, Iraq, and indeed other countries. For example, Hussein (2010) reported 10.15 % *Entamoeba spp*. infection in Duhok city among children examined in primary schools, which is lower than our findings in Duhok, Erbil and Sulaimaniyah cities. However, this was due to the sample collection season and persons examined because our sample collection was in the summer months and also the samples were collected from hospitalized persons, but Hussein (2010), reported results from the winter season, where the prevalence of the *Entameba* parasite is dependent on the conditions of the drinking water, socio-economic situation, temperature, and a number of other environmental factors which reported by Yilmaz *et al.* (1999). Omer and Bamarni, (2011) reported a 15 % infection rate by

Entamoeba spp. in their research in Duhok city, and whose findings show a lower rate of infection than in our records. The same reports on the epidemiology of the *Entamoeba spp*. have been given by other studies in Erbil City by Molan and Faraj (1989), who reported a 18.6 % *Entamoeba spp*. infection rate. Ali and Mohammed (2010) recorded 38.13% infection rate in Sulaimaniyah City, which is essentially similar to our findings across the entirety of Duhok, Erbil and Sulaimaniyah cities. Al-Ganabi (2002), reported 44.4 % *Entamoeba* infection rate among children in Baghdad (Iraq), in another study, Al-Ganabi (2005) further reported a 57.2 % *Entamoeba* infection rate, which his records are greater than our records. In Van city, Turkey, Taş et al. (2005) reported a low rate of *Entamoeba* infection, (1.1 %) than our findings. In Iran, Kia *et al.* (2007) reported a low rate (1.2 %) of *Entamoeba* infection as compared with our findings. In Sweden, Svenungsson *et al*. (2000) reported 1 % of *Entamoeba spp*. infection in their study and this is also lower than our findings. In this part of the microscopic diagnosis of *Entamoeba spp*. among collected samples, we found a greater infection rate of *Entamoeba spp*. among children (1-6 years) in Duhok, Erbil and Sulaimaniyah cities, which were 45.45 %, 41.17 % and 52.63 %, respectively; our findings agree with those of Al-Ganabi (2002) and Hussein (2010). Also, Ali and Mohammed (2010) reported a high infection rate of the *Entamoeba spp*. among children, but Amir et al. (2011), reported that there was no significant difference amongst infected persons of all ages. In our study, the infection rate of amoebiasis according to sex shows the high percentage of infection in male than female in Duhok, Erbil and Sulaimaniyah cities, Al-Ganabi (2000) found a similar situation, but Amir et al. (2011) found a higher infection rate among females than males. In the current study, it was used serological examination (ELISA test) for diagnosis of *E. histolytica* among all collected stool samples from hospitalized persons in Duhok, Erbil and Sulaimaniyah cities. According to our results, the *E. histolytica* infection rate was higher in Duhok City, which was 7.6 % (4/65) than Erbil and Sulaimaniyah cities, which were 4 % (2/50) and 4.2 % (2/47), respectively, and the total percentage of infection in all Duhok, Erbil and Sulaimaniyah cities was (9/162) 5.5 %, and our findings for infection rates of the *E. histolytica* are lower than those found by Omer and Bamarni (2011), which they reported 10.8 % of infection by *E. histolytica*, which examined by serological method

(ELISA test). These differences may be the effects of the time, conditions and environmental factors that are important for distribution of *E. histolytica* (Huston et al., 1999). Also, there are other studies which examined samples using the serological method, for example Haque et al. (2000) and Roy et al. (2005), which reported serological tests for diagnosis of *E. histolytica*. In another study, Hossain et al. (1983) reported 12 % infection rate in children examined by ELISA method. Also, Haque et al. (2001) reported 14.6% of infection by *E. histolytica* among children which both reports percentages are higher than our findings. Serological examination (ELISA test) is highly sensitive test (90 %) for diagnosis of *E. histolytica* infection which reported by Pillai et al. (1999) and Abdalla et al. (2000). The third line of our study was about molecular diagnosis of *E. histolytica* and *E. dispar*, in this diagnosis method we used Nested PCR amplification to find the *Entamoeba spp*. in collected stool samples, we used Nested PCR technique according to Romano *et al.* (2012). Tannich et al. (1991) used PCR method for diagnosis of *Entamoeba spp*. for the first time, and by this method they were able to differentiate between *E. histolytica* and *E. dispar*. According to the current study we showed that the most infection in Duhok, Erbil and Sulaimaniyah cities were by *E. dispar* and we found a high percentage of this species, for example the WHO (1997) reported a high rate of *E. dispar* infection as compared with *E. histolytica*, however, Petri et al. (2000) and Tachibana et al. (2000) reported a higher percentage of *E. histolytica* infection than *E. dispar* in some countries, such as Mexico and Japan. *E. dispar* is one of the non-pathogenic species of *Entamoeba*, and does not require treatment. To differentiation between these two species, it's very important to use a good method of diagnosis to distinguish between the non-pathogenic and pathogenic *Entamoeba spp*. In our study, we found 7.6 % *E. histolytica* infection rate in Duhok city, which is higher than in Erbil (4 %) and Sulaimaniyah (4.1 %) cities, the total number of infected persons in all Duhok, Erbil and Sulaimaniyah were nine (5.5 %). These findings show that Duhok city contains a higher number of persons infected with *E. histolytica* than Erbil and Sulaimaniyah cities, and it maybe as a result of the higher number of collected samples and drinking water (Huston et al., 1999). However, there are very small differences in the number of infected persons in Erbil and Sulaimaniyah. Lebbad (2010), reported the same results for the *E. histolytica* infection rate, he reported

5.7 % of infection rate in collected samples, which agree with our total results. There are others molecular studies which are same such as Fotedar et al. (2007) and Gutierrez et al. (2010). Also, there are more molecular studies about *Entamoeba* and differentiation between *Entamoeba spp*. In all parts of the world which are agree with our findings. By PCR-based method, the diagnosis of *Entamoeba spp*. are high sensitivity and specificity than other diagnostic methods such as microscopic and serological tests. Haque et al. (1998) reported higher sensitivity of diagnosis in distinguish and differentiation of *E. histolytica* and *E. dispar* parasites. In other molecular diagnostic method (Nested PCR), Roy et al. (2005) recorded higher infection rates of *E. histolytica* and *E. dispar*, and they found high sensitivity and specificity in differentiation of *E.histolytica*and *E. dispar* as compared with serological diagnostic method. Other part of our study was about PCR diagnosis of *E. dispar* in collected stools samples, we found different rates of *E. dispar* infection as compared with *E. histolytica*. In the current study, we found a high percentage of *E. dispar* in collected stool samples, these results showed that there are higher infection rates by non-pathogenic *Entamoeba* (*E. dispar*) than the pathogenic *Entamoeba* (*E. histolytica*). These results agree with reports by the WHO (1997), Markell et al. (1999) and Petri et al. (2000), which they found that the *E. dispar* was 10 times more prevalent than *E. histolytica*. In Duhok, Erbil and Sulaimaniyah cities, we found *E. dispar* infection rates of 21.5 %, 20 % and 27.6 %, respectively. The prevalence of *E. dispar* in Sulaimaniyah city was higher than in Duhok and Erbil cities, and the total infection percentage of *E. dispar* was showed 22.8 % in all Duhok, Erbil and Sulaimaniyah cities. In this study, we showed the high differences between *E. histolytica* and *E. dispar* infection rates, and there are other same studies which showed the same differences. Pechangou et al. (2015) reported a 25 % of *E. dispar* infection rate among HIV patients, which their findings are in close agreement with our findings. Noor et al. (2006) in Malaysia reported a 5.6 % of *E. dispar* infection and it’s lower than our findings. In Malaysia, Romano et al. (2012) worked in Nested PCR technique to diagnose and different *Entamoeba spp*., they found 19.2 % of *E. dispar* infection among examined persons, there findings are lower than our study records. In Iran, Hooshyar et al. (2004) reported a higher prevalence of *E. dispar*(92.1 %) than *E. histolytica* (4.95 %) among positive

Entamoeba spp. parasite. In another study in Iran, Nazemalhoseini Mojarad et al. (2006), found a 69.56 % of *E. dispar* infection among positive *Entamoeba spp*.. Gonin and Trudel (2002) reported a high rate of infection by *E. dispar* (69.4 %) among clinical samples, which is greater than our findings and disagree with our study. In this study, we found that some stool samples were infected with both *E. histolytica* and *E. dispar* (mixed infection), we found two infected samples with mixed infection in Duhok city (3 %) and one infected stool sample with mixed infection (2 %) in Erbil city, total mixed infection samples in our study reported in 1.8 % of infections. The same mixed infection records (*E. histolytica* and *E. dispar*) showed in several studies. In Poland, Myjak*etal.* (2000) found 5.2 % of mixed infection of *E. histolytica* and *E. dispar* in their study but their findings are disagreeing with our results. Hooshyar et al. (2004) reported 2.97 % of mixed infection in their study in Iran, which these results are near to our records. Romano et al. (2012) in Malaysia reported 11.5 % of mixed infection which the obtained records were higher than our results. Differences in rates of *Entameba* infection in different palaces and cities return to differences in environmental conditions such as variation of temperature, seasons, moisture and drinking water and other factors such as number of patient samples, patients ages, sample collection time, diagnostic method, persons immunological condition, type of children feeding, contaminated drinking water, simple life cycle and high resistance of cyst stage of *Entamoeba* parasite which are helpful for distribution of this parasite among persons (Huston et al., 1999). In the current study we found a higher percentage of *E. dispar* than *E. histolytica*, but in Malaysia Romano et al. (2012) and Noor et al. (2006), reported higher prevalence of *E. histolytica* than *E. dispar* and these reports are disagreeing with our findings. Finally, we have been able to show within the current study that molecular diagnosis (PCR) is the best sensitive method for the detection and differentiation of *Entamoeba spp*. in stool samples and the serological method (ELISA) placed in second place as sensitivity for diagnosis and differentiation of *Entamoeba spp*.

6. CONCLUSIONS

We can conclude the following points from this study:

1. In the current study, the prevalence of *E. dispar* was more than that of *E. histolytica*.
2. *E. histolytica* is morphologically similar to *E. dispar*, and we cannot differentiate between these two species of *Entamoeba* by microscopic examination methods alone.
3. The ELISA test is a rapid and simple method to diagnosis of *E. histolytica* in medical laboratories.
4. The molecular diagnosis method is highly sensitive and one of the best method to detect, distinguish and differentiate pathogenic (*E. histolytica*) and non-pathogenic (*E. dispar*) parasite.

Recommendation

The use of ELISA test in all medical laboratories and hospitals to detect the pathogenic species of the *Entamoeba* parasite.

1. Work to doing the more researches about epidemiology and prevalence of amoebiasis to help future studies and finding good diagnosis techniques, good prevention methods and good treatment.
2. Use of research and studies about *Entamoeba spp*. to strain determination and find responsible gene of the virulence in *E. histolytica.*
3. Good hand-washing, avoiding fecal-oral contact, good food preparation and drinking of healthy water are very important points to prevent amoebiasis infection.
4. Focussing on public health education methods and health system.
5. Data from the current study should be send to the general directorate of health by the university to inform all health centres to solve and prevent of this parasite.

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APPENDIX 1

(EXTENDED TURKISH SUMMARY)

KUZEY IRAK BÖLGESİNDE *ENTAMOEBA HİSTOLYTİCA/DİSPAR* YAYGINLIĞININ MİKROSKOPİ VE ELISA İLE ARAŞTIRILMASI VE BU TÜRLERİN NESTED PCR İLE DOĞRULANMASİ

HAZIRLAYAN: Arshad Mohammad ABDULLAH
Danışman: Prof. Dr. Hasan YILMAZ

1. GİRİŞ

Amebiasis, protozoon bir parazit olan *Entamoeba histolytica*'nın neden olduğu ve insanın bağırsak mukozası ve diğer organlarını etkileyen önemli hastalıklardan biridir. Bir protozoon parazit olan *Entamoeba histolytica'nın* neden olduğu amoebiasis, her yıl 50 milyondan fazla kişiyi enfenke etmekte ve yaklaşık olarak 100.000 kişinin ölümüne sebep olmaktadır (WHO 1997). Entamoebanın; *Entamoeba histolytica*, *E. dispar*, *E. moshkovskii, E. coli, E. hartmanni ve E. Polecki* olmak üzere 6 türü bulunmaktadır. Bu parazitlerin çoğu non-patojen olmakla birlikte insanlarda bağırsak lümeninde kommesal olarak yaşarlar. Amoebiasis'e neden olan *Entamoeba histolytica* patojen bir parazittir (WHO- Delialioğlu ve ark., 2008). Amoebiasis, kolon duvarının enfeksiyonu ve karaciğer, akciğer, beyin gibi konak dokularına hasarına yol açan ishal, karın ağrısı, kusma, bulantı ve gaz gibi belirtilerle karakterize olan bir parazittir. Bu semptomlar yetişkinlere kıyasla çocuklarda daha fazla görülür. (Salvioli ve ark., 1992). Fakat *E. dispar* ve *E. histolytica'nın* morfolojik farklılıkları mikroskopta ayırt edilemez (Garfinkel ve ark, 1989; Pillai ve ark, 1999). Bu iki türün ayrımı için serolojik veya moleküler tekniklere ihtiyaç duyulmaktadır. (Aguirre ve ark, 1995, Troll ve ark., 1997). *E. histolytica'nın* trofozoit ve kist olmak üzere iki formu bulunur. İnsanlara bulaş, kist içeren, insan dışkısıyla kontamine olan yiyeceklerin veya içme sularının alınmasıyla bulaşır. Kistler mide koşullarına dayanıklıdır ve mideden bağırsağa geçerek trofozoit formuna dönüşür ve kolona göç eden trofozoitler burada çoğalırlar (Tannich ve ark., 1991). Bazı durumlarda trofozoitler bağırsak mukozasına saldırarak hasar verir ve

dizanteriye neden olur. *E. histolytica'nın* bazı suşları karaciğerde, akciğerlerde ve beyin gibi organlara kan damarları yoluyla veya direkt temas ile geçerek apseler (ülserleşme) gibi ekstraintestinal enfeksiyonlara yol açar. Mobidite ve mortalite ile ilgili olan en yaygın bağırsak dışı enfeksiyonu amebik karaciğer apsesidir. Trofozoitler bağırsakta kistlerinden çıkarlar. Hem trofozitler hem de kistler dışkıya geçer. Enfektif aşama olan parazitin kist formu sağlıklı insanlara bu kistlerin kazara alınması sonucu bulaşabilir (Shandera ve ark., 1998). Bu parazitin labaratuvar tanısında mikroskobik yöntemlerden nativ-lugol direkt bakı, konsantrasyon yöntemleri ve Trikrom boyama yöntemleri kullanılarak parazitin tehişi yapılabilir (Haque ve Petri, 2006). *Entamoeba histolytica'nın* tanısında kullanılan IHA, IFA, ELISA gibi yöntemler mikroskobik yöntemlerden daha fazla duyarlıdır (Haque ve ark., 1995; Krogstad ve ark, 1978). WHO tarafından onaylanan PCR, klinik ve epidemiyolojik çalışmalarda dışkı, doku ve karaciğer apsesi aspiratı içeren birçok klinik türlerinde kullanılan en iyi metotdur (Calderaro ve ark., 2006; Hamzah ve ark., 2006).

Bu çalışmanın amacı, Duhok, Erbil ve Süleymaniye'de hastaneye yatırılan hastalarda *E. histolytica* ve *Entamoeba dispar'ın* mikroskopi (nativ-Lugol ve trikrom boyama ile), ELISA ve Nested-PCR yöntemleri kullanılarak prevalansının belirlenmesidir.

2. MATERYAL VE YÖNTEM

Bu çalışmada Ocak 2016- Mayıs 2016 tarihleri arasında Duhok, Erbil ve Süleymaniye hastanelerinde yatan 162 hastanın dışkı örnekleri incelendi. İlk olarak, örnekler ışık mikroskobu altında nativ-Lugol ve tricrom boyama yöntemleri kullanılarak incelendi. *E. histolytica* ve *E. dispar'ı* birbirinden ayırt etmek için ELISA ve PCR yöntemleri kullanıldı. ELISA yönteminde *E. histolytica* II kiti (TechLab made in USA) kullanıldı. PCR yöntemi için önce dışkı örneklerinde DNA izolasyonu yapıldı. DNA izolasyonu manuel olarak ve DNA izolasyon kiti (QIAgen, Germany) kullanılarak yapıldı. İzole edilen DNA'larin saflık dereceleri NanoDrop spektrofotometre (Thermo Scientific, USA) kullanılarak ölçüldü. Saf DNA'lar elde edildikten sonra Nested PCR yöntemi ile *Entamoeba* türleri tespit edildi. Birinci aşama PCR için F (5'-TAA GAT

GCA GAG CGA AA-3’) **ve** R (5’-GTA CAA AGG GCA GGG ACG TA-3’) primerleri kullanılarak *Entamoeba spp* SSUrDNA gen bölgesi çoğaltıldı. Nested PCR (İkinci aşama PCR)’da *Entamoeba* türlerine özel primerler kullanıldı. *E. histolytica* için F. (5’-AAG CAT TGT TTC TAG ATC TGA G-3’) ve R. (5’-AAG AGG TCT AAC CGA AAT TAG-3’) primerleri kullanılarak 439 bp.’lik bölge çoğaltıldı. *E. dispar* için F. (5’-TCT AAT TTC GAT TAG AAC TCT-3’) ve R. (5’-TCC CTA CCTATT AGA CAT AGC-3’) primerleri kullanılarak 174 bp.2lik bölge çoğaltıldı. Her reaksiyon, her primerden(10 mM) 2,5 µl, 12.5 µl Master mix ve 4 µl örnek DNA(Nested PCR için birinci aşama PCR ürünü kullanıldı)’sı içeren 25 µl’lik hacimlerde yapıldı. Reaksiyonlar ısıtıcı kapağı olan PCR makinesinde yürütüldü. Birinci aşama PCR, her bir örnek 92 °C’de 1 dakika 56 °C’de 1 dakika, 72 °C’de 1 dk ve Nested PCR, her bir örnek 92 °C’de 1 dakika, 48 °C’de 1 dakika, 72 °C’de 1 dakika olmak üzere toplam 30 döngü olarak yapıldı. Her iki PCR işleminde de ilave olarak birinci döngü öncesi 94°C’de 5 dk. denaturasyon, son döngüyü takiben de 72 °C’de 10 dk. ekstensiyon aşaması uygulandı. Nested PCR işlemi sonucunda elde edilen reaksiyon ürünlerinden 5 µl alınarak, sonuçları görüntülemek amacıyla, 100 bp’lik marker ile birlikte jel elektroforezde yürütülerek UV cihazında incelendi.

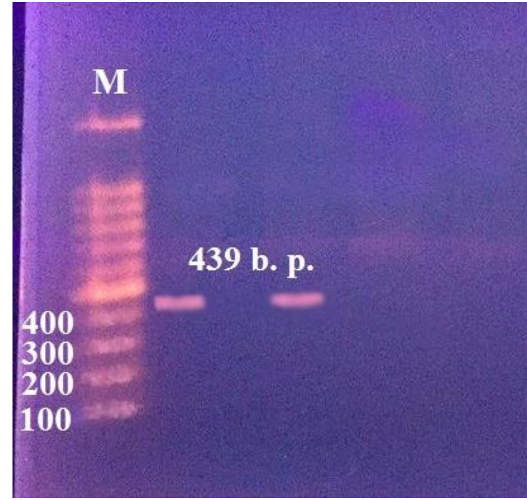
3. BULGULAR TARTIŞMA ve SONUÇ

Nativ lügol ve tricrom boyamaları ile dışkı örnekleri incelenen 162 hastanın 58’inde (%35.8) *Entamoeba spp*. saptandı. *Entamoeba spp*. Duhok, Erbil ve Süleymaniye şehirlerinde sırasıyla 22 (%33.8), 17 (%34) ve 19 (%40.4) kişide saptandı. İkinci aşamada bütün dışkı örnekleri ELISA ile incelendi ve toplam olarak sekiz (% 4.9) dışkı örneğinde *E. histolytica* saptandı. Duhok’ta 65 kişinin dördünde (6,15), Erbil’de 50 kişinin ikisinde (%4) ve Süleymaniye’de 47 kişinin ikisinde (%4.2) *E. histolytica* belirlendi. Daha sonra, tüm dışkı örnekleri Nested PCR ile incelendi (Şekil 1) ve örneklerin dokuzunda (%5.5) *E. histolytica*, 37’sinde (%22.8) *E. dispar* ve üçünde (%1.8) miks enfeksiyon (*E. histolytica* ve *E. dispar* birlikte) saptandı. Duhok şehrinde Nested PCR yöntemi ile beş hastada (%7.6) *E. histolyca*, 14 hastada (%21.5) *E. dispar* ve iki hastada (%3) miks enfeksiyon (*E. histolytica* ve *E. dispar birlikte*) saptandı. Erbil

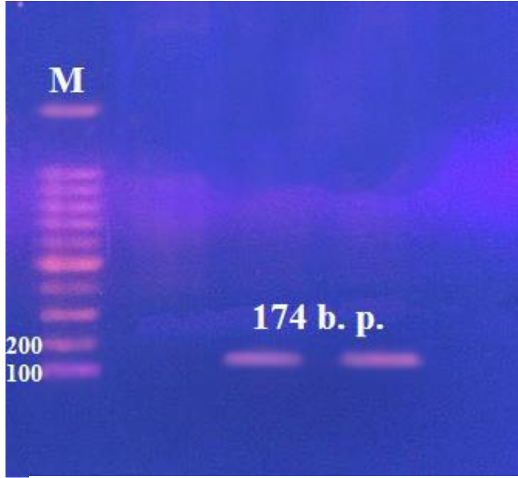
şehrinde Nested PCR yöntemi ile iki hastada (%4) *E. histolytica*, 10 hastada (%20) *E. dispar* ve bir hastada (%2) miks enfeksiyon (*E. histolyca* ve *E. dispar*) saptandı. Süleymaniye şehrinde Nested PCR yöntemi ile iki hastada (%4.2) *E. histolyca* ve 13 hastada (%27.6) *E. dispar* bulundu (Tablo 1). Çalışmada, Nested PCR yönteminin *E. histolytica* ve *E. dispar'ın* saptanması ve birbirinden ayırt edilmesinde diğer tanı yöntemlerinden çok daha duyarlı olduğu ve ayrıca ELISA yönteminin *E. histolytica'nın* saptanması için çok kullanışlı ve kolay uygulanabilir bir yöntem olduğu gözlendi.

Tablo 1. Enfeksiyon sonuçları (Mikroskobik, ELISA, PCR).

Şehir	Mikroskobi *E. spp*	ELISA *E.histolytica*	PCR *E.histolytica*	PCR *E. dispar*	PCR *E. h + E. d*
Duhok (65) (28♂/ 37♀)	22(33.8%) (12♂, 10♀)	4(6.1%) (3♂, 1♀)	5(7.6%) (3♂, 2♀)	14(21.5%) (8♂, 6♀)	2(3%)
Erbil (50) (32 ♂/ 18♀)	17(34%) (10 ♂, 7♀)	2(4%) (1 ♂, 1♀)	2 (4%) (1 ♂, 1♀)	10 (20%) (6 ♂, 4♀)	1(2%)
Süleymaniye(47) (29 ♂/ 18♀)	19(40.4%) (11 ♂, 8♀)	2(4.2%) (2 ♂, 0♀)	2(4.2%) (2 ♂, 0♀)	13(27.6%) (6 ♂, 7♀)	0
Toplam N= 162	58(35.8%)	8(4.9%)	9(5.5%)	37(22.8%)	3(1.8%)



A Şekil 3.1. A) *Entamoeba histolytica* (PCR ürünü, 439 bp.).



Şekil 3.2. B) *Entamoeba dispar* (PCR ürünü, 174 bp.). bp.).

Patient Information Form

Name of Patient

Age of Patient

Sex: Male ☐ **Female..** ☐

City:

Type of Parasite:

APPENDIX INDEX 3

DNA sequence specification sheets of *E. histolytica* and *E. dispar* (IDT)

IDT® INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET — WWW.IDTDNA.COM

04-Aug-2016 — Order No. 12052799 — Ref. No. 148931692

Sequence - Entamoeba histolytica EH-F — 25 nmole DNA Oligo, 22 bases

5'- AAG CAT TGT TTC TAG ATC TGA G -3'

Properties

*Tm (50mM NaCl)**: 50.3 °C
GC Content: 36.4%
Molecular Weight: 6,764.5
nmoles/OD260: 4.6
ug/OD260: 31.2
Ext. Coefficient: 217,000 L/(mole·cm)

Amount Of Oligo

6.1 = OD260 | 28 nmoles | = 0.19 mg
For 100 µM: add 280 µL

Shipped To

BERNARD LAM
NORGEN BIOTEK CORP
3430 SCHMON PARKWAY
THOROLD, ON L2V 4Y6
CANADA
9052278848
Customer No. 186735 PO No. PO7056

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): 1.10 at 25 °C
Strongest Folding Tm: 8.2 °C

Oligo Base Types	Quantity
DNA Bases	22

Modifications and Services	Quantity
Standard Desalting	1

Disclaimer

See on reverse page notes (I) (II) & (III) for usage, label license, and product warranties

Mfg. ID 207325519

Labels - Peel Here

IDT® INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET — WWW.IDTDNA.COM

04-Aug-2016 — Order No. 12052799 — Ref. No. 148931694

Sequence - Entamoeba dispar ED-F — 25 nmole DNA Oligo, 21 bases

5'- TCT AAT TTC GAT TAG AAC TCT -3'

Properties

*Tm (50mM NaCl)**: 45.9 °C
GC Content: 28.6%
Molecular Weight: 6,370.2
nmoles/OD260: 5.0
ug/OD260: 31.8
Ext. Coefficient: 200,500 L/(mole·cm)

Amount Of Oligo

5.4 = OD260 | 26.8 nmoles | = 0.17 mg
For 100 µM: add 268 µL

Shipped To

BERNARD LAM
NORGEN BIOTEK CORP
3430 SCHMON PARKWAY
THOROLD, ON L2V 4Y6
CANADA
9052278848
Customer No. 186735 PO No. PO7056

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): -2.35 at 25 °C
Strongest Folding Tm: 43.6 °C

Oligo Base Types	Quantity
DNA Bases	21

Modifications and Services	Quantity
Standard Desalting	1

Disclaimer

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Mfg. ID 207325521

Labels - Peel Here

148931694 B LAM Entamoeba dispar ED-F

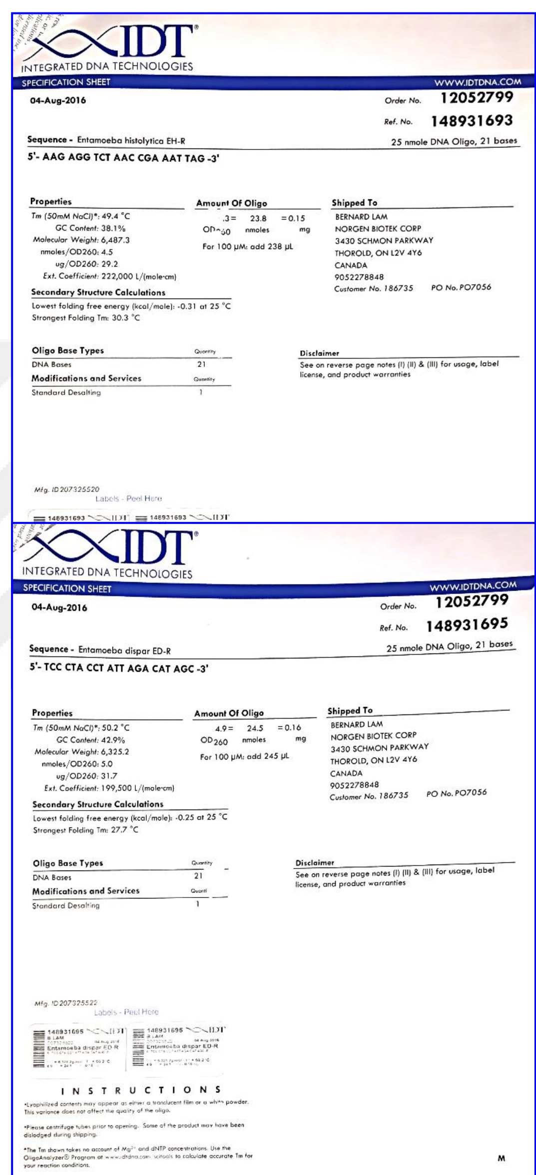
INSTRUCTIONS

•Lyophilized contents may appear as either a translucent film or a ...te powder. This variance does not affect the quality of the oligo.

•Please centrifuge tubes prior to opening. Some of the product may have been dislodged during shipping.

*The Tm shown takes no account of Mg^{2+} and dNTP concentrations. Use the OligoAnalyzer® Program at www.idtdna.com/scitools to calculate accurate Tm for your reaction conditions.

M



IDT® INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET — WWW.IDTDNA.COM

04-Aug-2016 — Order No. 12052799 — Ref. No. 148931693

Sequence - Entamoeba histolytica EH-R — 25 nmole DNA Oligo, 21 bases

5'- AAG AGG TCT AAC CGA AAT TAG -3'

Properties

*Tm (50mM NaCl)**: 49.4 °C
GC Content: 38.1%
Molecular Weight: 6,487.3
nmoles/OD260: 4.5
ug/OD260: 29.2
Ext. Coefficient: 222,000 L/(mole·cm)

Amount Of Oligo

.3 = OD260 | 23.8 nmoles | = 0.15 mg
For 100 µM: add 238 µL

Shipped To

BERNARD LAM
NORGEN BIOTEK CORP
3430 SCHMON PARKWAY
THOROLD, ON L2V 4Y6
CANADA
9052278848
Customer No. 186735 PO No. PO7056

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): -0.31 at 25 °C
Strongest Folding Tm: 30.3 °C

Oligo Base Types	Quantity
DNA Bases	21

Modifications and Services	Quantity
Standard Desalting	1

Disclaimer

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Mfg. ID 207325520

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148931693 IDT 148931693 IDT

IDT® INTEGRATED DNA TECHNOLOGIES

SPECIFICATION SHEET — WWW.IDTDNA.COM

04-Aug-2016 — Order No. 12052799 — Ref. No. 148931695

Sequence - Entamoeba dispar ED-R — 25 nmole DNA Oligo, 21 bases

5'- TCC CTA CCT ATT AGA CAT AGC -3'

Properties

*Tm (50mM NaCl)**: 50.2 °C
GC Content: 42.9%
Molecular Weight: 6,325.2
nmoles/OD260: 5.0
ug/OD260: 31.7
Ext. Coefficient: 199,500 L/(mole·cm)

Amount Of Oligo

4.9 = OD260 | 24.5 nmoles | = 0.16 mg
For 100 µM: add 245 µL

Shipped To

BERNARD LAM
NORGEN BIOTEK CORP
3430 SCHMON PARKWAY
THOROLD, ON L2V 4Y6
CANADA
9052278848
Customer No. 186735 PO No. PO7056

Secondary Structure Calculations

Lowest folding free energy (kcal/mole): -0.25 at 25 °C
Strongest Folding Tm: 27.7 °C

Oligo Base Types	Quantity
DNA Bases	21

Modifications and Services	Quantity
Standard Desalting	1

Disclaimer

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Mfg. ID 207325522

Labels - Peel Here

148931695 B LAM Entamoeba dispar ED-R

INSTRUCTIONS

•Lyophilized contents may appear as either a translucent film or a whi.. powder. This variance does not affect the quality of the oligo.

•Please centrifuge tubes prior to opening. Some of the product may have been dislodged during shipping.

*The Tm shown takes no account of Mg^{2+} and dNTP concentrations. Use the OligoAnalyzer® Program at www.idtdna.com/scitools to calculate accurate Tm for your reaction conditions.

M

CURRICULUM VITAE

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1. Name: Arshad Mohammad Abdullah

2. Date of birth: 01/01/1979 / Duhok- Iraq/ Email: arshadzanko@gmail.com.

B. Education:

1. Bachelor Degree & Master of Science Degree (M.Sc.): Duhok University/ College of Science Education/ Biology Det. /Parasitology.

2. PhD Student / Parasitology/ Van Yuzuncu Yil University- Turkey

C. Language skills:

Kurdish, English, Turkish, Persian, Arabic.

D. Employment history:

Assistant Lecturer in Faculty of Medical science, University of Duhok.

E. Conferences:

Participation in 4 international biological conferences.

F. Research April and Publications

1- Comparative study on the biochemical parameters in hydatid cyst fluids of sheep, goats, cattle and human cystic forms of *Echinococcus granulosus*.

2- The Epidemiology of Hydatidosis in Different Slaughtered Animals in Duhok abattoir.

3- Comparative study of enzyme activity in hydatid cyst fluids of *Echinococcus granulosus* in sheep and goats in Duhok City.

4- Antibacterial activity of some plant extracts against clinical pathogen.

5- Prevalence of Intestinal Parasites (*Entamoeba spp*. and *Giardia lamblia*) in Duhok and Erbil cities, Northern Iraq.

UNIVERSITY OF VAN YUZUNCU YIL
THE ISTITUTE OF NATURAL AND APPLIED SCIENCES
THESIS ORIGINALITY REPORT

Date: 28/03/2018

Thesis Title: Prevalence study of *Entamoeba histolytica/ dispar*, by microscopic and serological diagnosis (ELISA) using species verification by Nested PCR in North Iraq region.

The title of the mentioned thesis, above having total 16 pages with main parts and conclusion, has been checked for originality by Turnitin computer program on the date of 28/03/2018 and its detected similar rate was 8% according to the following specified filtering

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- Excluding the Materials and Methods
- Excluding the Bibliography,
- Excluding the Citations,
- Excluding the publications obtained from the thesis,
- Excluding the text parts less than 7 words (Limit match size to 7 words)

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Name and Surname: Arshad Mohammad ABDULLAH

Student ID#: 139102117

Science: Biology

Program: ……………………………………………………………….

Statute: M. Sc. ☐ Ph.D. ☐

APPROVAL OF SUPERVISOR
SUITABLE

(Title, Name-Surname, Signature)

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