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and Scientific Research  
University of Diyala  
College of Medicine**



**Molecular Detection of Some *Entamoeba*  
Species from Diarrheic Patients in Baqubah-  
Iraq**

**A Thesis**

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Fulfillment of the Requirements for the Degree of Master of Science in  
Medical Microbiology.**

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ وَمَا مِنْ دَابَّةٍ فِي الْأَرْضِ إِلَّا

عَلَى اللَّهِ رِزْقُهَا وَيَعْلَمُ مُسْتَقَرَّهَا

وَمُسْتَوْدَعَهَا كُلٌّ فِي كِتَابٍ مُبِينٍ ﴾

صدق الله العظيم

سورة هود، الآية (٦)

## SUPERVISOR CERTIFICATION

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# *DEDICATION*

*This thesis is dedicated*

*To My Father ..... With Respect*

*To My Mother ..... With Love*

*To My Brother..... With Gratitude*

*To My Sisters..... With love*

*To All My Friends..... With Special Thanks*

*Musab*

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## Summary

Diarrhea is one of the most common health complaints. It can range from a mild, temporary condition, to a potentially life-threatening one. Most cases of diarrhea are caused by an infection in the gastrointestinal tract. The etiological agents of diarrhea include viruses, bacteria and parasites.

The present study included 326 patients who attend the parasitology laboratories in Baqubah Teaching Hospital and AL-Batool Maternity Teaching Hospital/Diyala governorate, suffering from gastrointestinal complaints with acute diarrhea. Age ranged from  $>2$  year to  $\leq 19$  years. A questionnaire was applied from healthy subjects and patients. All samples of fresh feces were examined by light microscopy; the remaining samples were kept at  $-20^{\circ}\text{C}$  for DNA extraction were analyzed with conventional multiplex polymerase chain reaction (PCR).

The rates of the enteric protozoan detected by microscopy from patients with diarrhea were *E. histolytica* /*dispar*/*miscoviskii* 150(46.01%), *Giardia lamblia* 27 (8.28%), *Entamoeba coli* 10 (3.06%) *Balantidium coli* 4 (1.22%). From the total *Entamoeba* positive patients, approximately half of them were under 6 years old, with male percentage was slightly greater than female (58% versus 42%). The high frequency of *Entamoeba* was demonstrated in patients from rural areas 81(54%).

According to questioner 61 (40.66%) children's mother who are illiterate or incomplete primary school. The data analysis showed that 104 patients were drinking tap water, the infection rate increased (62%) when the hand washing with water only, and most the children who fed on artificial milk were found infected with *Entamoeba* (12%), and in present study microscopic revealed that there were 10/150 (6.66%) cases of co-infection between *Entamoeba spp.* and other parasites.

Molecular characterization of 150 amoebiasis patients was done by conventional multiplex PCR was performed for detecting *Entamoeba spp.* by amplification the gene small-subunit ribosomal RNA (SSUrRNA). Eighty six samples amplified out of 150 samples (57.33%). However, the amplification of these samples showed that 56 (65.11%) represented *E. histolytica* and 22 (25.58%) samples contained *E. moshkovskii*, while 8 (9.30%) represented *E. dispar*. There were 12 cases of mixed infection between *E. histolytica* and *E. moshkovskii*, and 5 cases of mixed infection between *E. histolytica* and *E. dispar*. The majority of the three types of *Entamoeba* positive cases were observed in patients 2-5 years age group, and the gender preference to get the infection with *E. histolytica*. The only demographic factor which had a significant association in this regard was the residence, in which 73.21% of subjects infected *E. histolytica* were rural resident which are far greater than those infected with *E. moshkovskii* or *E. dispar* (40.91% and 50% respectively). The most children's mother who are illiterate or incomplete primary school more prone to get the infection with *Entamoeba Spp.*

The study revealed that most patients drinking tap water found to have the highest *E. histolytica*, *E. moshkovskii*, and *E. dispar* infections were found (44/56, 12/22, and 6/8), also the infection rate increased with *E. histolytica*, *E. moshkovskii*, and *E. dispar* 60.71%, 59.09%, and 62.5% respectively when the hand washing with water only, and most the children who fed on artificial milk were found infected with *E. moshkovskii* (27.27%).

Clinical symptoms appeared to have a significant association with a particular species of *Entamoeba*. These included abdominal pain and fatigue. However, Pus cells and RBCs were detected in all cases of *E. histolytica* compared with 72.72% and 75% of cases with *E. moshkovskii* and *E. dispar* respectively, trophozoites were detected in 80.35% of fecal samples from patients infected with *E. histolytica*.

In contrast, cysts and bacteria were detected in different rates in fecal samples from patients infected with different species of *Entamoeba*, and *Monilia* was detected in only two fecal samples from patients with *E. histolytica*.

Furthermore, the genetic relatedness of the local isolates sequence were analyzed with MEGA 6 software through constriction phylogenetic tree. Isolate 1, 2, 4, 5, 11, 12 and 14 clustered with KP233840.1, KP233838.1, and KP233837.1 which are *E. histolytica* type of Iraqi isolation and also clustered with GQ423748.1 which is *E. histolytica* type of Phillipian isolation, as well as clustered with AB608092.1 which is *E. histolytica* type of Japanese isolation. Isolate 8, 9 and 13 clustered with KP722602.1, KP722603.1 and KP722605.1 which are *E. moshkovskii* type of Iraqi isolation. Isolate 6 and 7 clustered with KT825980.1 and KT825978.1 which are *E. dispar* type of Colombia isolation.

The present study aims to determine the rate of infection, risk factors of *Entamoeba* species, and then molecular identification and characterization co-infections, clinical features of different species of *Entamoeba*.



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### List of Abbreviations

Abbreviate	Key
Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
ALA	amoebic liver abscess
<i>A. lumbricoidis</i>	<i>Ascaris lumbricoidis</i>
<i>B. coli</i>	<i>Balantidium coli</i>
<i>B. hominis</i>	<i>Blastocystis hominis</i>
BLAST	Basic local alignment search tool
Bp	Base Pair
CAP	Cellulose Acetate Precipitin test
CIE	Counter Current Immunoelectrophoresis
<i>C. parvum.</i>	<i>Cryptosporidium parvum.</i>
CRD	carbohydrate recognition domain
dATP	Deoxyadenosine triphosphate
DCs	Dendritic cells
dCTP	Deoxycytosine triphosphate
dGTP	Deoxygunosine triphosphate
DFA	Direct fluorescent antibody
<i>D. fragilis</i>	<i>Dientamoeba fragilis</i>
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
<i>E. coli</i>	<i>Entamoeba coli</i>
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
<i>E. vermicularis</i>	<i>Entrobium vermicularis</i>
G	Gram
<i>G. lamblia</i>	<i>Giardia lamblia</i>
HIV	Human Immunodeficiency Virus
<i>H. nana</i>	<i>Hymenolipis nana</i>
IEC	intestinal epithelial cells
IHA	Indirect Haemagglutination
Ig	Immunoglobulin
LA	Latex Agglutination

Multiplex RT-PCR	multiplex Real time Polymerase chain Reaction
NCBI	National center for biotechnology information
NF $\kappa$ B	nuclear factor kappa B
No	Number
PCR	Polymerase chain reaction
PMBCs	peripheral blood mononuclear cells
PMN	Polymorphonuclear leukocytes
PMNs	polymorphonuclear leukocytes
Um	Micrometer
SNPs	single-nucleotide polymorphism
spp	Species
TNF-a	tumor necrosis factor alpha
tRNA	transfer Ribonucleic acid
WHO	World Health Organization
WS	Wash Solution
SSUrRNA	Small sub unit ribosomal RNA
IL	Interleukin
bp	Base bare
G=C	Guanine = cytocine
IFN $\alpha$	Interferon alfa
IFN- $\gamma$	Interferon gamma
A and T	Adinine and Thimine
CFT	Complement Fixation Test



# **Chapter One**

## ***Introduction***

## 1.1 Introduction

Diarrhoea is defined as the passage of three or more loose or liquid stools per day (or more frequent passage than normal for the individual), Diarrhoea is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms,

Acute diarrhea is defined as the abrupt onset of 3 or more loose stools per day and lasts no longer than 14 days; chronic or persistent diarrhea is defined as an episode that lasts longer than 14 days (WHO, 2017). Diarrhea is the main cause of morbidity and mortality among infants and young children, particularly in low-resource settings (Basmaci *et al.*, 2018). It is a symptom of infections caused by several bacterial, viral and parasitic organisms. However, with regard to developed countries, the prevalence of intestinal protozoan parasites is higher than that of intestinal helminthes (Rai *et al.*, 2017), every year over 350 million patients were infected with intestinal protozoan parasitic infection (Scanes and Toukhsati, 2018)

Amoebiasis is a common intestinal protozoan infection of the human gastrointestinal tract caused by *Entamoeba histolytica* which causes widespread mortality and morbidity worldwide through diarrheal disease and abscess collection in parenchymal tissues such as liver, lung, and brain. *E. histolytica* is a unicellular parasite. It moves by rapidly produce thick and hyaline pseudopodis. The prevalence of infection is unknown for most areas of the world due to the difficulty to characterize *E. histolytica* versus other amoebas with identical morphology, as *Entamoeba dispar*, and *Entamoeba moshkovskii* (Mohamed *et al.*, 2016). For many years *E. dispar* was considered a non-pathogenic, noninvasive parasite that did not cause disease. However, *E. dispar* has been associated with a few cases of amebic colitis and amebic liver abscesses, putting in question its status as a virulent ( Heymann, 2015 ). Both species occur in two forms: the hardy, infective cyst and the fragile, potentially pathogenic trophozoite (Alberta health, 2018). Another

four-nucleated morphologically identical organism, *E. moshkovskii*, has been observed in sewage as a free-living amoeba, but is also capable of colonizing the human intestine (Ngui *et al.*, 2012). Although, dysentery and extraintestinal disease have been proposed to be potentially associated with *E. dispar* and *E. moshkovskii* (Costa *et al.*, 2010). These findings complicated our understanding of the pathogenic behavior and public health importance of indistinguishable *E. histolytica/ dispar/ moshkovskii* complex (Oliveira *et al.*, 2015).

The majority of infections are asymptomatic (90% of cases) (Heymann, 2015). Symptoms, when occurred, it ranges from mild abdominal discomfort with diarrhea containing blood or mucous to acute or fulminating dysentery with fever, chills and bloody or mucoid diarrhea. Complications of prolonged infection include extraintestinal disease such as ameboma or abscesses in the liver, lungs, heart, brain, skin or other organ (Public Health Agency of Canada, 2014). Humans and other primates are the only known reservoirs (Stanley, 2003). Transmission is through the ingestion of fecally contaminated food or drinks, sexual exposure (usually anal sex) or through the unwashed hands of an infected food handler. The prevalence of *E. histolytica/dispar* in Iraq is 48% and 3.7% respectively. Najaf, Wasit, Basra, Diwaniya and Miasan provinces showed the highest prevalence rates, while the lowest prevalence was reported in Anbar, Diyala, Thiqr, and Erbil (Al Saqur *et al.*, 2017).

Microscopic examination of stool for cysts and trophozoites remains the most common test available for amoebiasis diagnosis. However, it lacks specificity for *E. histolytica*, easy diagnosis of amoebiasis now depends on the use of immune-chromatography and/or PCR is extremely sensitive and useful in differentiating *E. histolytica* from *E. dispar* and *E. moshkovskii* (Farthing *et al.*, 2013).

Symptomatic amoebiasis should be treated with antimicrobial. A follow-up stool should be done by the physician to ensure elimination of the organism ( Heymann, 2015). Contacts with positive stool specimens should be managed and treated as cases. The problem of treat or do not treat infection depends on whether it is pathogenic or not, and whether is recorded in endemic or non-endemic area (Alberta health, 2018). Recent Findings distribution of amoebiasis varies greatly in different regions of the world reaching up to 50% (El-Dib, 2017). The phylogenetic analysis of the *E. histolytica*, *E. dispar* and *E. moshkovskii* showed a variety of genotypes which may show the big variation in pathogenicity of humans (El-Dib, 2017).

### **1.2 Aims of the study**

Microscopical and molecular identification of different human species of *Entamoeba* and the possibility of causing symptoms by another species rather than *Entamoeba histolytica* by diagnostic study of stool samples taken from patients with clinical symptoms, investigate the relationship between *Entamoeba* species infection and some socio-environmental factors, and molecular characterization of *Entamoeba* species and phylogenetic analysis.