Republic of Iraq

Ministry of higher education and scientific research

University of Basrah

College of Science

Department of Biology

Histological and diagnostic study of Amoebiasis in Basrah governorate

Athesis submitted to the council of college of Science – University of Basrah in partial fulfillment of the requirements for the degree of Doctor of philosophy in

Biology / Parasitology

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2020 AD

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة البصرة
كلية العلوم
قسم علوم الحياة

دراسة نسجية وتشخيصية للإصابة بداء المتحولات الاميبي في محافظة البصرة

اطروحة مقدمة الى

مجلس كلية العلوم جامعة البصرة

وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في علوم الحياة

علم الطفيليات

من قبل

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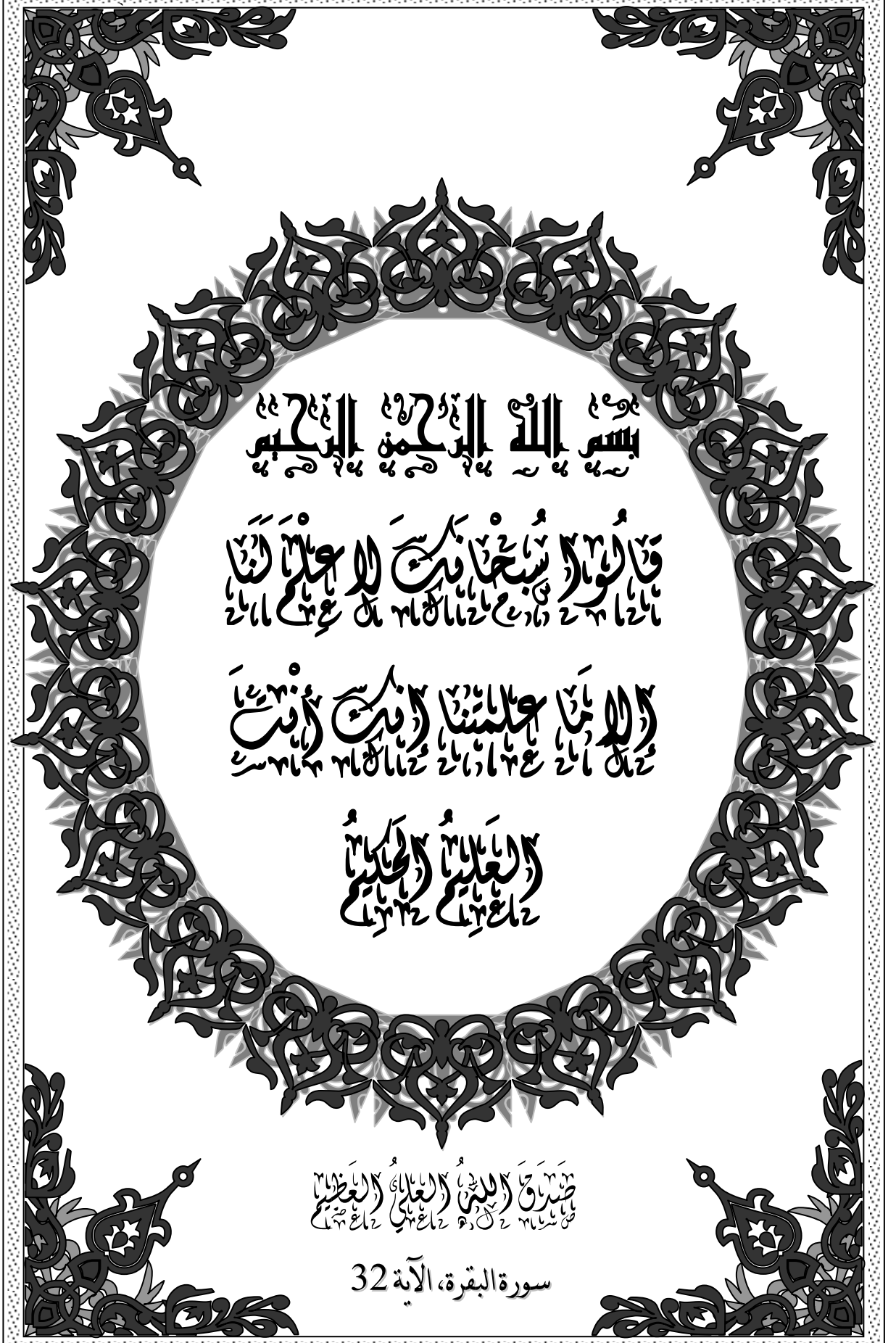
بسم الله الرحمن الرحيم
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صدق الله العلي العظيم
سورة البقرة، الآية 32

Dedication

This thesis is dedicated to…….

My anchor in this stormy sea of life ……. ***My mother***

The loving spirit who taught me the values of life…… ***My father***

Athraa

Acknowledgement

This work cannot be finished without the help of others……

First of all, I am grateful for Almighty God who gave me the strength to keep going.

I want to express my deep gratitude and heartfelt thanks to my supervisors**, Prof. Dr. Maha K. Al-Malak** and **Prof. Dr. Muslim A.A. Al-Tomah** for their listening, guidance , inspiration and continuous support throughout my PhD. Study.

My sincere thanks to the Dean of the College of Science for the support and for facilitating all my issues during the study. In addition , my gratitude goes to **Prof. Dr. Munaff J. Abd-Al-Abbas** the head of the Department of Biology for his continuous support.

My great thanks for **Assist**. **Prof. Dr. Shereen J. Al-Ali,** Department of Pathological Analyses, College of Science, University of Basrah for her scientific piece of advice ,continuous support that helped me overcome many crisis situations during my study. I have been amazingly fortunate to have her by my side.

Also my deepest thanks for **Prof. Dr. Adnan Issa Al-Badran** for his useful piece of advice. My great thanks to **Prof. Dr. Sabeeh H. Al-Mayah** from the College of Education / Pure Sciences / University of Basrah his useful scientific consultation.

I want to express my thanks to **Prof**. **Dr. Dhamia k. Suker** for allowing me to work at Cell Research and Biotechnology Laboratory.

Also my thanks go to **Dr. Rafid A. Dolab Alsaray** from the College of Pharmacy, **Prof. Dr. Mazin Auny Mahdi** and **Assist Prof. Dr. Jalal Jabbar Hassan** from Department of Physic / College of Science for their help with preparing and examine the electron microscope samples.

Special thanks for **Dr. Hussam S. Aziz and Assistant Lecturer Zahraa H. Al- Mabark** from Southern Technical University for their help to complete the qRT-PCR result of my thesis.

I offer my sincere thanks to my friend **Dr**. **Najwa M. J. A. Abu-Mijdad** for standing by my side when times get hard, for making me laugh when i didn't even want to smile. A warm thanks to my colleagues **Dr. Mohammed H. Minati** and **M.sc Zeena Hashim Abdul Wahid** for their support during my study. God bless them all.

Finally, I want to thank my beloved mother which is my rock in life and my family.

To everyone who support me even with a smile thanks a lot

Athraa

Summary

Amoebiasis is a gastrointestinal infection caused by a number of *Entamoeba* spp., and *Entamoeba histolytica* is the main species to cause the pathological symptoms of this disease. It is widely spread, causing mortality across the world through diarrhea.

This study was determined the prevalence of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* by three methods of diagnosis (microscopic examination, cultivation and PCR) that were compared to obtain an accurate diagnosis of *Entamoeba* spp. Total (n=150) stool samples related to patients (n = 100) and healthy controls (n= 50). Clinically diagnosed stool samples (n=100) were collected from patients attending the consultant clinics of different hospitals in Basrah during the period from January 2018 to January 2019. All samples were examined in a direct microscopic examination, The results showed that 60% of collected samples were positive.

Also all samples were cultivated on different media; the Brain heart infusion agar showed high efficiency and was most suitable in cultivating the parasite. Data and results of molecular study indicated by DNA extraction from stool samples and used in PCR technique with specific primers. The highest infection in Basrah patients was *E. moshkovskii* 15% followed by *E. dispar* 10% and *E. histolytica*, which was 5%.

The current study investigates the pathological changes of *Entamoeba* spp. infection in both rectum and cecum of experimental rats. The results showed the histological changes at the 7th, 14th, and 28th day post-infection for the three species. *E. dispar* and *E. moshkovskii* infection showed less pathological changes compared to *E. histolytica*. These changes include the attachment of the trophozoites to the mucosal layer,

significant surface epithelial changes such as dissociation and degeneration in the mucosal layer, and ulceration of the apical surface. Inflammatory cells infiltrate the varied regions, extending into the deep mucosa causing mild architectural alterations.

In the current study, Real-Time PCR technique was used to study the gene expression by using specific primers of tight junction genes (Cldn1, Ocln) for the large intestine cells (cecum) of rats affected by the three species separately.

The results showed that all tested samples gave positive results for the gene expression after 7, 14 and 28 days of infection with the three species, but the *E. moshkovskii* gave the highest level of expression in all periods.

The electronic microscope (Scanning and Transmission) was also involved in the current study to accurately determine the pathological changes resulting from infection of the three species. The results showed the adhesion of trophozoite and its effect on the surface layer of the large intestine, as well as the changes in the properties of this phase with the presence of filopoda and internal vesicles.

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List of Abbreviation

%	Percentage
+ Ve	Positive
µl	Micro liter
µm	Micrometre
AB	Absence microvilli
ALA	Amoebic liver abscess
Anova	Analysis of variance
AP	Amoebapore
AP-A	Amoebapore A
AP-B	Amoebapore B
AP-C	Amoebapore C
B	Bacteria
BDMA	Benzyl dimethyl amine
BHIA	Brain heart infusion agar
Bp	Base pair
C	Crypt opening
C_3	Component 3
Ca^{+2}	Calcium ion
cDNA	Complementary deoxyribo nucleic acid
CF	Complement fixation
Cldn1	Claudin-1
Cm	Free surface
C°	Centigrade
Cps	Cysteine proteases
Ct	Threshold cycle
CY	Dense cytoplasm
D	Desmosome
D.W.	Distilled water
DDSA	Dodecenyl succinic anhydride
Dm	Degraded mucus
DNA	Deoxyribonucleic acid
DPX	Dextrin plasticizer xylene
E	Eosin
E.	*Entamoeba*
E. Coli	*Escherichia coli*
ECM	Extracellular matrix
Ehcp	*Entamoeba histolytica* cysteine proteases
EL	Extensive lysis

ELISA	Enzyme-linked immunosorbent assay
EN	Enterocytes
F	Lipid droplets
FB	Intercellular fiber
FFPE	Formalin-fixed paraffin-embedded
Fig.	Figure
FV	Food vacuoles
G	Glycocalyx
G.	Gram
Gal/GalNAC	Galactose/n-acetylgalactosamine
GALT	Gut associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Genomic dna
Gids	Gastrointestinal disorders
H	Haematoxylin
H.	Hour
HK	Housekeeping gene
IBD	Inflammatory bowel disease
Ig	Immunoglobulins
IPIS	Intestinal parasitic infections
IS	Intercellular space
L	Lumen
L.	Dna ladder
LE	Lock egg medium
M	Mucosa surface
ME	Muscularis externa
Mg	Milligram
Min.	Minutes
Ml	Milliliter
Mm	Millimeter
MM	Muscularis mucosa
MNA	Methy nadic anhydride
Mt	Mitochondria
Mu	Mucus
MUC2	Mucin
MV	Microvilli
N	Nucleus
N.S.	Normal slain

Ng	Nanogram
No.	Number
Ocln	Occludin using
Oligo(Dt)	A short sequence of deoxy-thymine nucleotides
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PH	Power of hydrogen
PK	Proteinase K
qPCR	Quantitative polymerase chain reaction
R	Red blood cell
RBCS	Red blood cells
RNA	Ribonucleic acid
Rpm	Round per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real time polymerase chain reaction
S.	*Schistosoma*
S1	Isolate 1
S2	Isolate 2
S3	Isolate 3
SAW	Surface acoustic wave
Sec.	Second
SEM	Scanning electron microscope
SM	Submucosa
SPED	Specific primer for *E. dispar*
SPEH	Specific primer for *E. histolytica*
SPEM	Specific primer for *E. moshkovskii*
Spp.	Species (plural of specie1).
SPSS	Statistical package for social science
STEM	Scanning transmission electron microscope
T	Trophozoite
Tag	*Thermo aquaticus*
TBE	Tris borate-edta
TEM	Transmission electron microscopy
TJ	Tight junction
TJS	Tight junctions
Troph.	Trophozoite
TYI-S-33	Trypticase-yeast extract-iron-serum
UK	United kingdom
USA	United states of america
UV	Ultra violet

V	Multivesicular bodies
-Ve	Negative
WBC_S	White blood cells
WHO	World health organization
Yrs.	Years
ZO	Zonula occludens
ZO-1	Zonula occludens-1
ZO-2	Zonula occludens-2
ZO-3	Zonula occludens-3

1.1 Introduction

In many countries, amoebiasis is considered to be one of the major public health problems, it is caused by the genus *Entamoeba* which includes several species, such as *E. histolytica*, *E. dispar* and *E. moshkovskii*. One of the main reasons that this disease is widely spread in developing countries is the lack hygienic measurements and contamination water, in addition, the disregarding of parasitic infections in such countries increases the number of these infections (Watanabe and Petri, 2015).

Amoebiasis is a human gastrointestinal infection caused by *E. histolytica*, a primary parasite that causes widespread mortality and morbidity, it affects 40-50 million people around the world through diarrheal disease, *E. histolytica* is able to invade the intestinal mucosa and form an abscess in tissues such as the liver and lung . The symptoms of invasive amoebiasis include amoebic colitis and extra-intestinal manifestation represented by a potentially fatal liver abscesses. Notably, other species may infect humans including *E. dispar* and *E. moshkovskii* (Roure *et al* .,2019).

E.dispar is a morphology-like species of *E.histolytica* and colonizes the human intestine. It has recently been identified as a different species without any invasive capacity (Uslu *et al* .,2016). The recognition of *E. dispar* as a separate but strongly associated protozoan species has had wide effect for the epidemiology of amoebiasis, this non-invasive species is responsible for most of the asymptomatic infection in worldwide (Al-Areeqi *et al*., 2017).

E. moshkovskii has a genetic relationship with *E. histolytica*, *E. dispar* and its cyst and trophozoite forms are microscopically indistinguishable from them. *E. moshkovskii* is a prevalent *Entamoeba* in some environments that causes infection in humans (Khomkhum *et al*.,2019). It is revealed in fecal samples from patients with gastrointestinal symptoms in

limited studies from Australia, Bangladesh, India, Iran, Tanzania, and Turkey, therefore suggesting that this parasite could cause disease (Shimokawa *et al* .,2012).

The main laboratory diagnosis of *Entamoeba* spp. in human is microscopic examination of stool samples but it is not possible to distinguish between these types through it (Parija *et al*. , 2014). The morphological similarity between *Entamoeba* spp. is a major concern as it may lead to mistreating the patients,. therefore, the need of accurate diagnostic method is important, this method represented by the molecular diagnosis using polymerase chain reaction (PCR) technique which has been used in different regions around the world (Bahrami *et al*. , 2019).

Molecular diagnosis showed that the distribution of *E. dispar* was 10 times higher than that of *E. histolytica* worldwide, studies reported the relationship between *E. dispar* and the clinical symptoms (Ngui *et al*. ,2012).

Most molecular studies used PCR techniques to differentiate *E. histolytica* from *E. dispar*, whereas the diagnosis of *E. moshkovskii* was ignored, studies have shown that *E. moshkovskii* caused gastrointestinal disorders (GIDs) in human which is an appropriate host for this species (Fotedar *et al* . ,2008).

In Basrah many studies were conducted regarding *Entamoeba* spp. Al-Yaquob (2008) used the PCR technique for the detection and differentiation of *Entamoeba* spp. and compare this method with microscopic examination. Moreover, Al-Abodi (2015) refers to phylogenetic sequencing for species *E. histolytica* , *E. dispar* and *E. moshkovsk*ii in Al-Qadisiya .

Although *E. histolytica*, *E. dispar*, and *E. moshkovskii* are identical in appearance their pathogenicity differ from each other. Moreover, the pathogenicity of the *E. dispar* and *E. moshkovskii* is not well understood. Normally *E. histolytica* colonized in colon epithelium and the damage

caused by it result from many activities such as adhesion ,lysis and phagocytosis of the host cells. These activities can be carried out by several virulence factors found in the parasite including cysteine proteases that play a crucial role in *E. histolytica* tissue invasion (Caffrey *et al*.,2018; Rangel *et al* .,2019), the Galactose/N-Acetylgalactosamine (Gal/GalNAc) lectin (Verma and Datta, 2017), and amoebapores, a small peptides which cause pores the cell membranes of the target cells. The amoebapores enable a big outflow of extracellular Ca^{+2} which is linked to the release of amoebic proteases at the contact place with substrates degeneration and during *E. histolytica* infection an inflammatory response occurs. The parasite resists such response due to the degradation of complement component by the parasite (Saha *et al*.,2015,Burgess *et al*., 2017) Furthermore, the adherence of *E. histolytica* trophozoite result in the death of the host cell by apoptosis (Betanzos *et al*.,2019).

In general, proteases affect different types targets including mucin, extracellular matrix (ECM) components, tight junction proteins, immunoglobulins, complement and cytokines, then amoeba swallow the cell debris by phagocytosis (Cornick *et al*.,2016). The clinical aspects of amebiasis are intestinal or extraintestinal depending on the influence organ, Four forms of invasive intestinal amebiasis are clinical forms, all of which are acute in general: bloody diarrhea, colitis, amebic appendicitis, and ameboma of the colon. Invasive intestinal amebiasis responsible for 90% of dysenteric syndrome (Debnath *et al* .,2019). In many distinct clinical types, colorectal amoebiasis may found in different clinical from the asymptomatic case to severe necrotizing colitis. Therefore, the diagnosis should be accurate by taking a rectal biopsy as it may detect the existence of the parasites and at the same time eliminate the possibility of a carcinoma (Hardin *et al*.,2007).

In the intestine epithelia tight junction (TJ) service as intercellular cell seal preventing the pathogen from penetrating the epithelia ,these tight junctions (TJs) consist of a belt like region between the epithelial cells that separate the apical from the lateral plasma membrane and regulate ions passage as well as other molecules through the adjacent cells (Capaldo *et al*., 2017). TJs consist of integral protein such as occludin which was the best studied member of TJ protein ,in addition another TJ protein is claudin-1. These proteins interact with the actin cytoskeleton via TJ adaptor proteins like zonula occludens(ZOs) (Lee,2015). Many studies record that the parasite is able to alter gene expression of the host cells (Faust *et al*., 2011; Lopez *et al* .,2019).

The electron microscope provides a great understanding for the pathology of the parasite . Moreover scanning electron microscope (SEM) allows researchers to detect and localizes the parasite as well as specific surface exposed molecule (De Souza and Attias , 2018).The electron microscope was used to confirm *E. histolytica* invasion into the intestine(Espinosa and Martinez ,2000).

1.2 Aim of study

Since *Entamoeba* spp. are important enteric protozoa causing invasive and non-invasive disease, the study is designed to understand the histopathology, molecular biology and the invasion rout of the parasite. This will lead to the improvement of the diagnostic methods, prevention of the infection and give us different options of treatment.

In order to achieve the aim of the current study many objectives needed as follow:

1. The diagnisis of *Entamoeba* spp. from human stool samples using microscopical and molecular methods.
2. Cultivation of the parasite using basic and new cultivation media
3. Evaluate the histopathological changes of cecum and rectum in all experimental rats with induced amoebiasis during (7,14,28)days post infection.
4. Evaluation of the expression of tight junction genes (Claudin-1, Occludin) during *Entamoeba* spp. and clarify their linkage to amoebiasis during experimental infection.
5. Detection of the histological changes and cytopathology pathway during the experimental the amoebiasis using transmission and scanning electron microscope .

2.1 Historical background

Infection with *E. histolytica* was observed for the first time by Losch in 1875 as he notices the presence of amoebas within colons ulcers when he induced the infection in dogs by rectal inoculation using human feace. The disease caused by *E. histolytica* was confirmed by other researchers including Councilman and Lafleur and was distinguished from other non-pathogenic commensal such as *E. coli* by Schaudinn in 1903 (Meles and Bekele,2017).

The fact that *Entamoeba* spp. has more than one species was pointed out by Emile Brumpt in 1925 as *Entamoeba* has two species one (*E. dysenteriae*) causes the symptomatic infection and the second is *E. dispar* which causes asymptomatic infection . Louis Diamond in 1961 was allowed to study *E. histolytica in vivo* and *in vitro* (Pinilla *et al* .,2008).

In 1978 , Sargeaunt and Williams distinguished *E. histolytica* strains for the first time using isoenzyme electrophoresis which was a confirmation of *Entamoeba* being a complex species including pathogenic and non-pathogenic species . Diamond and Clark in 1993 reconfirm the existence of *E. histolytica* and *E. dispar* as a pathogenic and non-pathogenic species , respectively which are morphologically indistinguishable (Fotedar *et al* .,2007).

When Emile Brumpt described *E. dispar* in 1925 his description was dismissed by his colleagues as a synonym of *E. histolytica* until 1970 when evidence indicated Brumpt description of *E. dispar* as a separate species(Clark,1998).

E. moshkovskii or ″ Laredo strain″ was described for the first time by Tshalaia in 1941 in Moscow, Russia (Heredia *et al* ., 2012).

2.2 *Entamoeba* spp.

Intestinal parasitic infections (IPIs) infect 1/3 of world population. At least 50% of approximately 450 million infected people are children. Many factors affect IPI infection such as lack of health care , poverty, tropical wet climate and illiteracy (Gharibi *et al*.,2017) . *Entamoeba* spp. Include several species six of which infect human : *E. histolytica, E. dispar ,E. moshkovskii , E. hartmanni , E. coli* and *E. polecki* .

E. histolytica is the only species that causes pathological changes while the other are non-pathogenic species (Gomes *et al* .,2014).The diagnosis of *E. histolytica* depends on the microscopic examination which is an easy , useful and cheap tool . However , this method cannot distinguish *Entamoeba* spp. which are morphologically similar *E. dispar* and *E. moshkovskii* (Khan *et al* .,2019).

Accurate identification is achieved by molecular method such as PCR which is useful to identify between *E. histolytica* , *E. dispar* and *E. moshkovskii* in different samples (Cui *et al*.,2019).

2.2.1 *E. histolytica*

The pathogenic protozoa *E. histolytica* described as a unicellular organism belong the family Entamoebidae. Walker and Sellards in 1913 described cyst stages isolated from a symptomatic patients can cause disease .1969 the world health organization (WHO) define a amoebiasis as an infection caused by *E. histolytica* with or without clinical manifestation (Nowak *et al* ., 2015).

2.2.2 *E. dispar*

E. dispar is a non-pathogenic species of *Entamoeba* spp. Which is morphologically similar to *E. histolytica* this species was isolated from asymptomatic patients and identified as a separate species by isoenzyme patterns of amoeba . Both *E. histolytica* and *E. dispar* share nearly similar genetic sets that encode the majority of the parasite virulence factors . Approximately 12 % of the population worldwide are infected with both *E. histolytica* and *E. dispar* , the infection with *E. dispar* are substantially more common than *E. histolytica* . In order to differentiate between *E. histolytica* and *E.dispar* an amplification of gene segment is required from the parasite isolated from stool samples . Moreover , epidemiological studies suggest that the majority of the asymptomatic cases caused by *E. dispar* (Oliveira *et al* .,2015).

In 1997, Espinosa and colleagues inoculated $5x10^{5}$ trophozoites of *E. dispar* in hamsters for 7 days , after this period the authors notice the presence of focal inflammatory infiltration without formation of necrosis or granuloma in addition , *in vitro* study showed the secretion of toxic products by *E. dispar* in less amounts and less activity in compare with *E. histolytica* .

When *E. dispar* strains isolated from patients in the North and South of Brazil were inoculated in hamsters and rats in liver and cecum caused both hepatic and intestinal lesion were formed and they are similar to those of *E. histolytica* in addition to the presence of the trophozoite (Gomes *et al*.,2000).

Furthermore , molecular identification (DNA sequences) of *E. dispar* used to detect it in patients with amebic liver abscess. This case suggest that *E. dispar* may be participate in developing lesions in both intestine

and liver of human (Graffeo *et al* ., 2014). In Brazil, *E. dispar* strain cause amoebic liver abscess (Shibayama *et al* .,2007; Ximenez *et al* ., 2010).

Other studies demonstrate the formation of hepatic lesion induce by *E. dispar* trophozoite given to hamsters with the original intestinal flora of the host . Moreover , these lesions are similar to the hepatic lesions caused by *E. histolytica* suggesting a role of bacterial flora in influencing the pathogenicity of *E. dispar* (Costa *et al* ., 2006).

Studies using *in vivo* and *in vitro* experiments showed that different strains of *E. dispar* from different origin can cause pathological changes for the liver and intestine in combined with the normal flora of the intestine (Dolabella *et al* ., 2012). In addition to the pathological changes *in vivo* and *in vitro* that is caused by *E. dispar* these changes can found in some humans (Fotedar *et al*., 2007).

Experimental infection with *E. dispar* wister rats showed intestinal lesion with the destruction of mucosal and submucosal inflammation, moreover, the inflammation might reached the serosa(Oliveira *et al* .,2015). These pathological changes suggest that *E. dispar* induce pathological changes in human intestine (Espinosa *et al* ., 1998).

Previous studies showed the association of *E. dispar* and clinical symptoms and GIDs in travelers (Herbinger *et al* .,2011).In contrast , Dvorak *et al*.(2003) suggested that *E. dispar* (SAW760 and SAW1734) strains are non-virulent .

Similar finding was reported by Costa *et al*.(2000) when they inoculate hamsters with *E. dispar* trophozoites isolated from symptomatic and asymptomatic individual in Brazil. Furthermore, *E. dispar* was

reported to be frequently associated with gastrointestinal symptoms such as fever , abdominal pain and diarrhea (Pestehchian *et al* .,2011).

Although *E. dispar* have the ability to engulf Red Blood Cells (RBCs) *in vitro* but it does not do that in the colon ,in case of bleeding in the colon by another infection such as shigellosis (Ackers,2002; Boettner *et al* ., 2005).

2.2.3 *Entamoeba moshkovskii*

The third species of *Entamoeba* is *E. moshkovskii* which is also similar in morphology to *E. histolytica* and *E. dispar* (Bahrami *et al*., 2019). At the beginning *E. moshkovskii* was considered to be a free-living environmental strain but when isolated from a person lived in Laredo, Texas, this person was suffering from diarrhea, gastric pain and weight loss and the parasite was named as *E. histolytica* Laredo strain which have similar features to *E. moshkovskii* (Wilson *et al*.,2019).

Both the Laredo strain and *E. moshkovskii* are resistant to emetine and this feature also distinguish them from *E. histolytica* and *E. dispar*. Later on molecular study has confirmed the *E. histolytica* Laredo strain and *E. moshkovskii* is the same (Ali *et al* ., 2003). *E. moshkovskii* was first isolated from sewages but recent studies reported the presence of *E. moshkovskii* from fecal samples of human . Most of these studies were conducted in North America, Italy , South Africa , Bangladesh , India , Iran , Australia and Turkey Australia, and Turkey (Lopez *et al* ., 2015).

Although earlier studies did not show any association between *E. moshkovskii* and clinical symptoms , recent ones found this association in both Bangladesh and India and consider *E. moshkovskii* as pathogenic species causing gastrointestinal symptoms (dysentery)

(Soares *et al* ., 2019). Many studies such as Bahrami *et al.*(2019) reported the same association along with others from Bangladesh, Malysia and Tunisia (Ayed *et al* ., 2008 ; Anuar *et al* ., 2012). Other studies linked *E. moshkovskii* with GIDs in India and Australia (Fotedar *et al* ., 2008). Subsequently the association was reported between *E. moshkovskii* with diarrhea in human and mice (Shimokawa *et al* .,2018).

Shimokawa *et al*. (2012) report the *E. moshkovskii* and various symptoms such as diarrhea , colitis and weight loss in mice similar to *E. histolytica*. Moreover , the pathological changes in the intestine epithelium in mice infected with *E. moshkovskii* caused apoptosis (Khomkhum *et al*., 2019). In addition , reports showed that *E. moshkovskii* may cause subcutaneous abscess in individual in Indonesia (Sri *et al* ., 2018).

2.3 Biology and morphology of *E. histolytica*

E. histolytica can be found in the human body in forms , infective cyst and vegetative trophozoite.

1. Trophozoite

The vegetative stage has a diameter of 12-60 µm and surrounded by lipoprotein cell membrane , it can form ameboid pseudopodia to help it with it nutrition and movement. The cytoplasm is divided into ectoplasm and endoplasm which have fine grain ,the endoplasm contain cell organelles including lysosomes , Golgi apparatus , endosome , food vacuoles and glycogen mass. *E. histolytica* trophozoite do not have mitochondria, for this reason the energy is distributed anaerobically wins the protozoan of glucose that release the glycogen mass (Nowak *et al*., 2015).

The round nucleus contain the genetic materials which concentrated as small , dense karyosome locate in the center and a peripheral chromatin which is deployed evenly. The structure of the nucleus has been a characteristic feature of the family Entamoebidae as it has a highly polymorphic nuclei. The position and shape of the karyosome and to location of the chromatin are the criteria used to differentiate between the members of this family (Nowak *et al*., 2015) . The differences of nuclei structure of a amoebas show in Table 2.1.

Table 2.1 Structure of the nuclei of amoebas in the family Entamoebidae.

***Entamoeba* spp.**	**Peripheral chromatin**	**Karyosome**
E. histolytica ***E. dispar*** ***E. moshkovskii***	Fine granules, uniform in size	Small, compact, round, centrally situated
E. coli	Chromatin clumped	Large, not compact, round, always centrally or eccentric situated
E. polecki	No peripheral chromatin or chromatin clumped in large granules	Large, compact, round, always centrally situated
E. hartmanni	Morphology similar to *E. histolytica* , chromatin may appear as solid ring	Small, compact, round, always centrally or eccentric situated

2. cyst stage

This stage is also called the infective stage that cause the disease , Basically , the cyst is the same as a trophozoite but it is surrounded by specific membrane that allow the cyst to survive hard conditions in the environment outside the host body for several days . The specific membrane of the cyst consist of multi-layer , chitin which protect the cyst from harsh external environment (Stanley and Samuel ,2003). The cyst is round measured 10-20 µm in diameter , have 1,2,3 or 4 nuclei with karyosome with a peripheral placed chromatin similar to the trophozoite. The mature cyst contain glycogen found in cytoplasm like an irregular stains appear like a dark orange- brown stains when examin it using Lugol's iodine . In addition the cyst have blunt finished chromatodial bars (Ali *et al*., 2012). The characteristic of cysts of members of Entamoebidae family shown in Table 2.2.

Table 2.2 Morphological characteristics of cyst of the family Entamoebidae.

***Entamoeba* spp.**	**Shape of cyst**	**Glycogen mass**	**Nuclei**	**Chromatoidal bars**	**Size of cyst(µm)**
E. histolytica ***E. dispar*** ***E.moshkovskii***	Round	Glycogen irregularly distributed in the cytosol	1 to 4	large fingers, blunt finished	10-20
E. coli	Round to slightly oval	+	1-18	small	10-33
E. polecki	Round	+	1	+/-	5-17
E. hartmanni	Round	+	1-4	+/-	4-8

2.4 Life Cycle

Basically , several species within the genus *Entamoeba* can live in human but not all of them can cause disease . *E. histolytica* is the pathogenic species of this genus and it is well known of its pathogenicity both intestinal and extraintestinal infection . The importance of the other species came from confusing caused by the similarity of the *Entamoeba* spp. morphologically (Wilson *et al*., 2019).

During the parasite life cycle the main reservoir is an infected person who expel cysts within his feces and in the same time they became the source of infection . The way that food might be contaminated is via flies, which are vector to various infective stages including *E. histolytica*, *Salmonella* and *Shigella* . while water can be contaminated through releasing of wastewater into the rivers or lakes or the area of drinking water intakes. Therefore , factors such as bad sanitary condition , no running water and not following the hygienic rules lead to *E. histolytica* infection (Gałecki and Sokoł , 2019).

Many factors influence the prevalence of *E. histolytica* one of these factors is the weather as excessive rainfall , floods and droughts may help eliminate the cysts , but it also decrease the effectiveness of water treatment (Miraglia *et al* .,2009).

The infection with *E. histolytica* begin with ingestion the infective stage (cyst) orally from contained food or water (fecal – oral transmitted way) (De Valdoleiros *et al*., 2019) . The cysts is resistance to the low pH of the gastric juice of the stomach, the process of excystation happen in the small intestine releasing the trophozoite which then migrate to the large intestine. The trophozoite used binary fission to multiply and produce cysts by encystation then both stages pass through feces to the external

environment but because of the nature of the cyst wall it can survive from days to weeks and become the source of infection , while trophozoites are destroyed once they are outside the body and in case that trophozoites were ingested it will be killed by the gastric juice(Kantor *et al*., 2018).

Both cysts and trophozoite passed through feces , the cyst normally found in hard stool while the trophozoite found in diarrhea stool , *In vitro* the cyst is resistance to different chemicals in water and water chlorination and still have its ability to cause a disease but the cyst can be killed by boiling (Shirley *et al*., 2018).

Most of the cases trophozoite stay in lumen of the intestine (non-invasive infection) of asymptomatic individuals . In other cases , trophozoite invade the intestinal mucosa (intestinal disease) reaching the bloodstream to extraintestinal locations such as liver , brain and lungs (Wuerz *et al* ., 2012), this case called extraintestinal disease . It is now well known that invasive case caused by *E. histolytica* while the non-invasive ones caused by a separate species *E. dispar* . Although the 2 species are morphologically alike *E. histolytica* can ingest RBCs in a process called erythrophagocytosis (Fig. 2.1) (Haque *et al*., 2003).

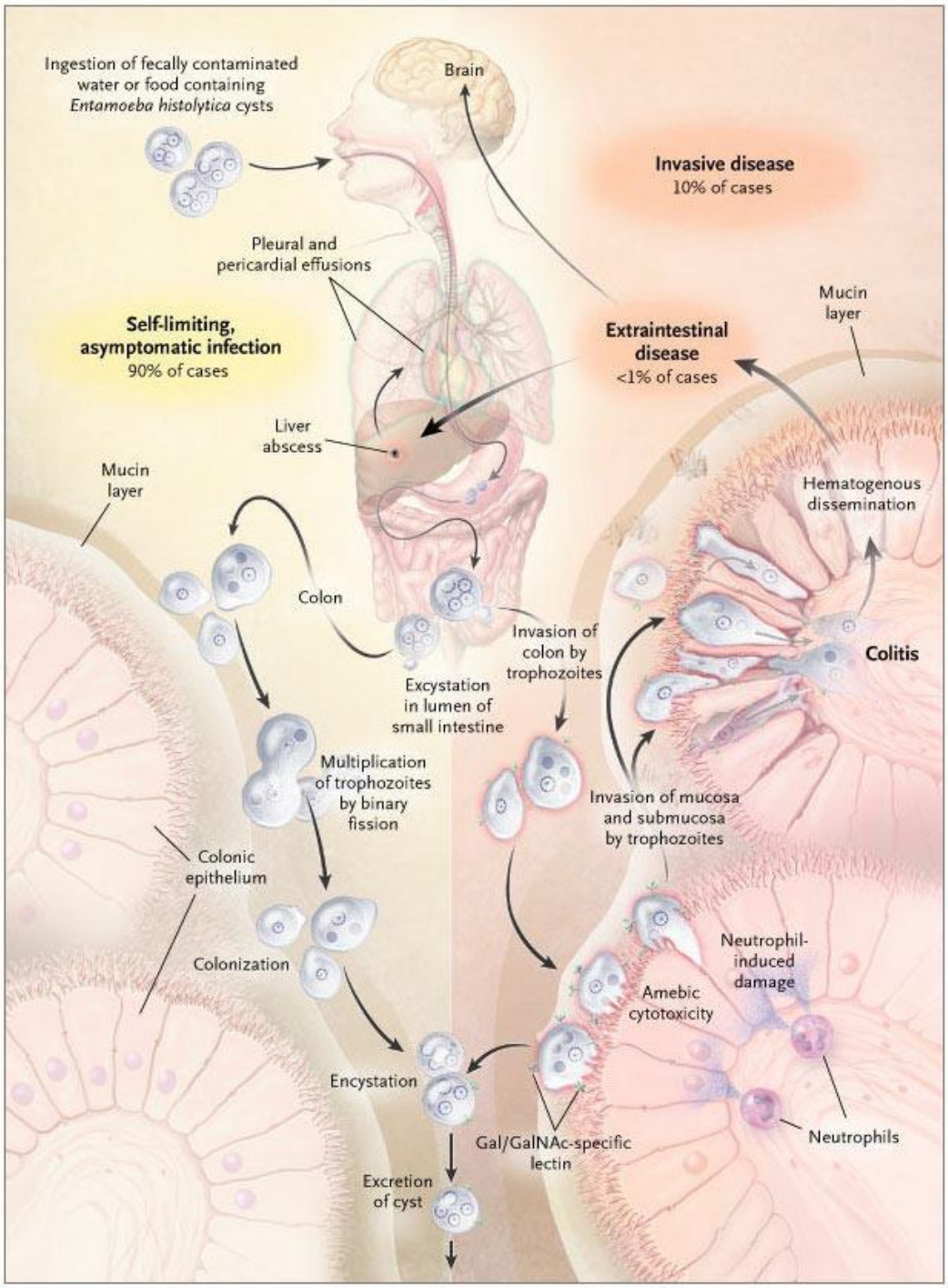


Fig. 2.1 *E. histolytica* life cycle (Haque *et al*., 2003).

2.5 pathogenesis of intestinal amoebiasis

Pathogenesis is the mechanisms that involve the initiation, evolution and ultimate outcome of the disease. It normally relates to factors of both the host and parasite (Kantor *et al* ., 2018).

The mechanism of amoebiasis pathogenesis is still now not fully understood as many factors may have an effect on the level of the pathological changes, one of these factors is the different strains of *E. histolytica* that can be isolated from asymptomatic and symptomatic individuals. These differences determine the various clinical symptoms of the disease that caused by the parasites virulence factors (Samie *et al.*,2012).

The pathological changes depend on many factors such as the severity of infection, the resistance of the host and the nature of nutrition. Moreover, the progress of the disease depend on the size of the parasite colony , the motility of intestine , the population size of the normal flora , the parasite adhesion, strain virulence ,the host environment, immune status ,age and gender (Bernin *et al*., 2014).

Many studies demonstrate the interaction between the intestinal normal flora of the host and *E. histolytica* may lead to the pathogenicity of the parasite and to generate more virulent strains (Dolabella *et al*.,2012). Galvan *et al*.(2008) illustrate that bacteria live in the gut increase the expression of Gal/GalNAC lectin in the trophozoite of *E. histolytica* leading to the increase of adhesion capacity . Furthermore the production of inflammatory cytokine was increased in the presence specific gut bacteria which may increase the damage of the epithelium allowing the trophozoite to invade the gut.

The clinical features of *Entamoeba* spp. infection ranged from asymptomatic infection or non-invasive infection which represent about 80-90 % of the total infections to amoebic colitis which also known as dysentery or diarrhea , the latest one involve the invasion of the gut and extend to extra-intestinal sites most commonly the liver causing liver abscesses (Fotedar *et al*.,2007).

The definition of asymptomatic amoebiasis is the type of infection when *E. histolytica* found in the stool without any sign of colitis. Furthermore , the persistence of asymptomatic infection which is the non- invasive kind progress to an invasive one when trophozoites penetrate the intestinal mucosa (Ali *et al*.,2008).

The amoebic colitis featured by a flask shaped ulcer in the gut , moreover, it can be associated by amoebic granuloma formation (ameboma)which can be define as an inflammatory and granulation occur in the intestinal wall surrounding the ulcer similar to colonic cancer (Kataria *et al*.,2018).The pathogen *E. histolytica* have may virulence factors , one of these factors is its ability to spread into different organs causing the extra-intestinal manifestation of amoebiasis .

The liver is the most frequent organ to be infected via the portal venous system (Lubbert *et al*.,2014).The spread of trophozoite through blood to other organs like lungs , spleen and brain rarely occur . The lungs are the second common organ to be infected after the liver as it follow an extension of liver abscess through the diaphragm to the lung . Regarding the spleen , the spleen gets infected because of the rupture of the abscess which extent to the peritoneum (Kaushik *et al*.,2013).The brain can be infected with *E. histolytica* via the invasion of trophozoite to the central nervous system via blood , this infection normally is deadly

(Maldonado *et al*., 2012). Bouali *et al*. (2014) diagnosed three cases of amoebic cerebral abscess in Tunisia two of the 3 causes were men at 33 and 43 years of age and the third was a 56 years old woman. There is another rare type of amoebiasis which is cutaneous amoebiasis, the type of infection caused by contaminated skin or can be caused by the continuous contact with exudates that contain trophozoite (Lamps, 2010).

During the invasive of the parasite , the trophozoite invade the epithelium by killing epithelial cells ,this action cause the abdominal pain, weight loss and bloody diarrhea with no fever which is a characteristic feature of amoebic not bacillary dysentery (Das *et al*., 2013; Petridou *et al*.,2017).

Although the normal incubation period takes 1 to 4 weeks , it can range from days to months or even years , about 4-10 % of *E. histolytica* carriers can develop clinical symptoms within a year (Hategekimana *et al*., 2017) . In human infection there are unknown triggers , that convert *E. histolytica* to its invasive phenotype , the virulence factors interact with host mechanism in a complex manner which increase the defenses of the host and resistance of the parasite (Faust and Guillen,2012).

2.6 Virulence factores

2.6.1 Proteases

E. histolytica have may proteolytic enzymes which sever as virulence factors such as collagenase and cysteine proteases (CPs) , these enzymes play a role in tissue invasion by the parasite and it cleave collagen , elastin, fibrinogen and laminin which are elements of the extracellular matrix (Betanzos *et al*.,2019).

CPs play an essential role in the pathogenesis of *E. histolytica* specially in invasion mechanism, the characterization of CP may give as a clear insights to amoebic invasion and allow us to find new ways to prevent such invasion leading to treatment and the control of both intestinal and hepatic amoebiasis (Que and Reed 2000).

Cps are proteolytic enzymes which are predominant in *E. histolytica* and associated with its pathogenicity . Many studies investigate the degradation of purified protein from the ECM , immunoglobulins (Ig) , complementray and mucin by these enzymes (Fig. 2.2 & 2.3) (Serrano *et al*.,2013).

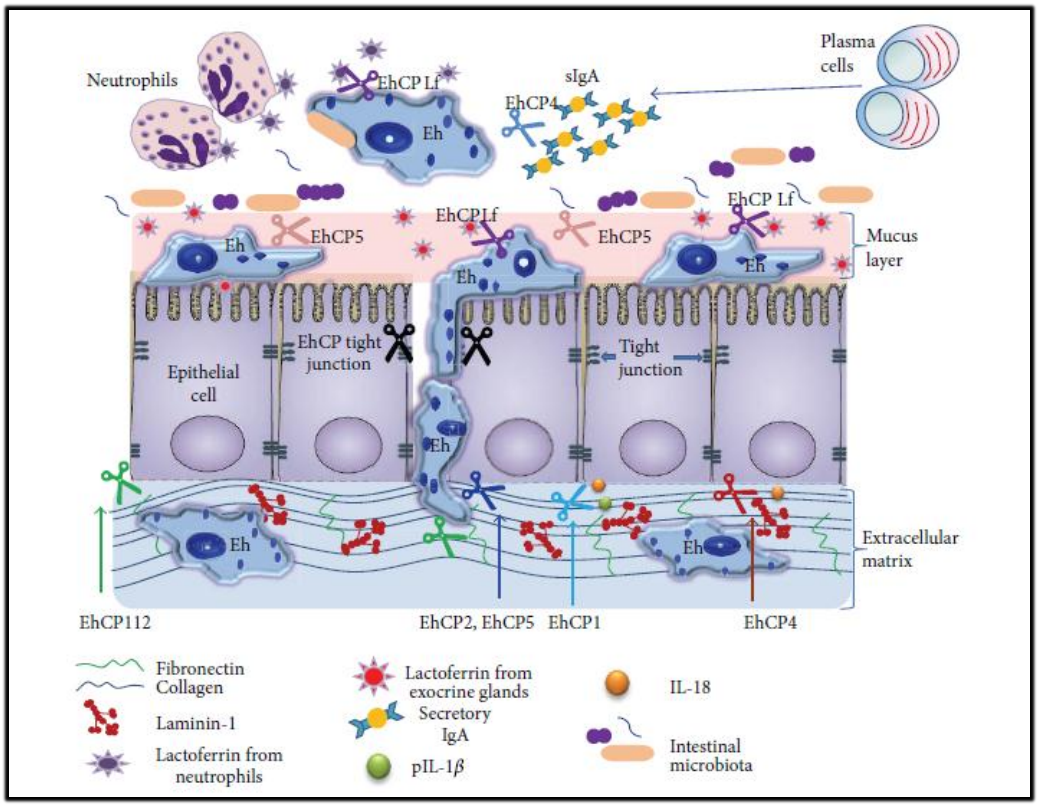


Fig. 2.2 Proteases from *E. histolytica* as virulence factors during intestinal amoebiasis (Serrano *et al*.,2013).

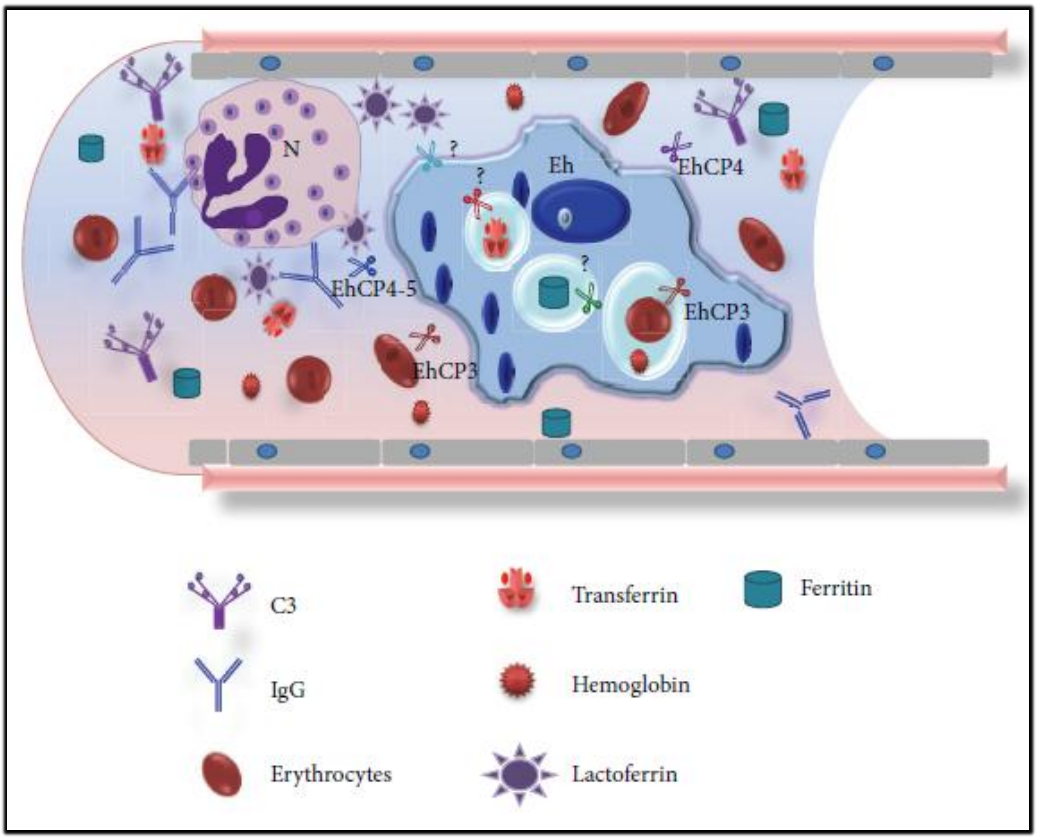


Fig. 2.3 *E. histolytica* proteases participating during trophozoite transit in blood vessels (Serrano *et al*.,2013).

The specific substrate of *E. histolytica* proteases is the similar to cathepsin B-like protease of the papain family (He *et al* .,2010). In *E. histolytica* proteases located different regions , these regions can be seen in Fig. 2.4.

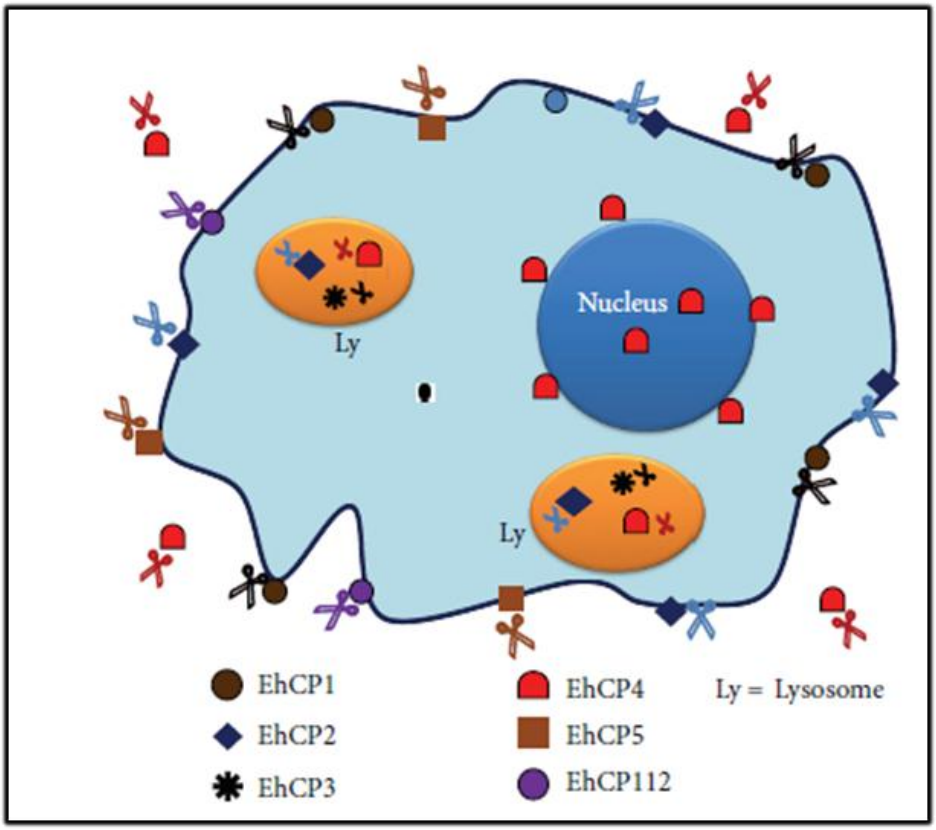


Fig. 2.4 Localization of proteases within *E. histolytica* (Serrano *et al*.,2013).

Genetically CPs are encoded by several genes in *E. histolytica* but only 3 peptidase genes are highly expressed these genes are : *ehcp1*, *ehcp2*, and *ehcp5* which encode to EhCP1, EhCP2, and EhCP5, these proteases are the main ones and consist 90% of the total expression of CP (Naiyer *et al*.,2019 ;Wilson *et al*., 2019).

EhCP3 was detected in *E. dispar* with percentage of 95 % and it is also found in *E. histolytica*. EhCP4 play a role in damaging the tissue integrity and escape from the immune system mechanisms it as it may participate in the inflammatory response within the amoebic lesion and it also degrades Component 3(C3) , it is an important virulent factor in *E. histolytica* (Que and Reed 2000).

The CPs secreted by *E. histolytica* cause degradation of mucus cleavage at the major cleavage site which is predicted to cause de polymerization of the mucus polymers , which damage the protective mucus gel(Betanzos *et al*.,2019).

After the mucus, distraction the TJs complex considered to be the first barrier that prevent the parasite to penetrate the intestine through the paracellular site . the TJs consist of proteins claudin and occludin. These proteins are associated with Zonula Occludens -1(ZO-1), Zonula Occludens-2 (ZO-2), and Zonula Occludens-3 (ZO-3) which are cytoplasmic proteins. *E. histolytica* have the ability to disturb the TJ complex by CPs (Figure 2.5) (Betanzos *et al*.,2013; Collins *et al*.,2017).

CPs occur in different parasites such as *Naegleria fowleri, Acanthamoeba* spp. , *Leishmania major* , *Trichuris muris* , *Schistosoma haematobium , S. japonicum,* and *S. mansoni,* (Caffrey *et al*.,2018; Betanzos *et al*.,2019) In addition , CPs can also found in plants such as pineapple (*Ananas comosus*) , Kiwifruit (*Actinidia deliciosa*) and papaya (*Carica papaya*) (Wang *et al*.,2014; Martin,2017; Liu *et al*.,2018).

Moreover , *Entamoeba* spp. secret another types of enzymes which are the glycosidases like sialidase, N-acetylgalactosamidase, and N-acetylglucosaminidase are required to eliminate the polysaccharides branches from mucin. This elimination will allow the parasite (trophozoite) to degenerate the mucous barrier and penetrate the epithelium which lead to high risk of invading other organs (Kantor *et al*.,2018). Another enzymes are phospholipase A_1,A_2 which participate in the metabolism of phospholipids. Moreover , Phospholipase

A_2 contribute to the cytopaphogenicity of *E. histolytica* (Vargas *et al*.,1995).

E. histolytica has a cytotoxic effect to various cells including ; Neutrophils, macrophages . Potentially , this cytotoxic activity of *E. histolytica* may results in its pathogenicity as it considered to be a major factor in the intensive damage of the tissue (Ralston and Petri ,2011).

2.6.1.1 Tight junction proteins

Tight junctions are multiprotein junctional complexes whose general function is to prevent leakage of solutes and water as well as seals the paracellular pathway (Fig. 2.5). The most important TJ proteins are claudins and occludins which are responsible of vital functions of the cells. In addition TJ include many other proteins such as cingulin and ZO-1, ZO-2, ZO-3 which act together as a framework connecting transmembrane proteins with the actin skeleton (Guillemot *et al*., 2008; Bhat *et al*.,2018) .

TJ abnormalities may cause disturbance in normal cell functioning leading to diseases like cancer, The abnormal TJ may result from inflammation , mutations or an aberrant signaling mechanism (Runkle and Mu, 2013).

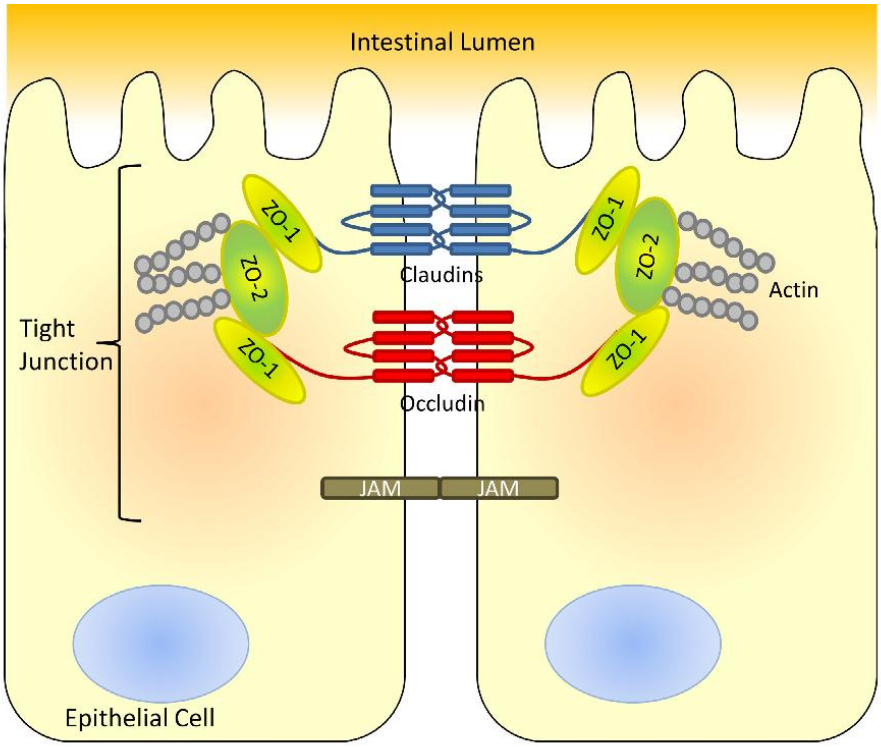


Fig. 2.5 Schematic representation of intestinal tight junction proteins. The location of tight junction proteins between two intestinal epithelial cell (Collins *et al*.,2017)

A. Tight Junction proteins (claudins and occludin)

1. claudins

Claudins are transmembrane proteins which have an essential role in maintaining epithelial cell functions, this group contains 27 proteins which selectively permeable depending on their function .The importance of this group came from the fact it mainly formed the TJs (Claudin,1,3,5,11,14 and 19) these claudins are controlling the cells water tight stability (Cording *et al*., 2013).

The elegant interaction between claudins and occludins hold all the cytoplasmic milieu proteins .Therefore , any malfunction in the claudins may result in diseases like Inflammatory Bowel Disease(IBD) (Lameris *et al*., 2013; Bhat *et al*.,2018). Claudins have various expression patterns

which vary according to cell types and tissues , their expression is important for cell membrane functions (Markov *et al*., 2010). Claudin-1 is expressed ubiquitously and in the gastrointestinal tract it exhibit a high variability in their expression in different parts of the tract (Webb *et al*., 2013).

They have two types of interactions , a cis-interaction inside the cell membrane and a trans-interaction with claudins found in the membranes of the adjacent cell . Both interaction are important to form TJ strands (Hashimoto *et al*.,2019).

2. Occludin

Occludin considered to be the first discovered transmembrane protein of TJs. Attempt to silence occludin *In vitro* result in increasing the permeability of the cell to small molecules (Runkle and Mu, 2013). The function of occludin in TJs is not fully understood but participates in paracellular permeability regulation and the adhesion of cells (Lavie *et al*.,2019).

It has two extra cellular Loops which interact with its equivalent on the adjacent epithelial cells in order to seal the TJs paracellular space (Goplen *et al*.,2013),it is also important for the stability of TJs and barrier function ,Moreover , when mice lack the expression of occludin they suffer from chronic inflammation , hyperplasia , and brain calcification (Lavie *et al*.,2019).

2.6.2 Gal/GalNAc Lectin

The binding of host Galactose N- acetyl D- galactosamine with Gal/GalNAc lectin of *Entamoeba* is a major molecule of the cell surface that participate in the adhesion of the parasite to the epithelial tissue,

basement membrane , human colonic mucin glycoprotein, Neutrophils and erythrocytes as in Fig. 2.6 (Soares *et al*.,2019).

Mucus layer help to block adherence of trophozoites to epithelial cells by providing ligands for the binding of Gal/GalNAc lectin, resulting in competitive inhibition but CPs are thought to bind less glycosylated regions of the Mucin (MUC2) polypeptide, facilitating solvation of the colonic mucus gel and invasion of the colonic epithelial cells, furthermore ,the interaction between lectin and precise quantities of mucin glycoproteins results in the formation of the cyst stage , in contrast, when the trophozoite is attached to the epithelium through lectin it lead to the death of the host cell through a contact -dependent cytolysis (Petri *et al* .,2002; Lidell *et al*.,2006).

Gal/Gal NAC lectin has been reported as a main vaccine candidate as well as it is a key molecule in the parasite pathogenicity and biology . This role came from their involvement in parasite attachment to the host cells and its resistance to complement (Tachibana *et al*.,2009; Saha *et al*.,2015).The prevalent of Gal/Gal NAc lectin adherence was less on the surface of *E. dispar* (Boettner *et al*.,2005).The expression of Gal/Gal NAc lectin genes are significant in *E. histolytica* in compare to *E. moshkovskii* (Wilson *et al*.,2019), as the differences in both parasites may came from the different nucleotide sequence of the Gal/ Gal NAc lectin encoding gene (Shimokawa *et al*.,2013).

2.6.3 Amoebapores

Amoebapores are defined as small peptides that consist of only 77 amino acids , they have the ability to induce toxic effect on the target cells as it inserted into the cell regardless of the interaction with specific membrane receptors. It has peptide –peptide interaction forming ion

channels within membranes causing lysis of cells, therefore , it plays a major role in damaging and destroying the host tissue during *Entamoeba* infection . Amoebapores are members of the saposin – like protein family which found inside the cytoplasmic granules of the amoeba trophozoite as a mature potentially active peptides .This protein family consist of 3 pore-forming peptides (Amoebapore A (AP-A), Amoebapore B (AP-B) and Amoebapore C (AP-C) and they are highly active at pH 5.2 (Bracha *et al*.,2002; Ghosh *et al*.,2019).

The participation of amoebapore results in a big outflow of extracellular Ca^{+2} and the entrance of amoebic protease in the cell cytoplasm which will activate effector caspases , this process will lead to the apoptosis of the host cell , for this reason , amoebapore contribute to the extracellular and intracellular killing of foreign cells (Fig. 2.6) (Huston *et al*.,2001; Saha *et al*. 2015). *E. histolytica* has 3 isoforms of amoebapore while *E. dispar* , *E. moshkovskii* have one homology (AP-A), furthermore , the isolation of amoebapore from the trophozoite of the 2 species *E. histolytica* and *E.dispar* revealed that *E.dispar* had less amount of this peptide but higher activity was in *E. histolytica* (Nickel *et al*.,2001;Al-Abodi,2015).

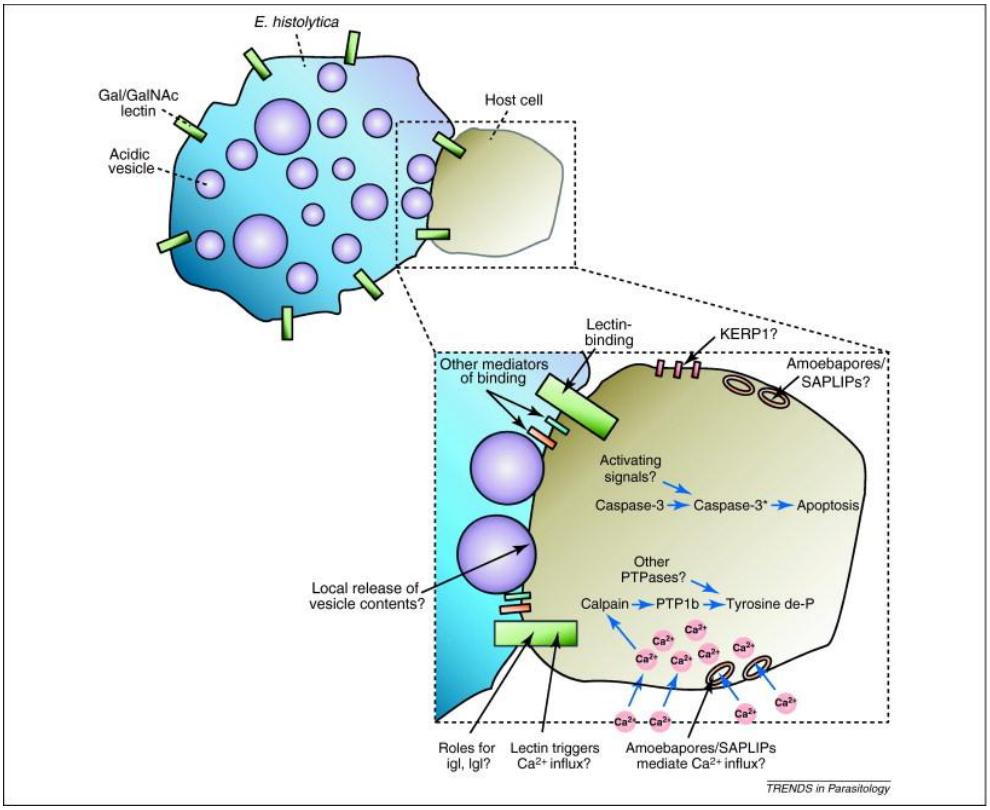


Fig. 2.6 Virulence factors for *E. histolytica* (Ralston and Petri, 2011).

2.7 Diagnosis methods

2.7.1 Clinical manifestations of parasitic diseases

Amoebiasis has a variety of clinical manifestations . Normally , These infections usually manifest with some degree of abdominal pain, and diarrhea. The diarrhea ranges from stools with a watery or mucus or bloody (Garcia *et al*.,2018).

2.7.2 Direct microscopic examination

Historically , the diagnosis of *E. histolytica* depend on microscopic examination of the parasite morphology , unfortunately , this type of examination do not differ among the members of (*E. histolytica* , *E.dispar* , *E.moshkovskii*) which are morphologically similar (Garcia *et al*.,2018).

It also does not allow the distinguish between the invasive *E. histolytica* and non-invasive species *E. dispar* unless the ingested RBCs can be seen inside the trophozoite of the invasive species . This type of examination has always been a definitive diagnostic method but it is not frequent to see these RBCs in the chronic type of amoebiasis **(**Proctor,1991; Soares *et al*.,2019).

The diagnosis of amoebiasis by microscopic examination need good training so the person perform the exam should by very skillful with a lot of experience to get accurate results and avoid misdiagnosis (Walsh,1986; Addib *et al*.,2007). The sample must be preserved using fixative agents such as polyvinyl alcohol or the sample can be kept cool at 4 C^{o}. The trophozoites may be still motile at this degree after 4h. (Jensen *et al* .,2000;Tanyuksel and Petri, 2003).

The microscopic examination can be performed with unstained sample and stained ones , the latest ones can be stained with Lugol's or D'Antonios iodine. The Iodine stain used to visualized the nucleus. Furthermore, several stain may be used such as Giemsa and methylene blue (Tan *et al*.,2010).

Several dyes were suggested for routine diagnosis of both *E. histolytica* and *E. dispar* such as Wheatley's trichrome or the modified iron hematoxylin which are used to prepare permanent smears. The Wheatley's trichrome is better in staining karyosome and chromatine granules when comparing with Iodine stain (Tan *et al*.,2010).

They are many factors that can affect the results of the microscopic examination , one of these factors is the difficulty in distinguish the non-motile trophozoite from the polymorphonuclear leukocyte , macrophage and other tissue cells . Finally , the presence of other species of

Entamoeba that are similar to *E. histolytica* such as *E. dispar* , *E. moshkovskii* , *E. coli* and *E. hartmanni* (Tanyuksel and Petri, 2003; Nowak *et al*.,2015).

2.7.3Cultural methods

Although culture is one of *Entamoeba* spp. diagnostic method , it's very difficult to perform with less sensitivity than the microscopic examination. The success rate of this method is 50-70 % (Fotedar *et al*.,2007).

There are 2 types of culture media which are used in cultivation *Entamoeba* spp. , xenic and axenic media . Xenic cultivation was first used in 1925 where the parasite is cultivated with unknown flora . Example of this type of media are Locke-egg (LE) medium and Balamuth's medium (Parija *et al*.,2014).

The axenic cultivation was first introduced in 1961 by Diamond . In this type of cultivation the parasite grew without the presence of any unknown flora . Example of this type of media are TYI-S-33, both these media are used of *E. histolytica* cultivation as well as *E. bengladeshi* and *E. moshkovskii* which are able to grow at 37 ^{o}C and 25^{o} C. Notably few strain of *E. dispar* can be cultivated in axenic culture. The disadvantages of cultivation method in *Entamoeba* diagnosis are it has less sensitivity than the examination it is difficult to perform and maintain and finally it is expensive method (Royer *et al*.,2012).

2.7.3.1 Culture media

1. Lock egg medium(LE) and Robinson's medium

Defined as diphasic media used for xenic cultivation , used to grow *Entamoeba* spp. , *Dientamoeba fragilis* , *Blastocystis hominis* , *Balantidium coli* (Bareja *et al*.,2015).

2. Trypticase-Yeast Extract-Iron-Serum (TYI-S-33)

It is also a diphasic axenic media used to cultivate *E. histolytica, Giardia intestinalis , Trichomonas vaginalis* . it can be xenic media for *Entamoeba gingivalis* cultivation (Gannon and Linke ,1991; Clark and Diamond ,2002).

3. Brain Heart Infusion Agar (BHIA)

This medium is a solid medium rich with nutrients . it can be used to cultivate many microorganisms such as several strain of bacteria , fungi and yeasts . BHIA is used in standard method for testing the water (recommended media) as well as in antimicrobial susceptibility test . Beef heart and calf brain infusion are rich with nutrients as peptone mixture provide nitrogen , vitamins , minerals and amino acids. All these elements assist various microorganisms to grow such as *Aspergillus brasiliensis, Saccharomyces cerevisiae , (*Hong *et al*., 2017; AL-Kafaween *et al*.,2019) *Leishmania, Trypanosoma* (Limoncu *et al*.,2004).

4.Buffalo milk agar

Milk is produced by many animals such as cows , goats, sheep and buffalos , but Buffalo milk is rich with lipids , proteins and lactose in compare with cow milk (Fangmeier *et al*.,2019). This medium utilized to grow *Leishmania donovani* and fungi (Muniaraj *et al*.,2007; Moubasher *et al*.,2018).

5. Sheep testes agar

The medium that contain the sheep testes increase the number of parasites and bacteria with high purity such as *Naegleria fowleri* (Othman *et al*., 2010;Abu-Mejdad and Al-Hilfy,2016). Normally , the meat of sheep's in enriched with proteins , amino acids , vitamin and other elements like nitrogen , carbon and potassium . In addition contain fat and salt (Oler *et al* .,2015).

6.sheep liver agar and chicken liver agar

The chicken liver is useful to isolate many microorganisms such as *Staphylococcus* spp. , *Escherichia coli* and *Salmonella* spp. The liver contain high percentage fatty acids , proteins , vitamin and minerals (Mohammed,2018).

2.7.4 Serological Tests

2.7.4.1 Antibody detection

The diagnosis of *E. histolytica* using serological tests is uncommon in developed countries. While it is not useful in developing countries where the infection is endemic because the antibodies stay detectable for years after treatment making the distinguish between present and past infection very difficult , Different immunological assays are good to diagnose *E. histolytica* such as Complement Fixation(CF) and Enzyme-Linked Immunosorbent Assay (ELISA) (Ohnishi *et al*.,2004).

ELISA is the most common used test in detecting amoebiasis . Hira *et al*.(2011) reported that this test has a sensitivity and specificity of 97.9% and 94 % respectively when it was used to diagnose amoebiasis in amoebic liver abscess (ALA) patients.

2.7.4.2 Antigen detection

The detection of antigens have many advantages because it gives specific identification of *E. histolytica* and distinguish it from *E. dispar* and *E. moshkovskii* . Furthermore, it has high degree of sensitivity and specificity with less expertise needed and numerous screening tools .

The common antigens used in this method are Gal/GalNAC lectin . Haque *et al*. (2000) found that 96-100 % of ALA patient have obvious level of Gal/GalNAC lectin antigen in both serum and pus in the liver abscess before receiving any dose of metronidazole.

The combination of serological tests may give the best method to diagnosis of amoebiasis(Tanyuksel and Petri,2003).

2.7.5 Molecular methods

The use of molecular method in the diagnosis of disease including amoebiasis had become an important approach to get more accurate diagnosis and it solved the problems of the traditional methods as it has better sensitivity , specificity and simplicity.

The precise distinguish of *E. histolytica* from the other non-pathogenic *Entamoeba* spp. is very important and crucial in both patients management and study the epidemiology of amoebiasis outbreaks. The diagnostic method using molecular based techniques have provide such accurate information making it the gold standard diagnostic method nowadays.

DNA samples extracted from fecal samples is the standardized procedure for molecular diagnosis of amoebiasis . Furthermore , the extraction of DNA from fecal samples is affected by many PCR

inhibitors that found in the samples such as bile salts and complex of polysaccharide (Holland *et al*.,2000).

The temperature affect the DNA samples as fecal or liver abscess pus must be transported and stored in cold temperature, as room temperature cause DNA degradation . The sensitivity of PCR depend on how proper the sample was stored and the duration of the storage , the optimal temperature is – 20 ^{o}C . Researchers found that freezing the fresh samples at – 20 ^{o}C give better results in terms of the PCR sensitivity (Lebbad and Svard, 2005).

Various methods of PCR were used for the detection of *Entamoeba* spp. The most studies genes are the small subunit rRNA gene (18S rRNA) and cysteine proteases. PCR targeting 18S rRNA is highly sensitive and better than the ELISA test that ever existed (Mirelman *et al*.,1997). There also differences between the results obtained from the microscopic examination and PCR as it will positive in microscopy examination and negative in PCR. These differences may occur when a narrow specificity primer is used instead of broader primers which may lead to detect new species like *E. bangladeshi* (Royer *et al*.,2012).

Despite all the advantages , there are some disadvantages represented by the time consuming of this method in case of large-scale experiment and it is costly (Parija *et al*.,2014).

2.7.5.1 Real-time PCR (RT-PCR)

Real time PCR is evolutional version of conventional PCR and has become a core of Laboratory diagnosis to many disease as it is a very sensitive method , it shortened time and minimize contamination (Fotedar *et al* .,2007).

This method used various chemestries such as hybridization probes and SYBR Green assay (Gomes *et al*.,2014). RT-PCR has been used to study intestinal and extraintestinal amoebiasis using different samples (Qvarnstrom *et al*.,2005).

Laboratories performing conventional PCR to diagnose amoebiasis can use SYBER Green chemistry as an alternative method . Roy *et al*.(2005) amplified 18 s rRNA in stool and liver abscess pus samples and compare them with both serological tests (antigen detection) and conventional PCR . RT-PCR has its disadvantages such as its expensive , application of this technique is limited in developing countries. (Rahman *et al*., 2008)

2.8 Epidemiology of amoebiasis

Parasite diseases contribute significantly to many illnesses causing death in some cases . These diseases can affect people in both developing and developed countries (WHO, 2008).

Amoebiasis caused deaths mostly in tropical and subtropical regions which lack standard hygienic conditions (Anuar *et al*., 2013). The reason of death by amoebiasis is mainly results from the severe complication during the course of infection(Intestinal – extra intestinal disease). Amoebic dysentery comes in the third places to cause deaths after Malaria and Schistosoma (Ghasemi *et al*., 2015).

In the last decade *E. moshkovskii* gain a lot of interest as it has the ability to infect humans (Shimokawa *et al*., 2012). Normally, *E. moshkovskii* found regions with high prevalence of *E. histolytica* (Heredia *et al*., 2012).

A high prevalence of *E. moshkovskii* infections was identified in Bangladesh by Ali *et al*. (2003) reported prevalence of *E. moshkovskii* is

21.1% among children are 2-5 years while *E. histolytica* has 15.6% prevalence. The fact is that infection with *E. moshkovskii* is not uncommon and has a higher prevalence than infection with *E. histolytica*. The same results were reported in India, Austoralia , Tunisia and Tanzania (Parija and Kairnar, 2005; Fotedar *et al*., 2008; Ayed *et al*., 2008; Beck *et al*., 2008).

Furthermore , several *E. histolytica* outbreaks was associated with sewage contamination. In 1993 , 730 student were infected with amoebiasis and the source of infection were an underground well water which was contaminated in Taiwan (Chen *et al*., 2001). As well as in Georgia , an outbreak was reported from water contamination (Barwick *et al*., 2002).

Amoebiasis cause around 100,000 death / year . The percentage of *E. histolytica* reaches 50% in some developing countries in Africa and Asia (Tengku and Norhayati ,2011). Bad sanitation , low socio-economic standards , contaminated water supply and overcrowding in developing countries all participated in increasing the prevalence of the disease (Kantor *et al*.,2018).

Different amoebic manifestation are distributed worldwide: amoebic liver abscess are common in South Africa while intestinal invasive amoebiasis are common in Eygpt, central and South of America , Asia (Ravdin *et al*., 2003).

Intestinal invasive amoebiasis is very similar in males and females . ALA rate differ from male and females , it is 10 times in male than female and normally the suitable age is between 18-50 years (Bernin *et al*.2014). The reason of this variation is unclear but the increased

consumption of alcoholic lead to increase liver degrade in males (Wuerz *et al*.,2012).

In Arabic countries , amobiasis is a common public problem in Saudi Arabia specially among children with a prevalence of 70.5% . Moreover , *E. histolytica , E.dispar ,Giardia lamblia* are the most common parasites in the patients (Al-Harthi and Jamjoom, 2007). In another study , the two main hospitals in Jeddah reported *E. histolytica* , *E. dispar* prevalence of 8.3% and 5.9% respectively.(Al-Braiken, 2008).

Most of the prevalence studies on intestinal amoebiasis were publish diagnosed intestinal amoeba according to microscopical examination . These studies reported a high infection prevalence's , for example , in Egypt the prevalence was 7.1% (Hassan *et al* ., 2012) , in Sudan was 42.9% (Mohamed *et al* ., 2016). Syria reported 8.75 % prevalence (Alhabbal , 2015) while in Nigeria the prevalence was 20 % in the infection of *E. histolytica* (Auta *et al* ., 2017). In Iraq the highest prevalence in the infection of *E. histolytica, E.dispar* was reported in Najaf, Wasit, Basrah, Diwania and Miasan provinces (18.6%,10.42%,10.2%,10.3% and 9.8% respectively). In contrast , the lowest prevalence's was found in Anbar , Diyala , Thi-Qar, Erbil and Nineveh (0.5%,1.9%,1.9%,1.2% and 2.1% respectively(Al- Saqur *et al*.,2017). In Babil , there was an association between *E. histolytica* and *Giardia lamblia* with diarrhea via prevalence of 17.9% of *E. histolytica* and 4.09 % for *G. lamblia* (AL-Khikani *et al*.,2019).

In Basrah , same correlation was found and the percentage of patient who have *E. histolytica* was 29.2% while 15% have *G. lamblia* (Al-Shaheen *et al*.,2007). In villages of southern Iraq , where drinking

contaminated water is common , *E. histolytica* was detected in 8% of tested water samples (Jarallah,2016).

2.9Drug therapy

Treatment of amoebiasis depend on the clinical stage of the disease (Table 2.3). The diagnosis must be accurate as treatment vary according to the form of the disease (intestinal and invasive). After diagnosis the infection by stool examination without further analysis regarding the species, this action will lead to mistreatment and the development of *E. histolytica* resistance against drug (Haque *et al*., 2006).

Drugs such as Paromomycin and Diloxanide furoate are efficient in eliminating the infective stage (cysts). Paromomycin does not absorbed in the bowel which will be effective of the parasite but the side effects of it will be abdominal cramps and nausea (Kikuchi *et al*.,2013). Metronidazole is used to treat invasive amoebic disease but it has no effect on cysts (Debnath *et al*., 2019). Regarding vaccination , no vaccine has been approved (Kantor *et al*.,2018).

Table 2.3 Drugs used to treat amoebiasis

Disease	Drugs
Amebic liver abscess	Metronidazole
Amebic colitis	Metronidazole
Asymptomatic intestinal Colonization	Diloxanide furoate, Paromomycin

2.10 Hygiene

In developing countries amoebiasis is common . Good practices help to prevent the spread of infection when living in areas of poor sanitation. *E. histolytica* differ from other disease which transmitted by the same route (fecal – oral route) (Mbagwu,2019).Generally , the disease can be controlled by :

1. Wash hand after using toilet and before meals or preparing one .
2. Not eating uncooked food , avoid eating unwashed vegetables and fruits .

Normally , water can be filtered plus dissolving tablets of iodine into that filtered water , in this case it will by safe to drink (Benetton *et al*.,2005; Adenusi *et al*.,2018).

3.Materials and Methods

3.1 Materials

3.1.1 Chemicals

The chemicals and their manufacture used in present study are listed in Table 3.1.

Table3. 1: Chemicals and their provider

Chemicals	Manufactures	Country
Absolute ethanol	Tedia	USA
Acetone	Scharlau	Spain
Agar	Oxoid	UK
Agarose	Biobasic	Canada
Ammonium sulfate	Merck	Germany
Ascorbic acid	Thomas	China
Benzoic acid	Fisher	USA
Brain heart infusion agar	LAB	UK
Bromophenol blue	Geneaid	Taiwan
Buffalo milk	Buffalo	Iraq
Calcium chloride	Thomas barker	India
Casein	Thomas	China
Chicken liver	Chicken	Iraq
Chloroform	Scharlab	Spain
Chromotrope 2R	Allied chemical	USA
Citric acid	Merck	Germany
Deionized water	Bioneer	Korea
Dextrin plasticizer xylene (DPX)	Sigma-Aldrich	USA
DNA ladder (100 bp)	Bioneer	Korea

Egg	Local source	Iraq
Eosin-Y water soluble	Sigma-Aldrich	USA
Epoxy 812 Embedding Medium kit	Sigma-Aldrich	USA
Erythromycin	S.D.I.	Iraq
Ethedium bromid	Biobasic	canada
Ether	Scharlab	Spain
Ferric ammonium citrate	Fluka	Switzerland
Formalin	BHD	England
Glacial acetic acid	Fine chem.Limited	India
Glucose	Scharlau	Spain
Glutaraldehyde	BDH	UK
Go Taq Green Master Mix	Promega	USA
GoTaq®2-StepRT-qPCR System	Promega	USA
Haematoxylin	Sigma Aldrich	USA
Human serum	Human	Iraq
Iodine crystals	Merck	Germany
L- cysteine	Sigma-Aldrich	USA
Lactic acid	Fluka	Switzerland
Light green SF	Biotech chemical	India
Magnesium chloride	Hopkin and Williams	England
Magnesium sulfate	Hopkin and Williams	England
Mercuric chloride ($Hgcl_2$)	IndiaMART	India
Nystatin	S.D.I.	Iraq
Osmium Tetroxide	Sigma-Aldrich	USA
Paraffin wax	Merk	Germany
Paraformaldehyde	Sigma-Aldrich	USA

Peptone	Oxoid	UK
Phosphate buffer saline	Oxoid	England
Phosphotungstic acid	Sigma-Aldrich	USA
Potassium chloride	Fluka	Switterland
Potassium iodide	Hopkin and Williams	England
Potassium phosphate monobasic	Sigma-Aldrich	USA
Potassium phosphate,dibasic	Sigma-Aldrich	USA
Presto ^{TM}Stool DNA Extraction Kit	Geneaid	Taiwan
primer	Alpha DNA	USA
Propylene oxide	Sigma-Aldrich	USA
ReliaPrep^{TM}FFPE Total RNA Miniprep System	Promega	USA
Reynold lead citrate	Thomas Scientific	USA
Sheep liver	Sheep	Iraq
Sheep testes	Sheep	Iraq
Sodium bicarbonate	BDH	UK
Sodium chloride	Fisher Scientific	UK
Sodium hydroxide	Fluka	Switzerland
Sodium phosphate dibasic	Fisher Biotech	USA
TBE buffer	Biobasic	Canada
uranyl magnesium acetate	Thomas Scientific	USA
Xylene	Scharlab	Spain
Yeast extract	Oxoid	UK

3.1.2 Equipments

The equipments used in this study are listed in Table 3.2.

Table3.2: Equipments and lab tools used in the present study .

Equipments	Company	Country
Autoclave	Hirayama	Japan
Centrifuge	Gemmy	Taiwan
Compound Light Photo Microscope	Leica	Korea
Deep Freezing	NUAIRE	Japan
Digital Camera	Apple	China
Distillator	GFL	Germany
Electrophoresis System	Fisher Scientific	USA
Eppendorf Centrifuge	Thermo	USA
Eppendorf Tube	Citotest	China
Fin Tips 10,100,1000µl	Citotest	China
Hood	Fisher Scientific	USA
Hotplate	Corning	USA
Incubator	Memmert	Turkey
Light Microscope	Human	Germany
Micropipettes(10,100,1000)µl	Citotest	China
Microtome	Leica	Korea
Mini Vortex Minifuge	Fisher Scientific	USA
Nanodrop	Quawell	USA
PCR Sprint Thermal Cycler	Thermo	USA
PCR Tube (Microcentrifuge	Citotest	China

Tubes)		
PH Meter	Fisher Scientific	USA
Real Time PCR	Chai Open qPCR	USA
Refrigerator	Vestal	China
Auto processor	Reichert-Jung	Korea
Sensitive Balance	Mettler Toledo	Switzerland
Slides	Citotest Lab Ware	China
Scanning transmission electron microscope	detector Zeiss Supra 55VP	Germany
UV Light Transilluminator	Wisd	Korea
Ultramicrotome	Rreichert-Jung	USA
Verity® Thermal Cycler For PCR	Applied Biosystem	USA
Vortex	Fisher Scientific	USA
Water Bath	Memmert	Germany

3.1.3 DNA Molecular Weight Markers

The Molecular weight markers used in agarose electrophoresis are listed in Table 3.3 . Appendix -I

Table 3.3 DNA Molecular Weight Markers

Markers	Provider/country	Fragment size (bp)
100bpDNA ladder	Bioneer/Korea	100,200,300,400,500,600,700, 800,900,1000,2000

3.2 Methods

3.2.1 Samples collection

In this study, total of 150 stool samples are related to (n = patients 100, n= healthy controls 50), Clinically diagnosed stool samples were collected from outpatients suffering from gastrointestinal disorders, abdominal pain and diarrhea, those patients were attending the consultant clinics of different hospitals in Basrah, including Al-mawani hospital, Al-Fayhaa, Al-Shifa, Ibn Ghazwan. In addition, some of the samples were obtained from private laboratories .The collection took place during the period from January 2018 to January 2019. All patients were 1 – 70 years old and from both sex. The samples were collected in a labeled sterile plastic containers and brought directly to the laboratory of parasitology in the Department of Biology/ College of Science/ University of Basrah. Each sample was divided into two parts, the first one for direct microscopic observation and culturing while the second part kept at -20C° for DNA extraction (Fig. 3.1)

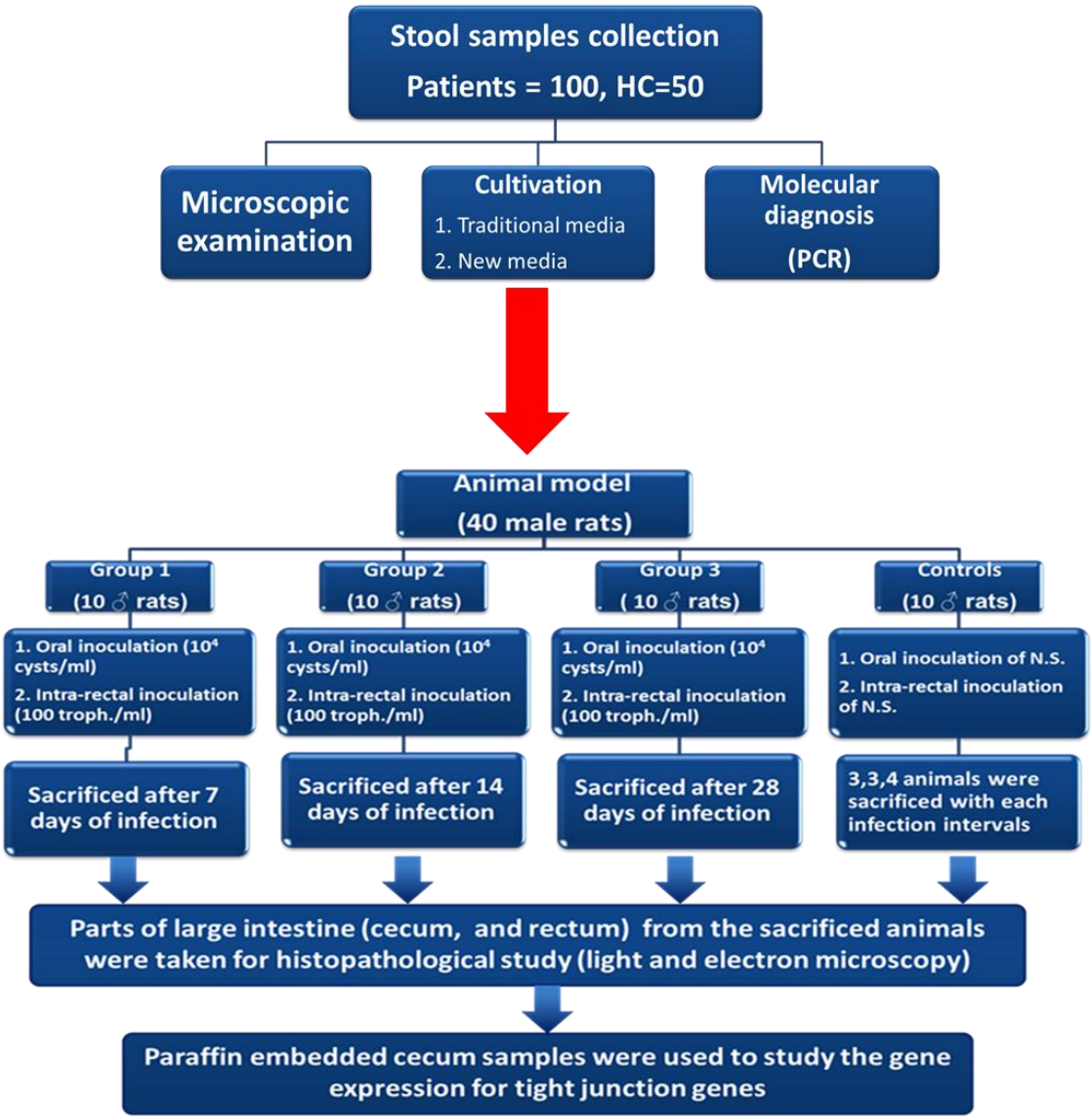


Fig. 3.1 Flowchart representing the design of the study

3.2.2 Microscopic examination of *Entamoeba* spp.

The microscopic examination of 150 stool samples were performed by direct wet smear method which include a diagnostic examination for trophozoite and cyst stages of *Entamoeba* spp. and concentration methods (Garcia and Ash , 1979 ; Garcia *et al*., 2018).

3.2.2.1 Diagnostic examination of *Entamoeba* spp. trophozoite stage:

Normal saline (N.S.) was used in this method, a drop of N.S.(0.9%) was placed on the edge of a clean slide, a small part of the stool sample taken by a wooden stick was transferred and mixed well with the N.S. ,the mixture was covered with a coverslip which placed carefully to avoid the formation of air bubbles, then sample was examined using light microscope under magnification power 40x & 100x.

3.2.2.2 Diagnostic examination of *Entamoeba* spp. Cyst stage:

Entamoeba spp. Cysts were diagnosis by used Lugols iodine solution which prepared as follow :

Iodine crystals	1 g.
Potassium iodide	2 g.
Distilled water (D.W.)	100 ml

The potassium iodide was dissolved in D.W. then the iodine crystals were added gradually. The solution was filtered and stored in a sterile dark container. The smear was prepared the same way as it was prepared for trophozoite examination but using the Lugols iodine solution instead of N.S. to stain the cyst nucleus.

3.2.2.3 Concentration Methods

The isolation of *Entamoeba* spp. from stool samples were performed by using simple sedimentation and formalin-ether technique.

1.Simple sedimentation technique

This technique included the following steps with some modifications, briefly:

1. Normal saline (2 ml) was added to 1g. stool sample and mixed well , the sample was centrifuged at 4000 rpm for 1 min. and the supernatant was discharged.

2.Then (2 ml) of N.S. was added to the sediment, the sample was centrifuged at 4000 rpm for 1 min. and the supernatant was discharged.

3.Also (2 ml) of N.S. was added to the sediment and mix well then filtered using medical gauze,

4. Normal saline (2 ml) was added to the supernatant then centrifuged at 4000 rpm for 1 min. and the supernatant was discharged.

5. Normal saline(1 ml) was added to the sediment and mixed well.

6. One drop of the mixture was transferred to a clean slide and examined under the light microscope.

7. *Entamoeba* spp. cysts and trophozoite were counted using Neubauer chamber slide .

2. Formalin-ether technique

This technique included the following steps**:**

1.About (10 ml) of formalin 10% was added to 1g. stool sample and mixed well, left for 15 min. then the mixture was filtered using medical gauze.

2. Normal saline (2 ml) was added to the supernatant then centrifuged at 4000 rpm for 2 min. and the supernatant was discharged.

3. Formalin 10% (2 ml) was added to the sediment and mixed well.

4.Then(2 ml) of ether was added to the mixture and mixed well.

5. The sample was centrifuged at 4000 rpm for 2 min. which give four layers, the sediment layer at the bottom of the tube which contains the parasite, formalin layer, debris/ fat layer and ether layer then the supernatant was discharged .

6. Normal saline (1 ml) was added to the sediment and mixed well.

7. One drop of the mixture was transferred to a clean slide and examined under the light microscope.

8. *Entamoeba* spp. cysts and trophozoite were counted using Neubauer chamber slide .

3.2.3 Staining of *Entamoeba* spp. by trichrome stain (Garcia and Ash , 1979).

In order to get a precise identification for *Entamoeba* spp. The parasites were stained by trichrome stain including (Basic and Modified methods) as follow :

1.Schaudinns fixative:

A.Saturated mercuric chloride solution

Mercuric chloride ($HgCl_2$)	110 g.
D.W.	1000 ml

The mercuric chloride ($HgCl_2$) was dissolved using a water bath 50°C until the formation of the crystals.

B. Schaudinns fixative (stock solution)

Mercuric chloride saturated aqueous solution	600 ml
Ethanol 95%	300 ml

Before use, glacial acetic acid was added 5 ml / 100 ml of stock solution.

2. Trichrome staining

Chromotrope 2R	0.6 g.
Light green SF	0.3 g.
Phosphotungstic acid	0.7 g.
glacial acetic acid	1 ml
D. W.	100 ml

One ml of glacial acetic acid was added to the first 3 components, the mixture left for 15-30 min. then D.W. was added.

3. Modified D'Antonis iodine

Potassium iodide	1 g.
Iodine crystals	1.5 g.
D.W.	100 ml

Both potassium iodide and D.W. were mixed well, the iodine crystals were added with contentious stirring until the solution is homogenized then the solution was filtered and ready to use.

3.2.3.1 Basic trichrome staining procedure

1. Sample was concentrated as previously explained (section 3.2.2.3)
2. Drops of Schaudinns fixative was added to the smear and kept for 30 min. ,with take care to the smear kept not dry.

3. Drops of ethanol 70% was added to the smear and kept for 5 min. and remaining ethanol 70% was discharged then drops of ethanol 70% that containing D'Antonis iodine was added to the smear and kept for 3 min.
4. Drops of ethanol 70% was added to the smear and kept for 5 min. and remaining ethanol 70% was discharged then new drops of ethanol 70% was added to the smear and kept for 3 min.
5. Drops of trichrome stain was added to the smear and left for 10 min.
6. Drops of ethanol 90% acidified (1% acetic acid) was added to the smear and kept for 3 sec.
7. Drops of ethanol 100% was added to the smear and kept for 5 min. and remaining ethanol 100% was discharged then new drops of ethanol 100% was added to the smear and kept for 5 min.
8. Then xylene drops was added to the smear and kept for 5 min.
9. Finally Dextrin plasticizer xylene (DPX) was added to the smear and covered with a coverslip.

3.2.3.2 Modified trichrome staining procedure

1. Sample was concentrated as previously explained (section 3.2.2.3)
2. Schaudinns fixative (1 ml) was added to the sediment (1ml) and kept for 30 min. then centrifuged at 2000 rpm for 1 min. and the supernatant was discharged.
3. Ethanol 70% (1ml) was added to the sediment and kept for 5 min. then centrifuged at 2000 rpm for 1 min. and the supernatant was discharged then ethanol 70% (1ml) that containing D'Antonis iodine was added to the sediment and kept for 3 min. then centrifuged at 2000 rpm for 1 min. and the supernatant was discharged.

4. Ethanol 70% (1ml) was added to the sediment and kept for 5 min. then centrifuged at 2000 rpm for 1 min. and the supernatant was discharged then new (1ml) ethanol 70% was added to the sediment and kept for 3 min. then centrifuged at 2000 rpm and kept for 1 min. and the supernatant was discharged.

5. Trichrome stain(1ml) was added to the sediment and kept for 10 min. then centrifuged at 2000 rpm for 1 min. and the supernatant was discharged

6.The smear prepared from sediment by adding drops on the edge of slide, then drops of ethanol 90% acidified (1% acetic acid) was added to the smear for 3 seconds.

7. Drops of ethanol 100% was added to the smear and kept for 5 min. and remaining ethanol 100% was discharged then new drops of ethanol 100% was added to the smear and kept for 5 min.

8. Drops of xylene was added to the smear for 5 min.

9.After that, DPX was added to the smear and covered with a coverslip.

3.2.4 Cultivation of *Entamoeba* spp.(Clark and Diamond,2002)

In order to obtain a clear growth of *Entamoeba spp*. 150 stool samples (100 patients and a 50 for healthy controls) were cultivated in different media.

3.2.4.1 Basic culture media:

Locke's egg medium, Robinson's medium and Trypticase yeast extract iron serum (TYI-S-33) medium (Von brand *et al.*,1943; Robinson,1968; Diamond *et al*.,1978) **. Appendix-II**

3.2.4.2 New culture media: including

1.Brain heart infusion agar (Hi media, India)

This medium prepared from mixing 5.2g Brain heart infusion agar with 100 ml D.W.

2. Sheep testes agar

Sheep testes extract: five g. of sheep testes was taken and added it to 150 ml D.W. ,then put it in a mixture for 15 min. and filtrated it.

This medium prepared from mixing 1.5g agar with 100ml sheep testes extract.

3.Buffalo milk agar

This medium prepared from mixing 1.5g agar with 100ml buffalo milk.

4. Sheep liver agar

Sheep liver extract: five g. of sheep liver was taken and added it to 150ml D.W., then put it in a mixture for 15 min. and filtrated it.

This medium prepared from mixing 1.5g agar with 100ml sheep liver extract.

5.Chicken liver agar

Chicken liver extract: five g. of chicken liver was taken and added it to 150ml D.W. ,then put it in a mixture for 15 min. and filtrated it.

This medium prepared from mixing 1.5g. agar with 100ml chicken liver extract.

All the prepared media above were mixed and sterilized by autoclave then stored at 4°C.Then 15 ml from each media was added to culture media tubes .

Antibiotic

Two type of antibiotic were added to all the media above

- Erythromycin 0.5g/100ml
- Nystatin 0.5g/100ml

both antibiotic were sterilized by millipore filtration and stored at 4°C.

Phosphate Buffered Saline(PBS) (pH 7.4) (Sambrook *etal*.,2001; Haida and Hakiman,2019)

prepared from the following components :

Nacl	4g.
Kcl	0.1g.
KH_2PO_4	0.1g.
Na_2PO_4	0.46g.
D.W.	500ml

All the components were mixed, adjusting pH to 7.4 then sterilized by autoclave and stored at 4°C.

3.2.4.3 Inoculation of *Entamoeba* spp. in culture media

Samples (n=150) were cultivated by using the different culture media. Each media was supplied with the inoculation of Nystatin 0.25mg/ml, Erythromycin 0.25mg/ml, PBS (pH 7.4) 2 ml, Human serum 7 ml and suspension of parasite 1 ml (which contains 500 trophzoite). The tubes were incubated at 35.5-36 ◦C for 48 h. the presence of the parasite was confirmed by light microscope after 48 hour. The numbers of the parasite were calculated by using Neubauer chamber (Elnazeer *et al*., 2016).

3.2.4.4 Preparation of sub-culture for parasites

After the parasite was confirmed .The culture suspension that contain the parasite was collected in a sterile tube , centrifuge at 4000 rpm for 10 min., repeated centrifugation for several times then supernatant was discharged and 1ml of sediment was added to new culture tube. This process repeated each 72 h.

3.2.5 Molecular diagnosis of *Entamoeba* spp.

At first, molecular methods were used to diagnose all samples (n=150, a 100 for patients and a 50 for healthy as controls). After that, the molecular analysis was repeated for samples that were succeeded by cultivated methods (n=10).

3.2.5.1 DNA extraction

DNA was extracted from stool samples according to the procedure of Presto ™Stool DNA Extraction Kit.

1. Sample lysis.

ST1 Buffer (800µl) was added to stool samples (180-220 mg) that is in beadbeating tube containing beads and vortexed for 10 min. at room temperature then incubated at 70◦C for 5 min, The beadbeating tubes were centrifuged at 4500 rpm for 2 min. after that the supernatant (500 µl) was transferred to a new 1.5 ml centrifuge tube.

2. PCR inhibitor removal

Insoluble particles and PCR inhibitors may be removed by adding 150 µl of ST2 Buffer then vortexed , incubated at 4◦C for 5 min. and centrifuged at 4500 rpm for 3 min. Inhibitor removal column placed in centrifuge tube , the clear supernatant (500µl) was transferred to the inhibitor removal column and centrifuged at 4500rpm for 1 min. then

discard the column. Clear supernatant is transferred to a new 1.5ml centrifuge tube.

3. DNA Binding

ST3 Buffer (800µl) was added to the clear supernatant and mixed , Genomic DNA (GD)column placed in collection tube, then sample mixture 700µl was transferred to the GD column ,centrifuged at 4500 rpm for 1 min. then discarded the supernatant, this step was repeated with the remaining sample mixture .

4. Wash

ST3 Buffer (400µl) was added to the GD column and centrifuged at 4500 rpm for 30 sec. then discarded the flow-through after that 600 µl wash buffer was added to GD column, centrifuged at 4500 rpm for 30 sec., discarded the flow-through, another wash buffer 600 µl was added to GD column, centrifuged at 4500 rpm for 30 sec. then discarded the flow-through and centrifuged at 4500 rpm for 3 min. at room temperature to dry the column .

5.Elution

The dry GD column was transferred to a new 1.5 ml centrifuge tube, then the elution buffer (50 µl)was added into the center of column and left for 2 min. to allow elution buffer to be completely absorbed then centrifuged at 4500 rpm for 2 min. then the eluent DNA was stored at -20 C.

Note: The concentration of the eluent DNA was determined by using Nanodrop spectrophotometer measuring (A260/A280 ratio)

3.2.5.2 Visualization of genomic DNA on agarose gel electrophoresis

The extracted DNA was visualized using agarose gel electrophoresis (Sambrook *et al.,* 2001) as following procedure:

1. Preparation of 0.8% agarose gel

The agarose gel 0.8% was prepared by adding 0.2g. of agarose to 25 ml of Tris Borate-EDTA (TBE) buffer ,boiled using a hot plate then it was allowed to cool and 0.2 µl of ethidium bromide (0.1 mg/ml) was added**.**

2. Casting of the horizontal agarose gel

The gel tray was assembled to casting tray and the combs were positioned at one end of the tray, then the agarose solution was poured into the gel tray and allowed to cool at room temperature then combs were carefully removed after that TBE buffer was added until the buffer reached up to over the surface of the gel.

3. Loading and running genomic DNA in agarose gel

- Six µl of DNA was mixed with 2 µl of bromophenol blue and loaded inside wells.
- The cathode was connected to the wells sides of the unit and the anode to the other side.
- The gel was run at 60V until the bromophenol blue dye migrated to the end of the gel.
- DNA bands were examined under UV transilluminator and photographed by a digital camera**.**

3.2.5.3 Diagnosis of *Entamoeba* spp. by polymerase chain reaction (PCR)

The PCR amplification reaction was performed to the final volume of 25 µl. The reaction was optimized to combine the specific primer for *E. histolytica* (SPEH):forward primer 5´-ATGCACGAGAGCGAAAGCAT-3´ and reverse primer 5´-GATCTAGAAACAATGCTT CTCT-3´; for *E. dispar* (SPED):forward primer 5´-ATGCACGAGAGCGAAAGCAT-3´and reverse primer 5´-CACCACTTACTATCCCTACC -3´ ; for *E. moshkovskii*(SPEM):forwardprimer5´-ATGCACGAGAGCGAAAGCAT-3´and reverse primer 5´-TGACCGGAGCCAGAGACAT-3´ (Table 3.4) (Hamzah *et al*.,2006). The thermal cycler program is described in Table 3.5.

Table 3.4 Component of PCR reaction for *Entamoeba* spp. identification.

Reagents	Volume µl
DNA template(50-70ng)	3
Forward primer	1.5
Reverse primer	1.5
Go Taq Green Master Mix	12
Nuclease- free water	7
Total volumes	25

Table3.5 Thermal cycler conditions for *Entamoeba* spp. identification.

Steps	Temperature	Time	No.of cycles
Initial denaturation	95°C	4 min.	1
Denaturation	95°C	35sec.	

Annealing	55°C- for *E. histolytica* 53°C- for *E. dispar* 52.7°C- for *E.moshkovskii*	35sec.	35
Extension	72°C	1min.	
Final extension	72°C	7min.	1

3.2.5.4 Visualization of PCR products on agarose gel electrophoresis

PCR products (6 µl) were visualized as procedure found in section 3.2.5.2 except the concentration of agarose 2% (0.5g) and using 100 bp DNA Ladder (4 µl) . The expected bands are 166bp for *E. histolytica* gene, 752 bp for *E. dispar* gene and 580bp for *E. moshkovskii* gene, all the bands were examined under UV transilluminator and photographed by a digital camera**.**

3.2.6 Calculate sensitivity to diagnostic methods for clinical samples (n=100)

For the three diagnosis methods (direct microscopic examination, cultivation and molecular diagnosis) the sensitivity to detect *Entamoeba* spp. were calculated according to the equation below: (Roudsari *et al*., 2011).

$$\text{Sensitivity} = \frac{\text{No. of true positives}}{\text{No. of true positives + No. of false negative}} \times 100$$

$$= \frac{\text{No. of true positives}}{\text{Total number of sick individuals in population}} \times 100$$

3.2.7 Experimental model of amoebiasis

3.2.7.1 Animals model

Forty experimental male rats *Rattus norvegicus*, age 8-10 weeks , 110-120g weight, were purchased from College of Veterinary Medicine/ University of Basrah. The rats which were used for breeding , examined to be sure that the animals were healthy from any intestinal parasitic infection including amoebiasis. The rats were housed in plastic cage supplied with soft- wood sawdust in the animals house of college of science .The animals were fed with standard food. The rats divided into four groups, each group with (10) rats, the control group was treated with 0.9% Nacl solution (normal saline) and the other experimental groups were treated to include the intestinal amoebiasis as illustrated in Fig. 3.1.

3.2.7.2 Histopathological examination

A. Light microscope study

Histopathological preparation for light microscope was performed using standard laboratory procedures (Drury *et al.* ,1967).

1.Fixation: Tissues (large intestine parts) included cecum and rectum samples were removed from control and experimental animals, rinsed into N.S. , then the tissues cutting to suitable sizes and fixed directly into 10% formalin for 24 h.

2.Washing: The samples were washed with tap water.

3.Dehydration : The samples were dehydrated through a graded ethanol series (70%-80%-90%) 2 h./each concentration then transferred to ethanol 100% in two changes one hour for each.

4.Clearing: The samples were treated with chloroform for 12-24 h.

5.**Infiltration**:All the specimens were infiltrated with melting paraffin wax in 60°C with three changes every 2 h. for three time, then left in melting paraffin wax at 60°C overnight on hot plate of auto processor.

6. Embedding and Sectioning: The samples were embedded with paraffin after orientation the specimens into the right sectioning direction. Sections (7 µm) thickness were made using rotary microtome and float in a 45°C water bath, then mounted on slides, left overnight on a hot plate at 45°C.

7.Staining : The slides were placed in xylene in order to dissolve paraffin wax, then rehydration in graded series of ethanol (100, 90, and 70)% for two min. in every concentration ,the slides were rinsed with water for two min. after that stained in haematoxylin stain for five min., washed with water then placed in ethanol 70% acidified (1% HCL) for three sec., washed with water and stained in eosin stain for five min., washed with water then dehydrated in graded series of ethanol (70, 90, and 100)% for one min. in every concentration , The slides were placed in xylene for five min., left to dry after that DPX was added to the slides and covered with coverslip, examined under the compound light photo microscope .

B. Electron microscope study

Cecum samples after 28 days of amoebiasis infection were cut to small pieces (1 cm) and prepared for ultrastructural study by transmission and scanning electron microscope (TEM and SEM) according to (Humana,2007) as follows:

B1 .Transmission electron microscope (TEM)

1. Primary fixation

Cecum samples were cut into small pieces ($1mm^2$) and fixed directly in mix equal volumes of 2% paraformaldehyde**,** 2.5 % glutaraldehyde for 24h. then the specimens were rinsed three times with N.S. for 10 min. per each rinse

2. Secondary fixation

The samples were fixed in 2% aqueous Osmium tetraoxide(OsO_4) for 1 h. then it was rinsed three times with N.S. for 10 min. per each rinse.

3. Dehydration :

Tissues were dehydrated through graded acetone series (30,50,70,80 and 90%) for 30 min. each concentration then transferred to 100% in three changes 30 min. for each. Then the samples were immersed in a mixture of equal volumes of acetone 100% and propylene oxide for 5 min. after that transferred to pure propylene oxide in three times for 10 min. per each change.

4. Infiltration :

Tissues samples were infiltrated with epoxy resin mixture according to the Epoxy 812 Embedding Medium kit (Table 3.6) as followed:

It was infiltrated 1:2,1:1 and 2:1 (part epoxy resin: part propylene oxide) for 1 h. each change , after that the samples were transferred in fresh resin 100% in two times for 30 min.

Table 3. 6 The components of Epoxy resin mixture used in TEM

Reagents	Volume
Agar 100 epoxy resin	20ml (24g)
Dodecenyl succinic anhydride (DDSA)	22ml(22g)
Methy nadic anhydride (MNA)	5ml(6g)
Benzyl dimethyl amine (BDMA)	1.4 ml(1.5g)

5. Embedding and sectioning:

The samples were embedded with epoxy resin for 24-48h. at 60◦C. Sections (80nm) were using ultramicrotome and float in water bath that contains D.W. and drops of toluene, then mounted on copper grid that contain film and carbon.

6. Staining :

Sections were stained with stains (Table 3.7 & 3.8) as followed:

Samples were stained with drops of uranyl acetate(1%) for 20 min., rinsed with 50% ethanol after that stained in Reynold lead citrate stain (2ml) for 10 min., washed with D.W. then it was examined and imaged by scanning transmission electron microscope (STEM).

Table 3. 7 The components used in the Preparation of Uranyl acetate

Reagents	Quantity
Uranyl acetate	0.05g
D.W.	10ml

Table 3. 8 The components used in the Preparation of Reynold lead citrate

Reagents	Quantity
Lead nitrate	1.33g
Trisodium citrate	1.76g
D.W.	50 ml

B2 . Scanning electron microscope

The tissues were prepared the same way as it was prepared for transmission electron microscope examination from step 1 to step 3 then the samples mounted on stub , dried by freeze dry at electron microscope system and was coated with gold after that it was examined and imaged by scanning transmission electron microscope (STEM).

Note: This part of the study was carried out in both Electron Microscope Unit Manager, Pharmacy College and Department of Physics in College of Science, Basrah University.

3.2.8 Molecular analysis of TJ gene claudin-1 and occludin

Specimens from cecum related to infected rats with amoebiasis at the period 7,14 and 28 days post infection that are paraffin embedded were used to study gene expression for tight junction protiens by qRT- PCR method as follows :

3.2.8.1 Total RNA extraction

Total RNA were extracted from paraffin embedded samples of the cecum according to the procedure of ReliaPrep^{TM}FFPE Total RNA Miniprep System.

A. Preparation of Solutions

Prior to RNA extraction the required solutions were prepared as follow :

1. **Wash Solution(10 reaction size):** prepared from adding 12ml of 95–100% ethanol to the bottle containing 3ml of concentrated wash solution
2. **DNase I:** Nuclease-Free Water (275µl) was added in the DNase I vial
3. **Lysis Buffer (10-reaction size) :** prepared from adding 10µl of blue dye to each vial of lysis buffer then vortex to mix.

B. Procedure

1. Preparation of formalin fixed and paraffin embedded (FFPE)sections.

Sections 5–50µm thickness were cut from FFPE blocks using a microtome then put the sections in a 1.5 microcentrifuge tube .

2. Deparaffinization Using Mineral Oil

Mineral oil (500µl) was added to the sample then incubated at 80°C for 1 min. and vortexed to mix.

3. Sample Lysis

Lysis Buffer(100µl) was added to the sample , centrifuged at 10,000 rpm for 15 sec. where two phases will be formed, a lower blue (aqueous) phase and an upper (oil) phase, upper phase was discarded ,then 10µl Proteinase K(PK) was added directly to the lower blue phase and mixed by pipetting, incubated at 56°C for 15 min., then at 80°C for 1 h. The tubes were removed from the 80°C heat block, and placed on ice for 1 min. to cool them , then tubes were kept at room temperature for 2 min.

4. DNase Treatment

The DNase treatment mixture was prepared from **$MnCl_2$, 0.09M** (13µl), **DNase Buffer(** 7µl), **DNase I enzyme** (10µl).The mixture 30µl was added directly to the lower blue phase of the sample then incubated for 15 min. .

5. Nucleic Acid Binding

BL Buffer(325µl) was added to the sample ,then isopropanol 100% (200µl) was added , vortexed briefly to mix then centrifuged at 10,000 rpm for 15 sec. Two phases will be formed, a lower blue (aqueous) phase and an upper (oil) phase. The binding column was placed in a collection tube, transferred the entire lower blue (aqueous) phase of the sample to the binding column. The mineral oil (upper phase)was discarded and

centrifuged at 10,000 rpm for 30 sec. , the flowthrough was discarded then reinserted the binding column into the collection tube.

5. Column washing and elution

Wash solution (500µl) was added to the binding column, centrifuged at 10,000 rpm for 30sec. , the flow through was discarded and reinserted the binding column into the same collection tube .The previous step was repeated. The binding column was centrifuged at 16,000 rpm for 3 min., the binding column was transferred to a clean elution tube , Nuclease-Free Water(30–50µl)was added to the column . Centrifuged at 16,000 rpm for 1 min. discarded the binding column then the elution tubes were stored at –70 °C.

Note: The concentration of the eluent RNA was determined by using Nanodrop spectrophotometer measuring (A260/A280 ratio).

3.2.8.2 cDNA synthesis

Total RNA of 400 ng from each sample was used to generate Complementary deoxyribonucleic acid (cDNA) according to the procedure of GoTaq®2-Step RT-qPCR System as described in Table 3.9

Table 3. 9 Volumes for a single reaction of step1 cDNA synthesis

Reagents	Volumes (µl)
RNA template	1–5
Oligo deoxythymine (dT) Primer (0.5µg/µl)	1
Random Primers (0.5µg/µl)	1
Nuclease-Free Water	Top up to 10
Total reaction	**10**

Each tube was closed tightly then placed in heat block70°C for 5 min., put in ice for 5 min., each tube was centrifuged for 10 sec., the tubes were kept until the reverse transcription reaction mix2 was added as in Table 3.10. The thermal cycler program is described in Table 3.11.

Note: The synthesized cDNA was stored at -20°C

Table 3. 10 Volumes for a single reaction of step2 cDNA synthesis

Reagents	Volumes µl
Nuclease-Free Water	1.5
Go Script™ 5X Reaction Buffer	4
$MgCl_2$	2
PCR Nucleotide Mix	1
Recombinant RNasinR Ribonuclease Inhibitor	0.5
GoScript™ Reverse Transcriptase	1
Total reaction	**10**

Table 3. 11 Thermal cycler conditions for cDNA synthesis

Steps	Temperature	Time	No. of cycles
Annealing temperature	25°C	5 min	1
Incubation	42°C	1 h.	
Inactivate the reverse transcriptase	70°C	15 min.	

3.2.8.3 Quantitative Real –Time PCR(qRT-PCR)

A total of 120 ng of cDNA of each samples was used to measure the gene expression of Claudin-1(Cldn1) and Occludin(Ocln), using GAPDH (Gapdh) as a housekeeping gene, primers for Cldn1: forward primer 5'-TGTCCACCATTGGCATGAAG-3'and reverse primer 5'-GCCACTAATGTCG CCAGACC-3'; for Ocln :forward primer 5'-CTACTCCTCCAACGGCAAAG-3'and reverse primer 5'-AGTCATCCACGGACAAGGTC-3'; for housekeeping gene(HK) Gapdh : forward primer 5'-TGGAGTCTACTGGCGTCTTC-3'and reverse primer 5'-TCCACACCCATCACAAACATG-3' (Li *et al.*,2015). The components for a single reaction and the thermal cycler condition described in Table 3.12 & 3.13

Table 3. 12 Components for a single reaction for qRT-PCR

Reagents	**Volumes µl**
GoTaqR qPCR Master	10
Forward Primer	1
Reverse Primer	1
cDNA template	6
Nuclease-Free Water	2
Total reaction	**20**

Table 3. 13 Thermal cycler condition for qRT-PCR

Steps	Temperature	Time	No. of cycles
GoTaq® DNA Polymerase activation	95°C	2 min.	1
Denaturation	95°C	15Sec.	40
Annealing and extension	60°C	1 min.	

The data was analyzed by calculating the expression level of genes interesting using cycle threshold number (CT) value Relative quantify of the expression of Cldn1 and Ocln genes were obtained using GAPDH as a housekeeping gene , the expression level of each gene was calculated according to a Livak method (Livak and Schmittgen ,2001) as following steps:

$$\Delta CT_{infection} = CT_{target\ gene} - CT_{HK\ gene}$$

$$\Delta CT_{control} = CT_{target\ gene} - CT_{HK\ gene}$$

$$\Delta\Delta CT = \Delta CT_{infection} - \Delta CT_{control}$$

$$\text{Gene expression (E)} = 2^{\wedge}- \Delta\Delta CT$$

$$\text{Fold change} = \frac{\text{Exp. Of infection}}{\text{Exp. Of control}}$$

- $\Delta CT_{infection}$ =ΔCT of the infected group
- $\Delta CT_{control}$ = ΔCT of the control group
- $CT_{(target\ gene)}$= values of CT of the target genes (Cldn1 and Ocln)
- $CT_{(HK\ gene)}$ = values of CT of the housekeeping(Gapdh)

3.2.9 Statistical analysis

Two-way ANOVA were performed to evaluate the difference in gene expression levels of Cldn1 and Ocln among *Entamoeba* spp. (*E. histolytica, E. dispar and E. moshkovskii*) for three periods of infection using SPSS under a probability of $P \leq 0.05$.Categorical variable are expressed as number and percentage.

4.Results

4.1 Sample collection

The study subjects (n=150) , males were 73 while the females were 77 , the age range was 1-70 years .

4.1.1 Prevalence of *Entamoeba* spp. infection according to microscopic examination

In general, the present study showed that 60% was positive samples in direct microscopic examination, regarding to sex , in our study the infection was found in 56.6% of male patients and 43.4% of female patients (Table 4.1).

Table 4. 1 Frequency of *Entamoeba* spp. infection between sex.

Sample type	Total No.	Percentage (%)	Sex	
			Male(%)	Female(%)
Infection (patients)	100	60+ ve	34 (56.6%)	26 (43.4%)
		40-ve	17 (42.5%)	23 (57.5%)
Healthy control	50	0	22 (0%)	28 (0%)

The results showed that percentage of *Entamoeba* spp. infection was different according to age groups. The highest percentage of *Entamoeba* spp. infection was in group1 (1-10 years) and group 7 (61-70 years) with 31.6% and 23.3% and the lowest percentage was associated with group 3 (21-30) and group 4 (31-40) that the results revealed to 1.6 % for both groups as shown in Table 4.2

Table 4. 2 Frequency of *Entamoeba* spp. infection among age groups

	Age groups						
	Group 1 1-10 years	**Group2 11-20 years**	**Group3 21-30 years**	**Group4 31-40 years**	**Group5 41-50 years**	**Group6 51-60 years**	**Group7 61-70 years**
+ ve samples (n=60)	19	10	1	1	7	8	14
Percentage	31.6	16.6	1.6	1.6	11.6	13.3	23.3

The results showed a difference in the percentage of infection between the months of the year, The highest infection was in July 18.3%, while the lowest infection was in December 1.6% (Table 4.3)

Table 4. 3 Frequency of *Entamoeba* spp. infection between the months of the year.

	Months											
	January	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
+ ve sample (n=60)	**2**	**3**	**4**	**6**	**7**	**8**	**11**	**8**	**5**	**3**	**2**	**1**
Percentage	**3.3**	**5**	**6.6**	**10**	**11.6**	**13.3**	**18.3**	**13.3**	**8.3**	**5**	**3.3**	**1.6**

There were differences in the stool appearance. It differ from bloody, mucous, fatty, and semisolid. The mucus samples were the most frequent with 31.6% followed by semi-solid samples 23.3%.

4.2 Diagnostic of *Entamoeba* spp.

4.2.1 Direct microscopic examination

Direct microscopic examination showed a positive results, the trophozoite was clearly shown as a static colorless or moving using normal saline method (Fig. 4.1) .Moreover, the cysts were diagnose by Lugol's Iodine stain(Fig. 4. 2), the diagnostic method by both basic and modified trichrome stain showed the *Entamoeba* spp. trophozoite as in Fig. 4.3& 4.4

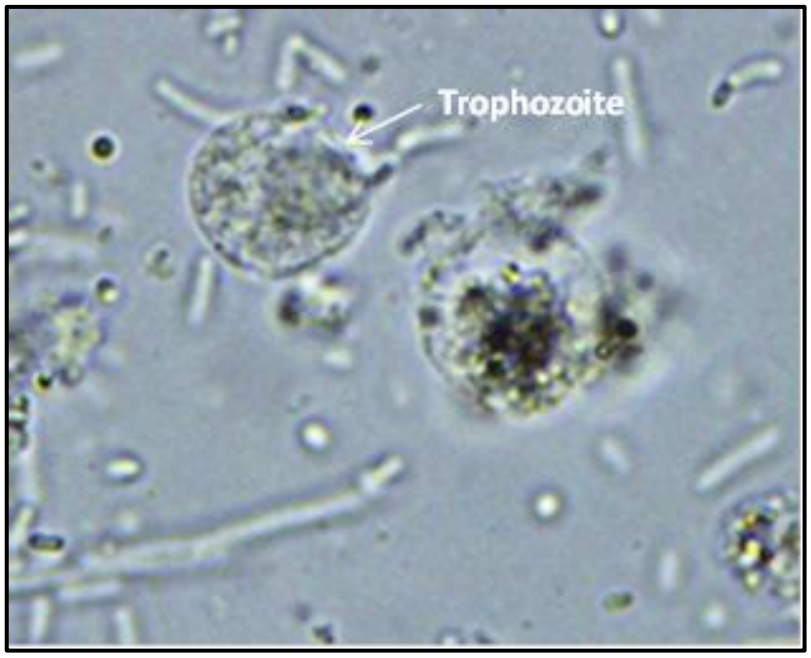


Fig. 4.1 Direct examination of *Entamoeba* spp. trophozoite by normal saline method . trophozoite with irregular chromatin in the nucleus 100x.

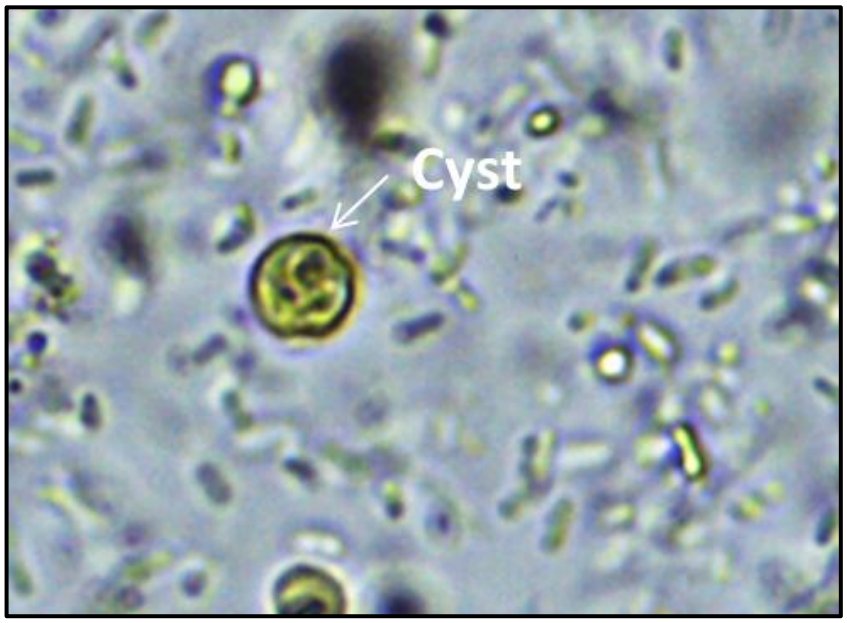


Fig. 4.2 Direct examination of *Entamoeba* spp. cyst by Lugol's Iodine method. Cysts have round shape with one nucleus 100x.

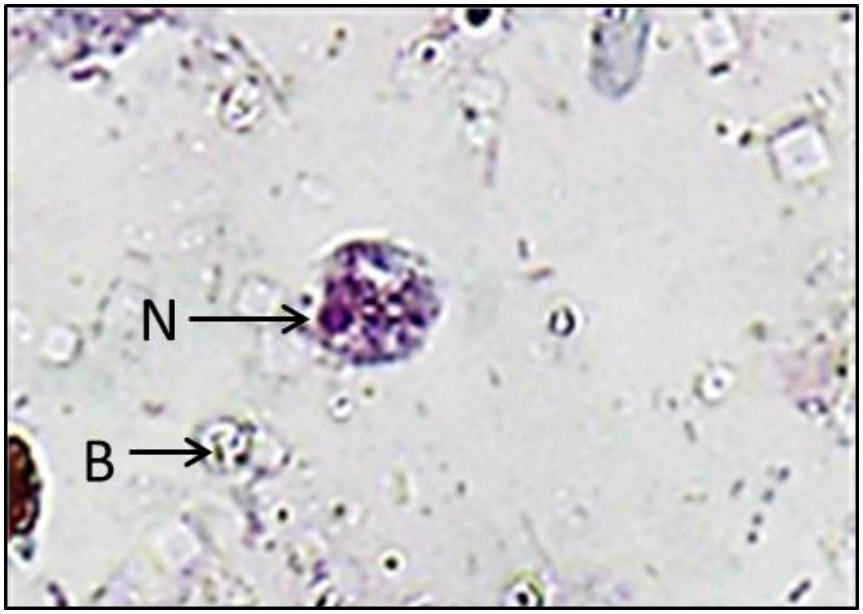


Fig. 4.3 Direct examination of *Entamoeba* spp. trophozoite by basic trichrome stain. Characteristic features of trophozoite, the nucleus (N). Note the bacteria (B) in the samples 100x

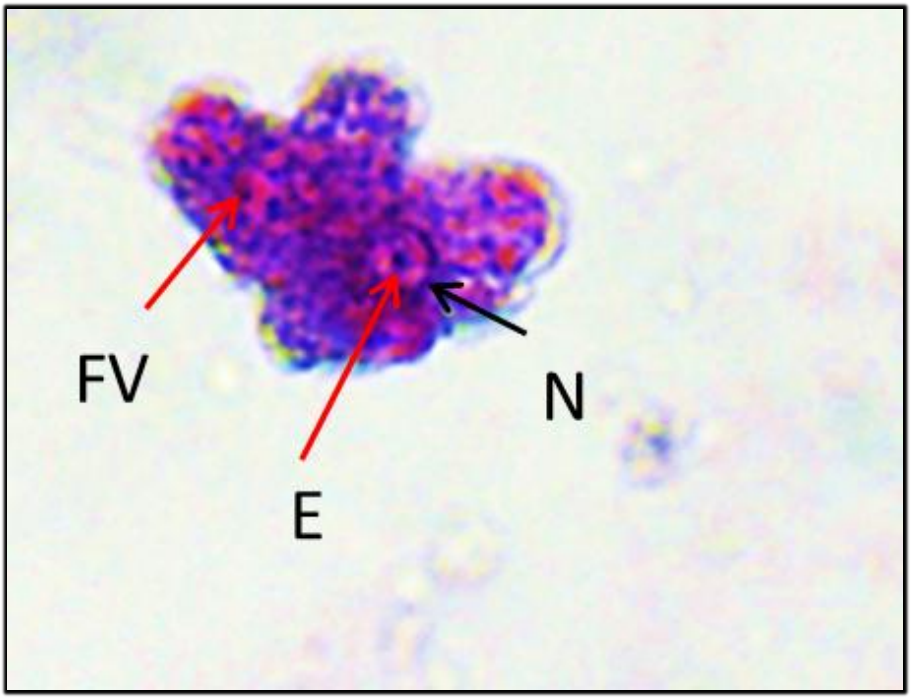


Fig. 4.4 Direct examination of *Entamoeba* spp. trophozoite by modified trichrome stain. Characteristic features of trophozoite, the nucleus (N), endosome (E) and the food vacuoles (FV).Note reduction of bacteria the samples 100x.

4.2.2 Cultivation of *Entamoeba* spp.

1. Cultivation of *Entamoeba* spp. for clinical samples

Generally, clinical samples (n=100) were cultivated, however, results revealed to 10% of total sample n=100 was positive during culturing and these samples was also positive diagnosed in direct microscopic examination

2. Cultivation of *Entamoeba* spp. for control samples

Control isolates (50) were negative in culture.

4.2.2.1 Parasite cultivation in basic media

In the present study three culture media were used in order to obtain the best medium for *Entamoeba* spp., cultivation included (Locke's egg medium, Robinson's medium and Trypticase yeast extract iron serum medium). The best growth period for each culture medium after incubation for 48h. was determined by confirming that the parasite stags alive for the longest period , Locke's egg medium showed that this period reaches a maximum to 4 days. with parasite alive ,while Robinson's and TYI-S-33 media recorded a maximum to 2 days that the parasite still alive (Table 4.4). From these results it is clear that Robinson's and TYI-S-33 media are less efficient than Locke's egg medium in maintaining the parasites growth.

4.2.2.2 Parasites cultivation in new culture media

The new five culture media (Brain heart infusion agar, Sheep testes agar, Buffalo milk agar , Sheep liver agar , Chicken liver agar) were compared with Locke's egg medium which have the best results. The results showed that the growth of the parasites varied according to the culture media . Brain heart infusion agar was the best in maintaining the growth parasite for 10-11 days .Secondly, the Sheep testes agar that have a maintaining period of 7-8 days. Thirdly, the Buffalo milk agar with 4-5 days maintaining period while both Sheep liver agar and Chicken liver agar culture media showed the least efficiency in maintaining the parasite growth (Table 4.4).

Table 4. 4 Maintenance periods of *Entamoeba* spp. trophozoite on variable culture media

Samples	Culture media	Maintenance period (days) after incubation for 48h.											
		1	2	3	4	5	6	7	8	9	10	11	12
S1	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	-	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S2	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	-	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S3	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S4	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-

S5	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-	-
S6	Locke's egg medium	+	+	+	-	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	-	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S7	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	-	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	-	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-	-
S8	Locke's egg medium	+	+	+	-	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	-	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-	-
S9	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	-	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-

	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S10	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	-	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-	-

+ positive growth , -Negative growth

4.2.3 Molecular diagnosis of *Entamoeba* spp.

4.2.3.1 Total DNA extraction

The genomic DNA showed in Fig. 4.5.

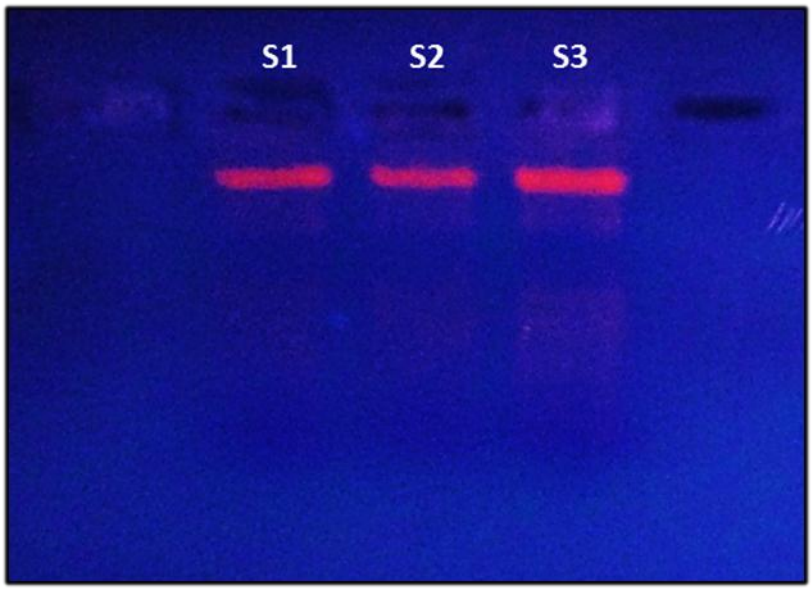


Fig. 4.5 Genomic DNA bands of 3 isolates stool .The DNA was electrophorized using 0.8% agarose gel**.**

4.2.3.2 Polymerase Chain Reaction of *Entamoeba* spp. from clinical samples by specific primers

A. Diagnosis of *E. histolytica*

Five isolates out of 100 samples (5%) were diagnosed as ***E. histolytica*** using the specific primer. The gene band size was 166bp as shown in Fig. 4. 6

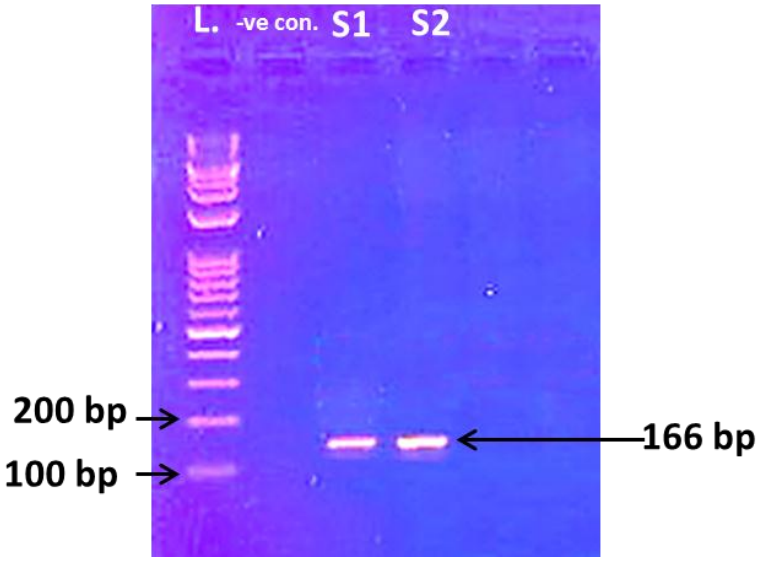


Fig. 4.6 SPEH bands (166bp) of 2 isolates for *E. histolytica* using agarose gel electrophoresis (2%) of PCR products:Lane 1: L (100bp) DNA ladder, Lane 2: negative control (-ve con.),Lane 3: isolate 1(S1),Lane 4: isolate 2(S2).

B. Diagnosis of *E. dispar*

Ten isolates out of 100 sample (10%) were diagnosed as ***E. dispar*** using the specific primer. The gene bands size was 752bp as shown in Fig. 4. 7

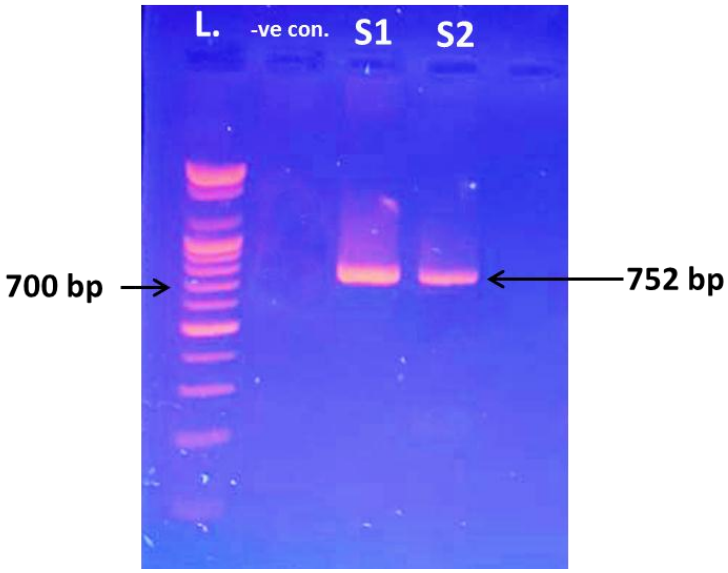


Fig. 4.7 SPED bands (752bp) of 2 isolates for *E. dispar* using agarose gel electrophoresis (2%) of PCR product : Lane 1: L (100bp) DNA ladder, Lane 2: negative control (-ve con.) ,Lane 3: isolate 1(S1),Lane 4: isolate 2(S2) .

C. Diagnosis of *E. moshkovskii*

Fifteen isolates out of 100 sample (15%) were diagnosed as *E. moshkovskii* using the specific primer. the gene bands size was 580 bp as shown in Fig. 4.8

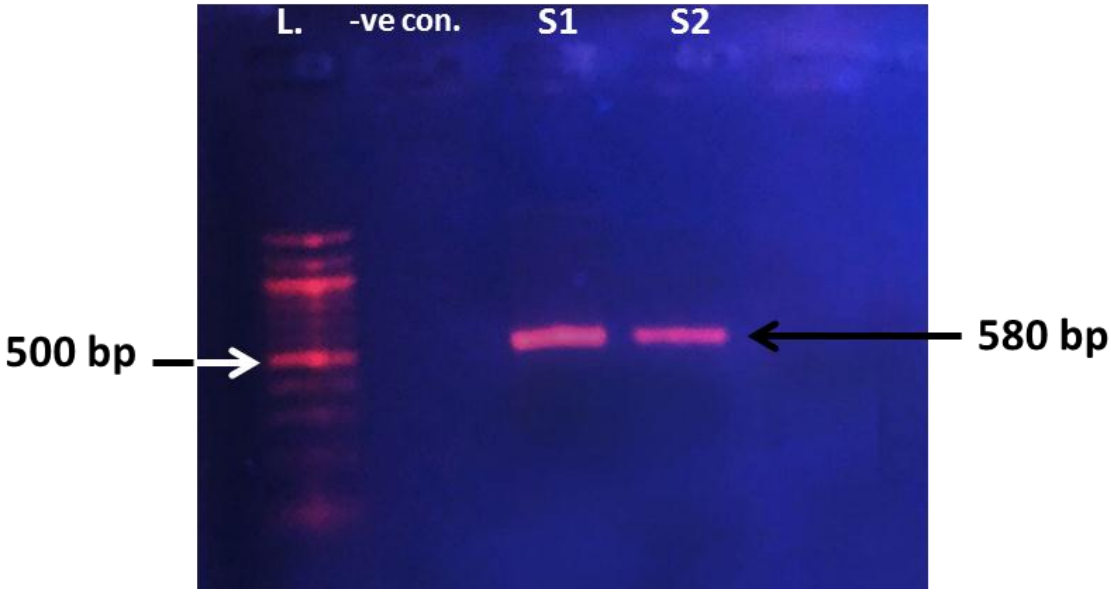


Fig. 4.8 SPEM bands (580bp) of 2 isolates for *E. moshkovskii* using agarose gel electrophoresis (2%)of PCR products :Lane 1: L (100bp) DNA ladder, Lane 2: negative control (-ve con.) ,Lane 3: isolate 1(S1),Lane 4: isolate 2(S2) .

4.2.3.3 Polymerase Chain Reaction of *Entamoeba* spp. from cultivated samples

Out of 10 culture isolates 3 isolates were identified as *E. moshkovaskii* while 7 isolates were negative .

4.2.3.4 Polymerase Chain Reaction of *Entamoeba* spp. from controlled samples

Control isolates (50) were negative.

4.3 Sensitivity of diagnostic methods for clinical samples

In terms of the sensitivity between the three methods of diagnosis that be used in our study (microscopic examination, cultivation and molecular diagnosis).The highest sensitivity was reported for microscopic examination (60%) while the lowest sensitivity value was reported for cultivation (10%),the molecular diagnosis showed a sensitivity of (30%).

4.4 Histopathological study

4.4.1 Light microscope study

The histological changes in cecum and rectum of experimental rat with induced infection of the three *Entamoeba* spp. illustrated as follow:

4.4.1.1 Histological changes in *E. histolytica* experimental infection

1.Cecum

Cecum sections from large bowel of control rats showed that the smooth surface of the mucosa layer doesn't contain villi, this layer is composed of two types of cells that can be distinguished from each other the absorptive cells and the mucous cells, in addition ,simple tubular (crypts of Lieberkuhn) intestinal glands were extend from the surface to the muscularis mucosa, these glands were lined by mucous cells and absorptive cells . The lamina propria is a thin layer of loose connective tissue and it was rich in cells (lymphocytes).The submucosa consists of loose connective tissue with blood vessels, the tunica muscularis composed of strands (smooth) muscle fibers extend up-ward, these layers surrounding the lumen are filled with feacal materials (Fig. 4.9 & 4.10) .

Histological analysis of cecum sections of rats infected with amoebiasis – *E. histolytica* at 7 days post-infection showed trophozoite attached to the surface of the epithelial layer, causing destruction of mucosa, degeneration of interglandular epithelium, as well as increased number of goblet cells. Furthermore infiltration of inflammatory cells within lamina propria was noticed (Fig.4.11,4.12,4.13& 4.14) .

The development of lesions at 14 days post-infection showed some amoeba's trophozoites within cellular debris and amorphous substance . Complete destruction of the mucosa and cytolysis of the apical portion and

degeneration of the epithelium lining of the crypts, the results indicate the presence of deep intestinal crypts as well as infiltration of inflammatory cells which include the base of crypts and muscularis mucosa, in addition blood vessels that found in the submucosa layer also smooth muscles were observed in muscularis externa , in addition to the heavy deposition of amorphous material on the surface layer (Fig.4.15,4.16,4.17,4.18,4.19,4.20&4.21).

The results clarified the changes of infected cecum at 28 days post-infection with *E. histolytica* and revealed that the trophozoites found within the intestinal tubular crypts, the stage showed an obvious ovale structure with clear nucleus (Fig.4.22). Mucosa fold also flat mucosa in other area and increased mucous cells at this layer can also be noticed. Moreover, there were many changes in the intestinal crypts include degeneration of interglandular epithelium ,dilated ,hyperplasia and free lymphocytes can be seen. Muscularis mucosa showed a destruction effects, Inflammation in variable regions, also edematous in the submucosa layer (Fig.4.23,4.24&4.25).

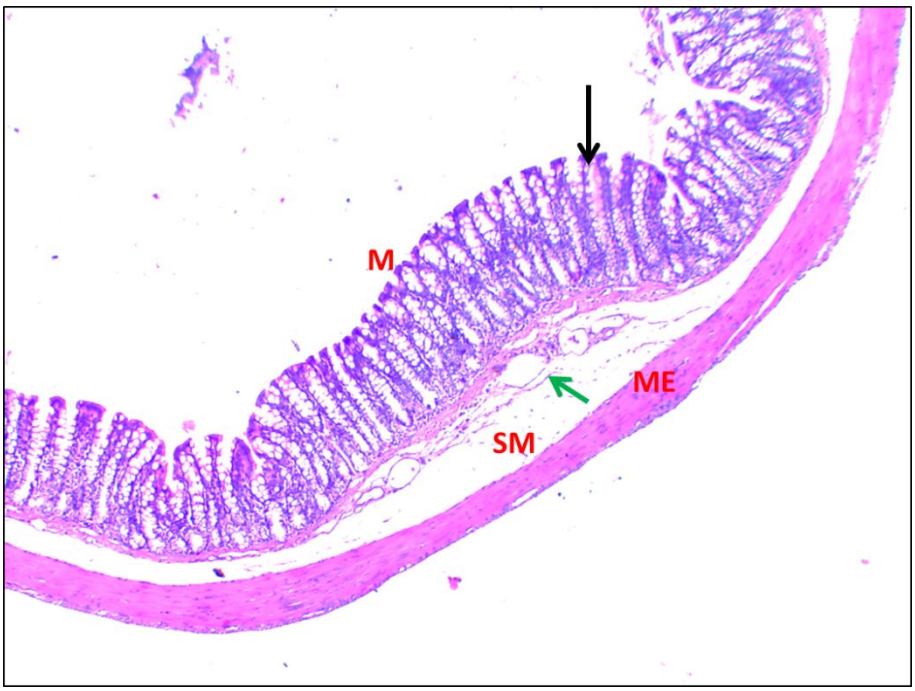


Fig. 4.9 Cecum section of control rat(normal structure).showed the mucosa surface (M) straight crypts (➔)submucosa (SM) with blood vessels (➔) and muscularis externa (ME) H&E stain (98x).

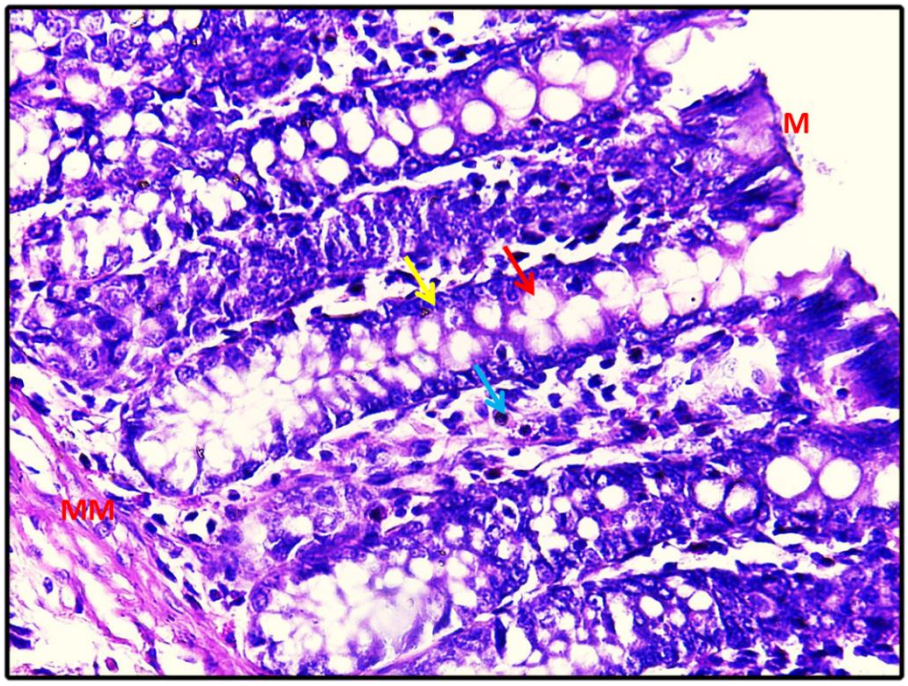


Fig. 4.10 Cecum section of control rat(normal structure).showed the smooth surface of mucosa (M)long –striated glands of Lieberkuhn lining with goblet cells (➔)absorptive cell(➔)extend down to the muscularis mucosa(MM)lamina propria with few lymphocytes(➔)H&E stain(379x).

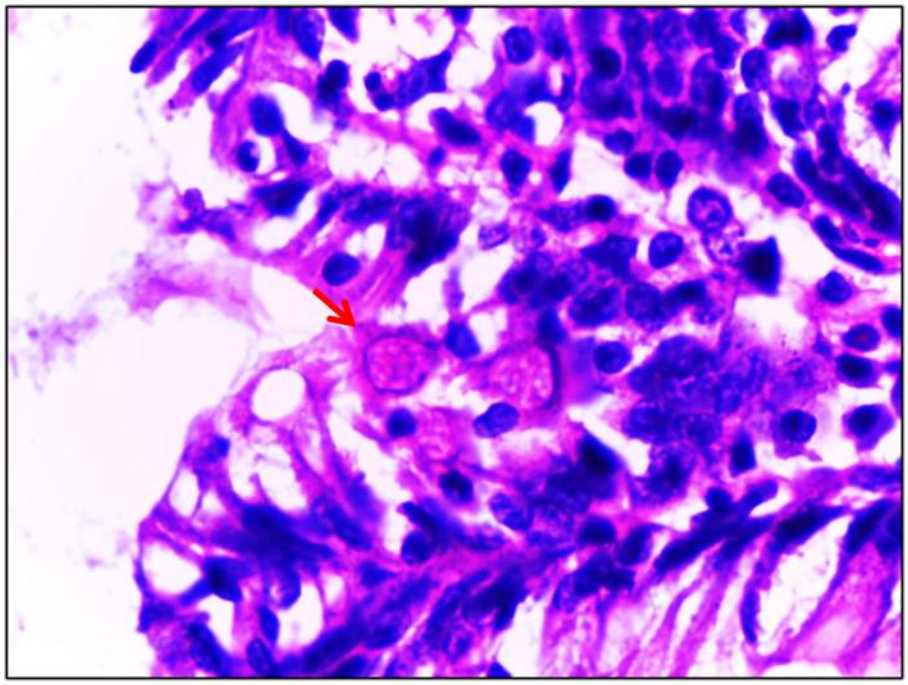


Fig. 4.11 Cecum section of infected rat with *E.histolytica* 7 days post-infection .Showed the trophozoite(➔) attached to the surface epithelial layer. H&E stain(948x).

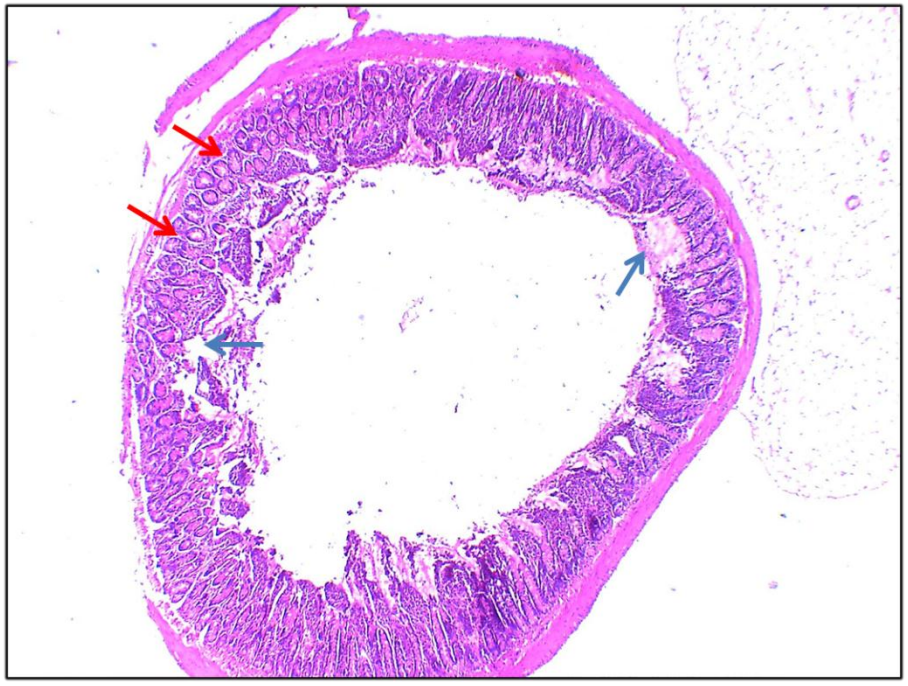


Fig. 4.12 Transverse cecum section of infected rat with *E.histolytica* 7days post-infection. Showed destruction of epithelial layer (➔)intestinal crypts (➔) H&E stain(40x)

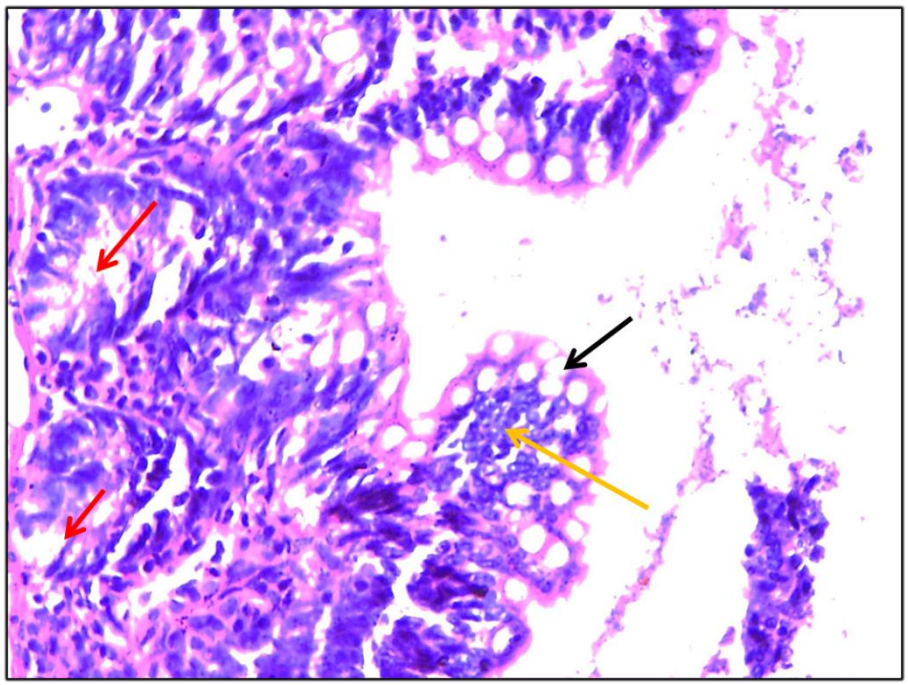


Fig. 4.13 Degeneration in cecum section of infected rat with *E.histolytica* 7days post- infection. Showed degeneration of most crypts(➔) increased number of goblet cells (➔)heavy infiltration of inflammatory cells (➔) . H&E stain(379x).

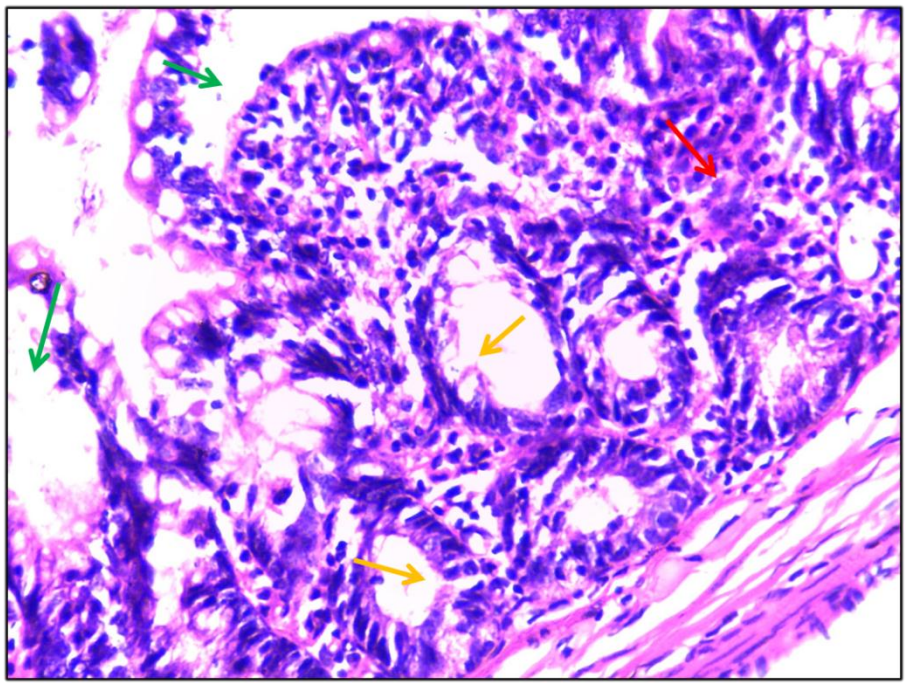


Fig. 4.14 Destruction in cecum section of infected rat with *E.histolytica* 7days post- infection. Showed destruction of mucosa (➔)degeneration of interglandular epithelium(➔)heavy infiltration of inflammatory cells within lamina propria (➔)H&E stain(379x).

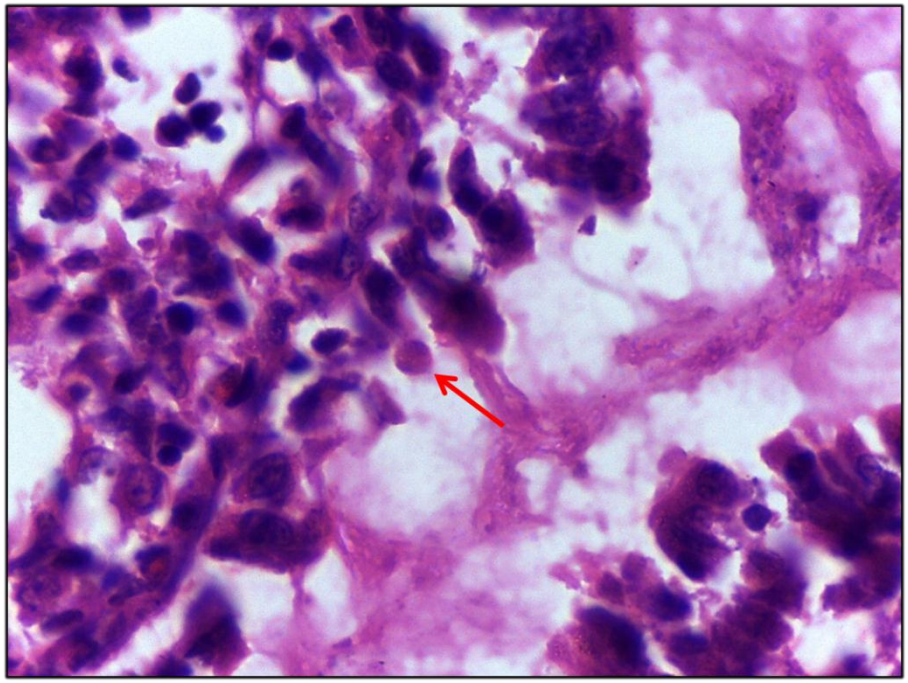


Fig. 4.15 Cecum section of infected rat with *E.histolytica* 14 days post-infection. Showed the trophozoite(➔)within cellular debris and amorphous substance. H&E stain. (948x).

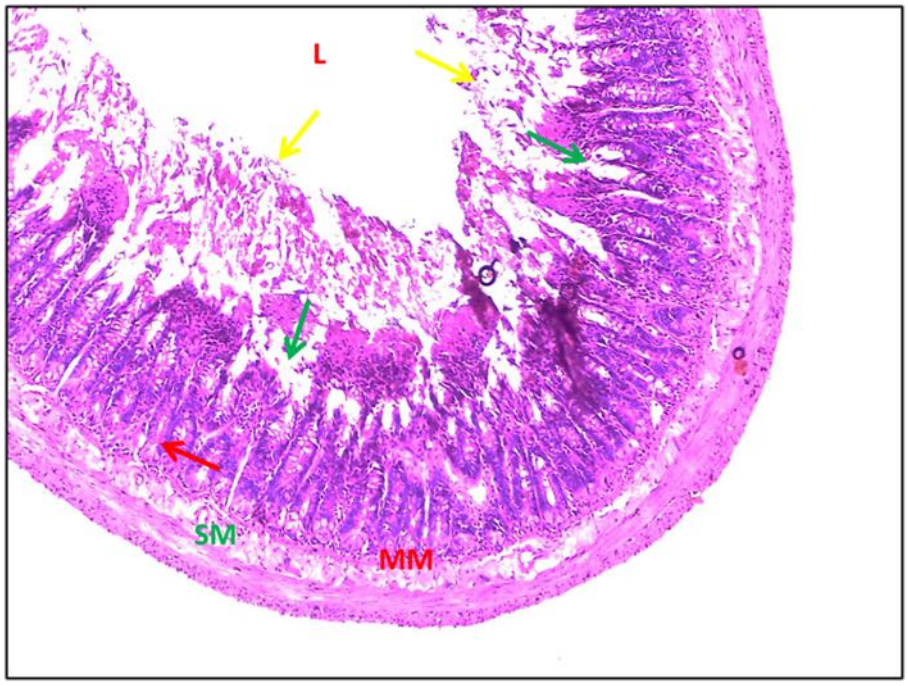


Fig. 4.16 Destruction in cecum section of infected rat with *E.histolytica* 14 days post- infection. Showed destruction of mucosa(➔) cellular debris and fecal(➔)in the lumen(L)deep intestinal crypts(➔)and inflamed muscularis mucosa (MM)and submucosa (SM). H&E stain(98x).

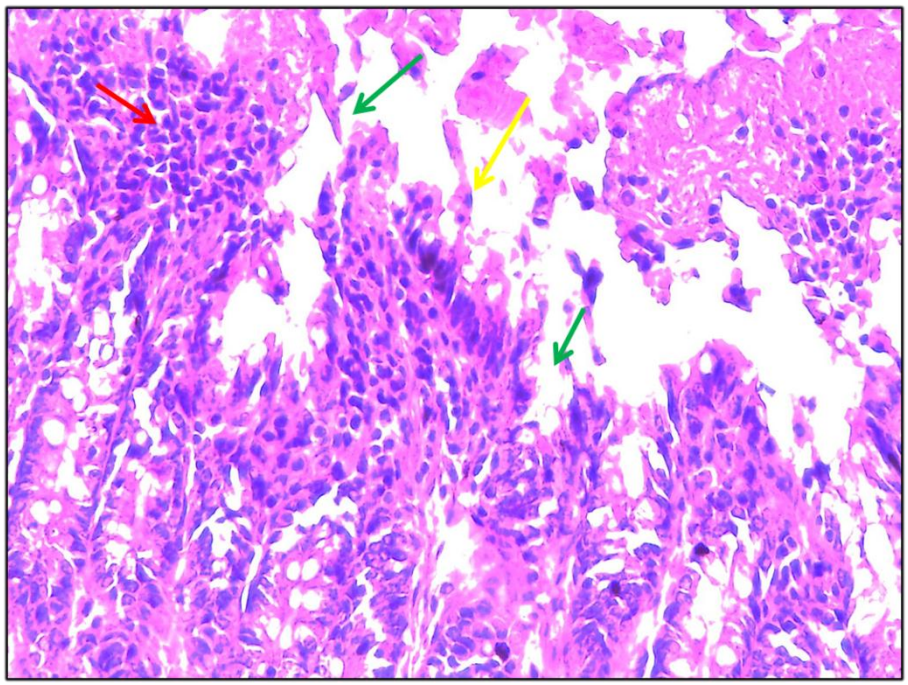


Fig. 4.17Transverse cecum section of infected rat with *E.histolytica* 14 days post- infection. Showed complete destruction of surface mucosa (➔) heavy infiltration of inflammatory cells (➔)cellular debris (➔) and the tissue lost its normal architecture. H&E stain(379x).

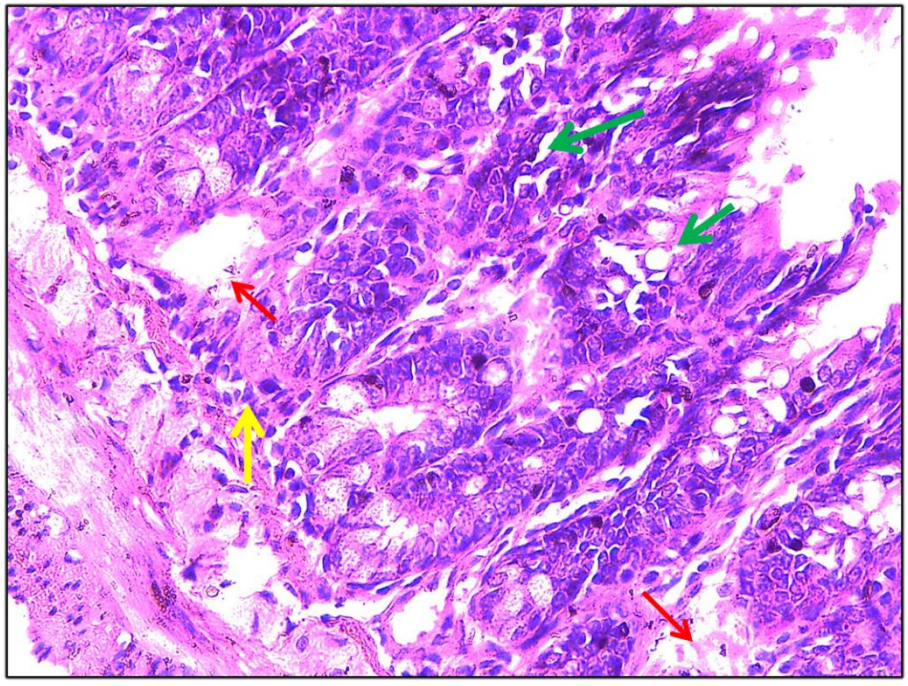


Fig. 4.18 Transverse cecum section of infected rat with *E.histolytica* 14 days post- infection. Showed cytolysis of the apical portion of crypts (➔)degeneration of the epithelium lining of the crypts (➔) infiltration of inflammatory cells (➔). H&E stain(379x).

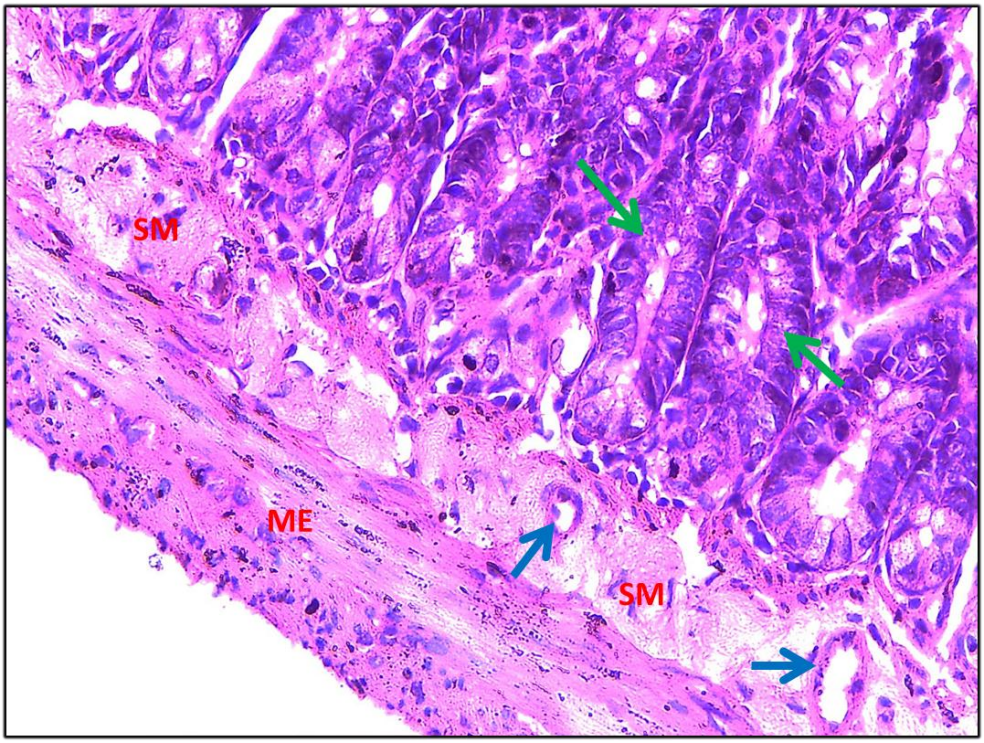


Fig. 4.19 Transverse cecum section of infected rat with *E.histolytica* 14 days post- infection. Showed elongated tubular glands (➔) with normal layer composed submucosa (**SM**)blood vessels(➔)and muscularis externa(**ME**) . H&E stain(379x).

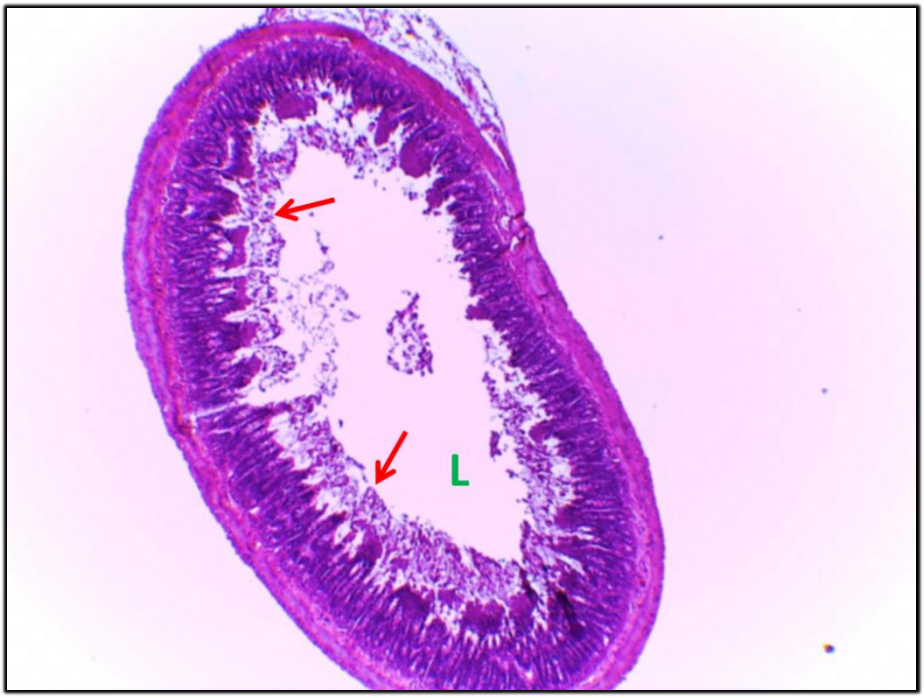


Fig. 4.20 Cecum section of infected rat with *E.histolytica* 14 days post-infection. Showed lumen(**L**) with debris and fecal (➔)H&E stain(40x).

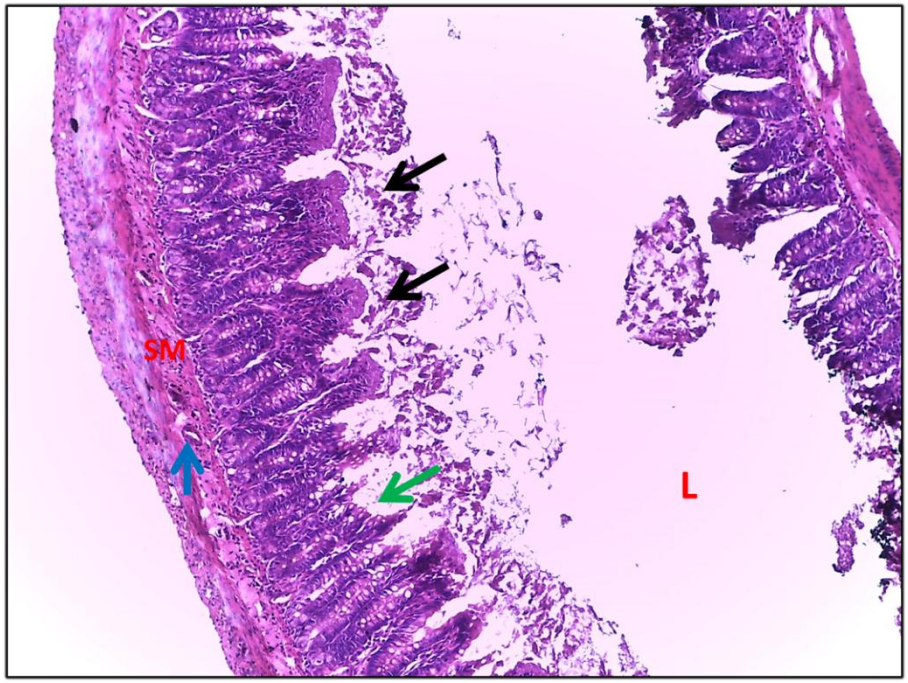


Fig. 4.21 Cecum section of infected rat with *E.histolytica* 14 days post-infection. Showed cecum lumen (**L**) amorphous material (➔)covering the mucosa, partial degeneration of crypts apical portion (➔)and submucosa(**SM**)with blood vessel (➔)H&E stain(98x).

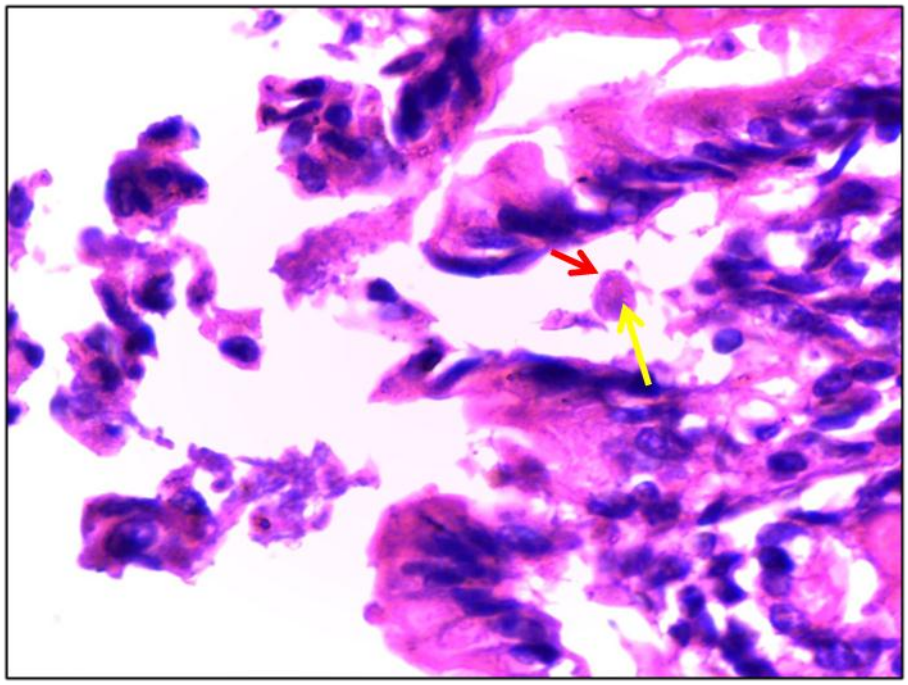


Fig. 4.22 Cecum section of infected rat with *E.histolytica* 28 days post-infection. Showed ovale, regular trophozoite (➔)with obvious nucleus (➔) within the intestinal tubular crypts. H&E stain(948x).

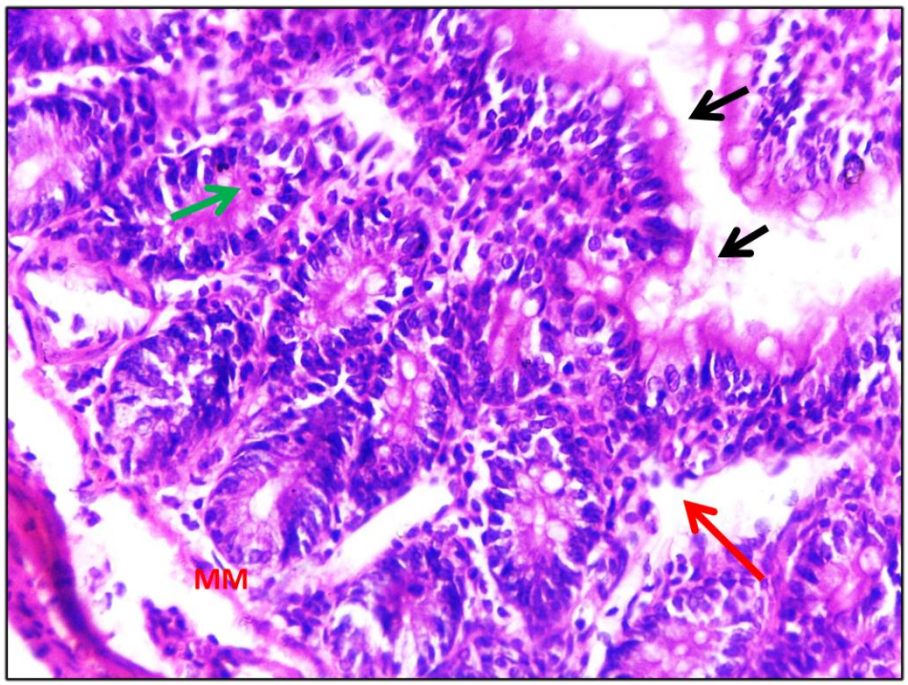


Fig. 4.23 Transverse cecum section of infected rat with *E.histolytica* 28 days post- infection. Showed mucosa folds(➔)free lymphocytes in the crypts(➔)edematous of muscularis mucosa(**MM**)and degeneration of lining epithelium of crypts (➔). H&E stain(379x).

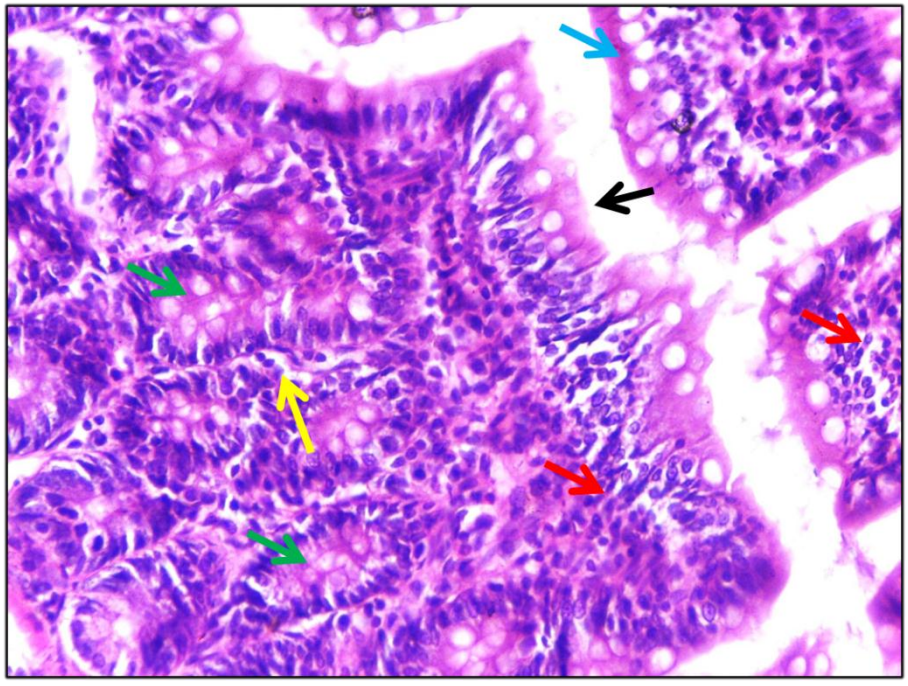


Fig. 4.24 Transverse cecum section of infected rat with *E.histolytica* 28 days post- infection. Showed flat mucosa(➔) heavy inflammatory cells (➔)beneath the mucosa ,crypts hyperplasia(➔)the inflammatory cells (➔) extending among the crypts , goblet cells hyperplasia (➔). H&E stain(379x).

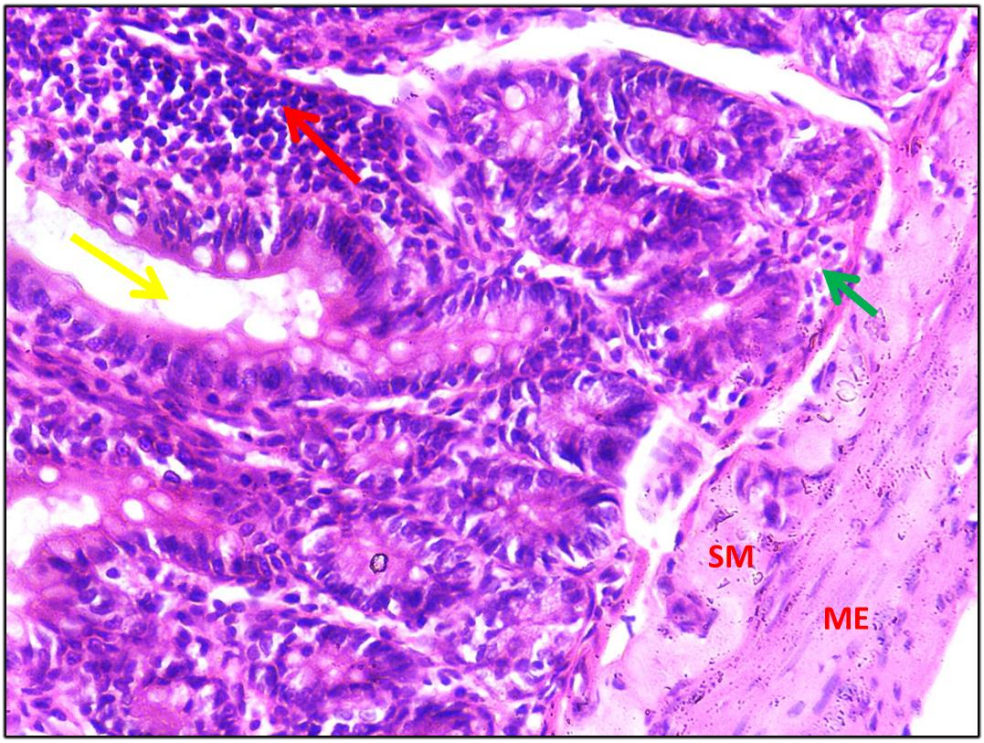


Fig. 4.25 Transverse cecum section of infected rat with *E.histolytica* 28 days post-infection. Showed aggregation of inflammatory cells (➔), inflammatory cells near the muscularis mucosa(➔)intestinal crypts were dilated (➔) with normal layer submucosa(SM), muscularis externa (ME)H&E stain(379x).

2.Rectum

Rectum sections of the control rats showed the lining of mucosa by simple columnar epithelium, the intestinal glands were longer and filled with goblet cells, these glands were extend from the surface to the muscularis mucosa, the longitudinal folds were contained a core of submucosa covered by the mucosa, The submucosa consists of loose connective tissue with a highly vascular, the muscularis externa differential to an inner circular smooth muscle layer and outer longitudinal muscle layer (Fig. 4.26 &4.27).

Microscopic photography showing the histological changes of infected rectum at 7,14 and 28 days post-infection revealed the attachment of the trophozoites to the mucosa layer. Furthermore, thick mucosa was lined by simple columnar epithelium ,the presence of branch intestinal –hyperplasia-

destruction of some crypts and heavy infiltration of inflammatory cells were obvious at 7 days post-infection (Fig. 4.28,4.29&4.30).

At 14 days post-infection the mild inflammation can be found, in addition to degeneration in the submucosa and hyperplasia in the crypts, thick wall of muscularis externa can be seen (Fig.4.31&4.32).

The findings of infected rat's rectum at 28 days post-infection showed the trophozoites within the mucosa and atrophied, contracted lumen in addition to the mucosa with ulceration of the apical surface as well as crypts were appeared lining abundant goblet cells. Other changes include edematous submucosa can be seen (Fig.4.33&4.34).

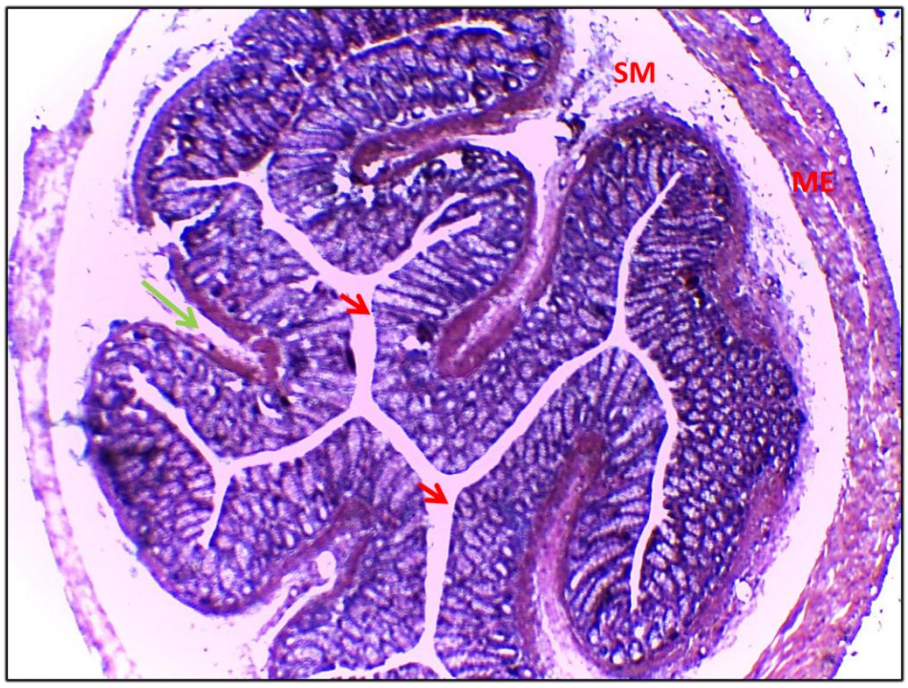


Fig.4.26 Transverse rectum section of control rat(normal structure).showed the mucosa was lining by simple columnar epithelium (➔)large ,longitudinal folds (➔)each fold composed of submucosa(**SM**)and mucosa, the muscularis externa (**ME**)differentiated to an inner circular smooth muscle layer and outer longitudinal muscle layer. H&E stain (98x)

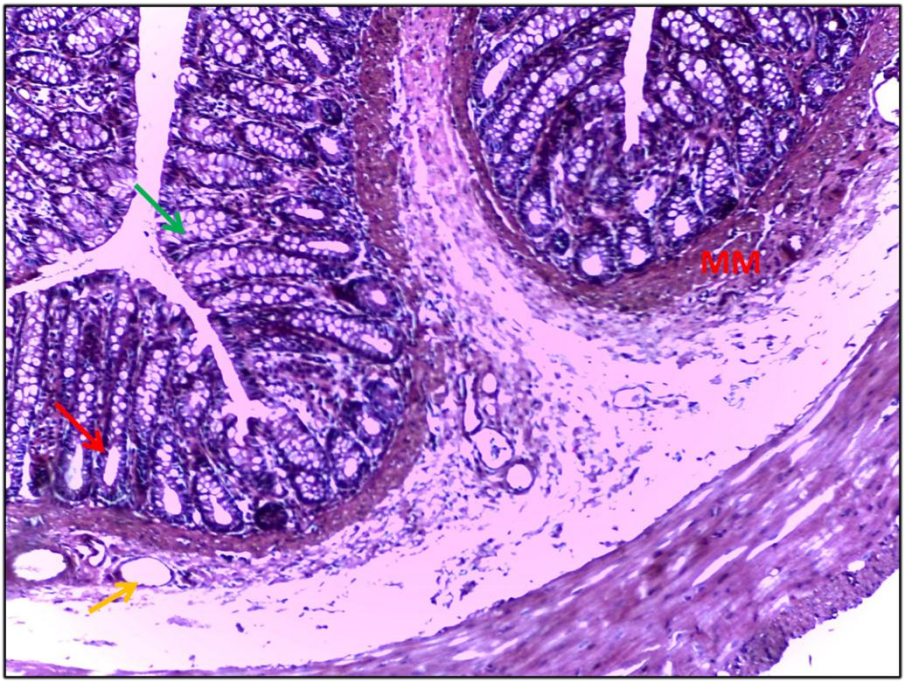


Fig.4.27 Transverse rectum section of control rat(normal structure).showed the longitudinal, deep crypts(➔)goblet cells(➔) muscularis mucosa(MM)blood vessels in submucosa(➔)H&E stain(379x).

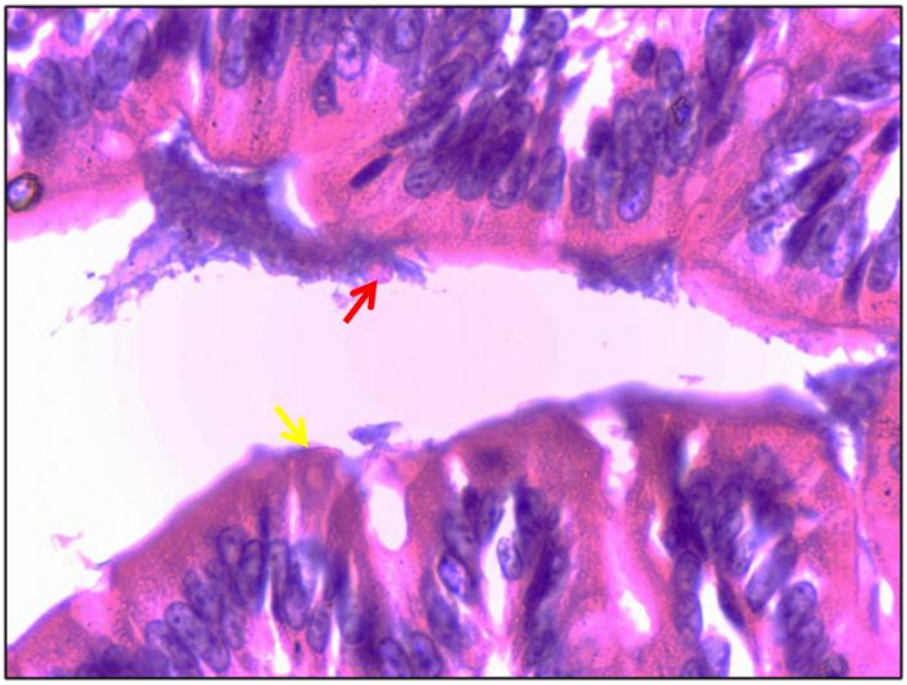


Fig.4.28 Rectum section of infected rat with *E.histolytica* 7 days post-infection. Showed the attachment of trophozoite (➔)at the surface mucosa (➔). H&E stain(948x).

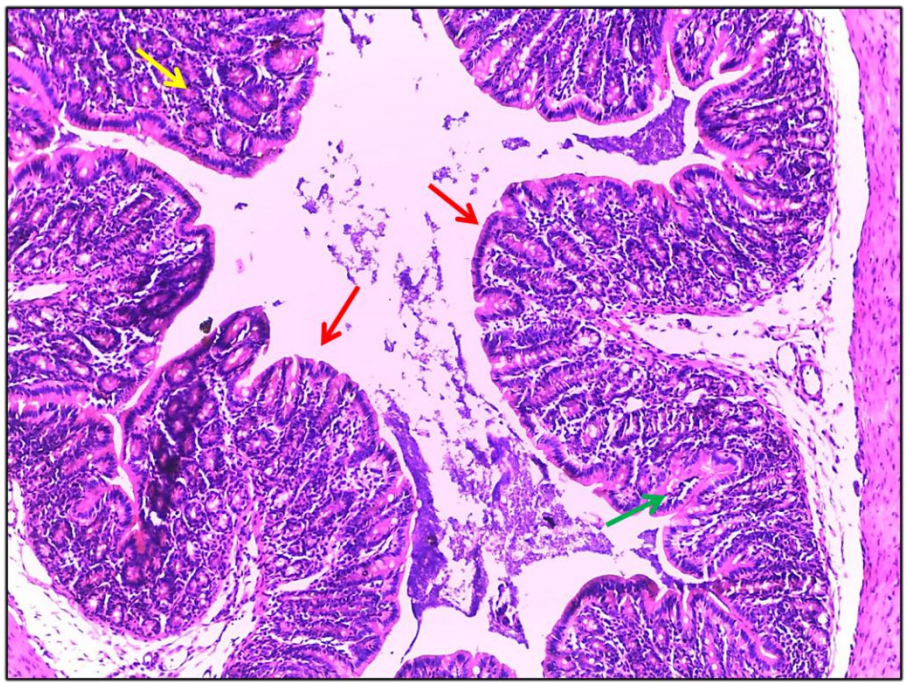


Fig. 4.29 Rectum section of infected rat with *E.histolytica* 7 days post-infection. Showed the mucosa layer ( )crypts hyperplasia ( )branched intestinal crypts ( ). H&E stain(98x)

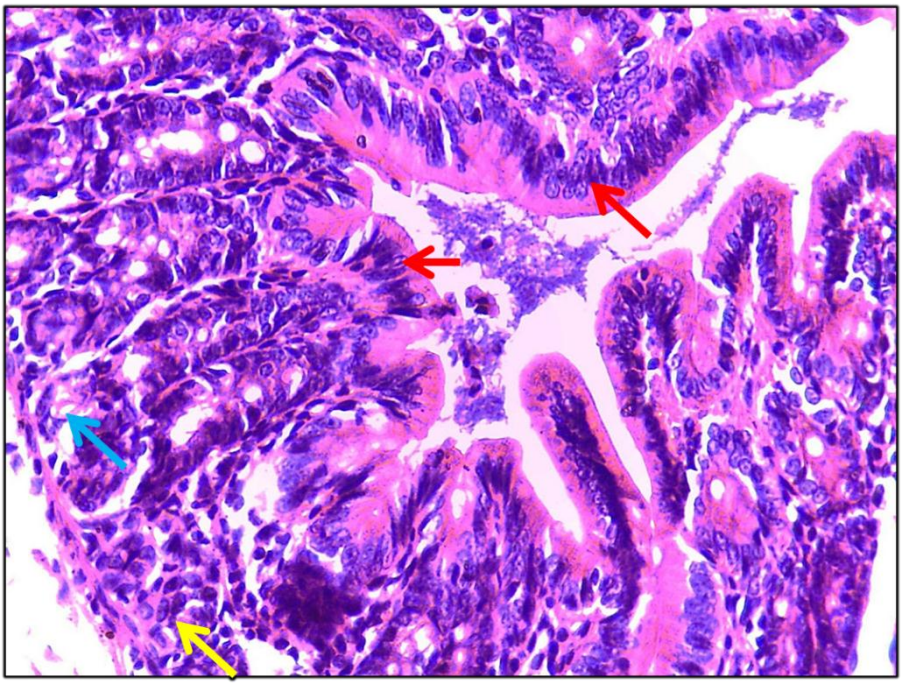


Fig. 4.30 Rectum section of infected rat with *E.histolytica* 7 days post-infection. Showed thick mucosa was lined by simple columnar epithelium( )destruction of some crypts( )infiltration of inflammatory cells ( ). H&E stain(379x).

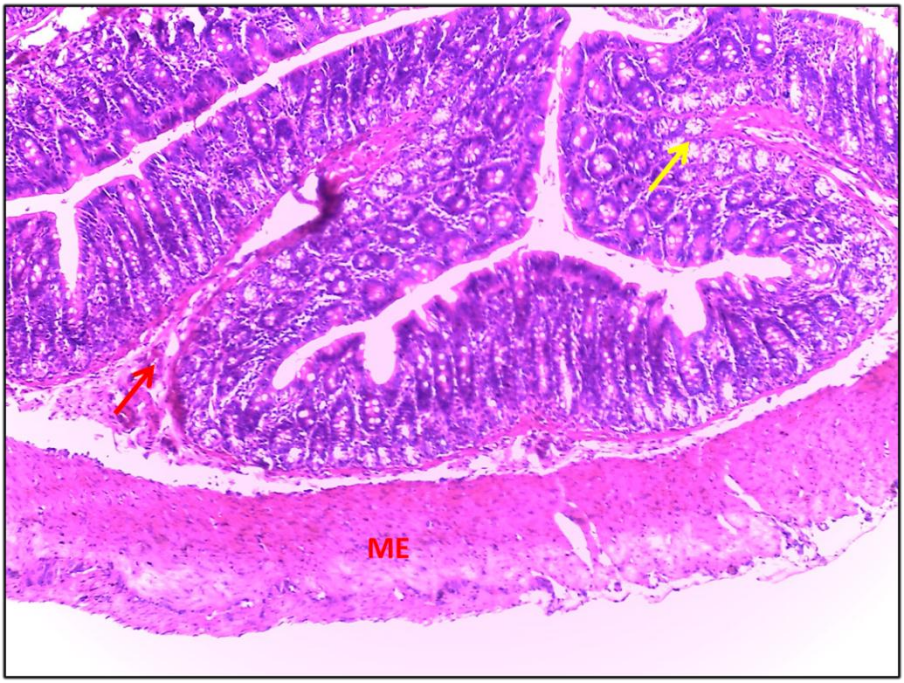


Fig.4.31 Rectum section of infected rat with *E.histolytica* 14 days post- infection. Showed the degeneration in the submucosa (➔)and in the crypts (➔) thick wall of muscularis externa (**ME**). H&E stain(98x).

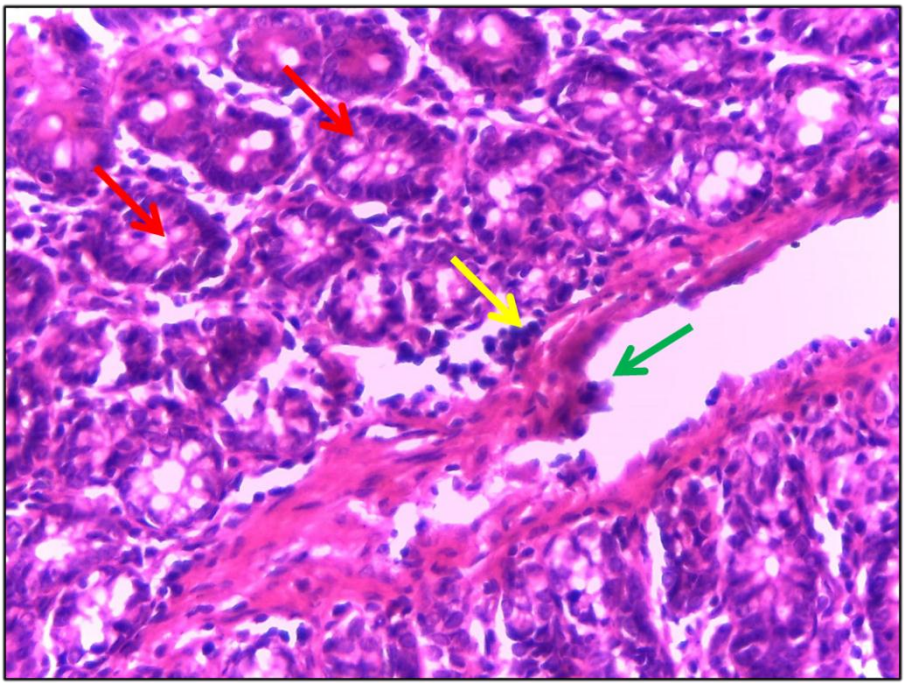


Fig. 4.32 Rectum section of infected rat with *E.histolytica* 14 days post- infection. Showed the degeneration in the submucosa (➔)with mild inflammation(➔) crypts hyperplasia (➔). H&E stain(379x).

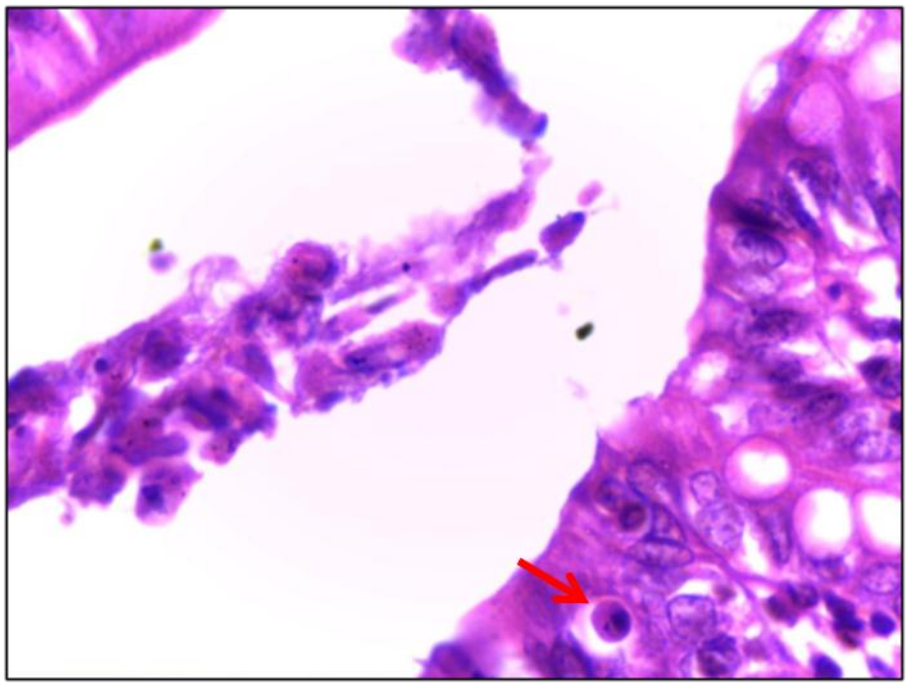


Fig. 4.33 Rectum section of infected rat with *E.histolytica* 28 days post- infection. Showed the trophozoite within mucosa(➔)H&E stain(948x).

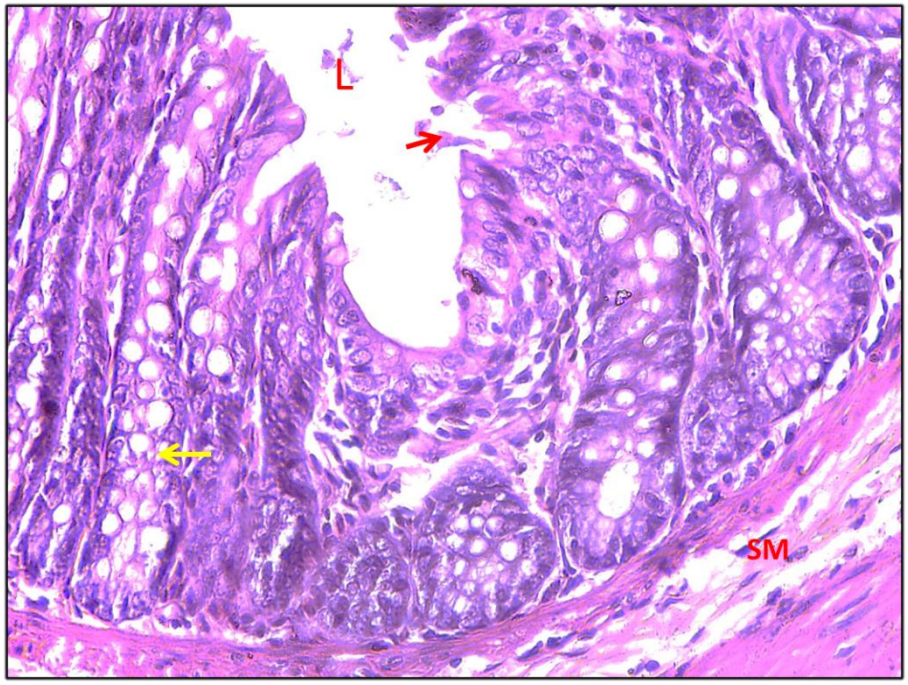


Fig. 4.34 Rectum section of infected rat with *E.histolytica* 28 days post- infection. Showed atrophied, contracted lumen (L)the mucosa with ulceration of the apical surface(➔)goblet cells hyperplasia (➔)edematous submucosa (SM)H&E stain(379x).

4.4.1.2 Histological changes in *E. dispar* experimental infection

1.Cecum

Histological changes found in sections of cecum that associated with *E. dispar* 7 days post-infection showed mild changes in the mucosa layer architecture represented by flat surfaces, in addition, to elongated crypts that lined with high number of goblet cells. Moreover, infiltration of inflammatory cells that distributed within lamina propria can be seen (Fig.4.35).

Two week post-infection showed that the mucosa was modified into longitudinal folds with the trophozoites attached to the epithelial layer (Fig.4.36 &4.37), where it was flat in other area . Elongated deep crypts and moderate inflammation in the lamina propria with regular muscularis mucosa can be seen. This period also showed lymph nodule with active germinal center and the submucosa consists of loose connective tissue with blood vessels(Fig.4.38&4.39).

Light microscopic examination on cecum sections from infected rats with *E. dispar* at 28 days post-infection revealed large number of trophozoites found on the surface layer with typical shape. Other changes include unclear mucosal layer and deep crypts with mild degeneration of some crypts base ,we can see also a regular muscular mucosa and submucosa(Fig.4.40&4.41).

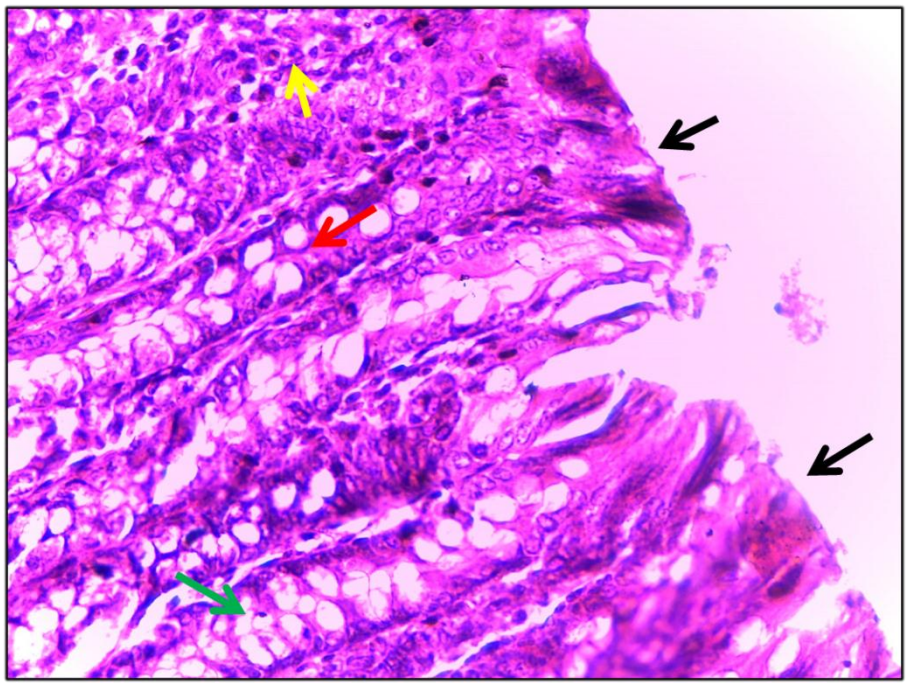


Fig. 4.35 Flat surface in cecum section of infected rat with *E.dispar* 7days post- infection. Showed flat surface of mucosa(➔)elongated crypts (➔)lined with goblet cell (➔) infiltration of inflammatory cells (➔) . H&E stain(379x).

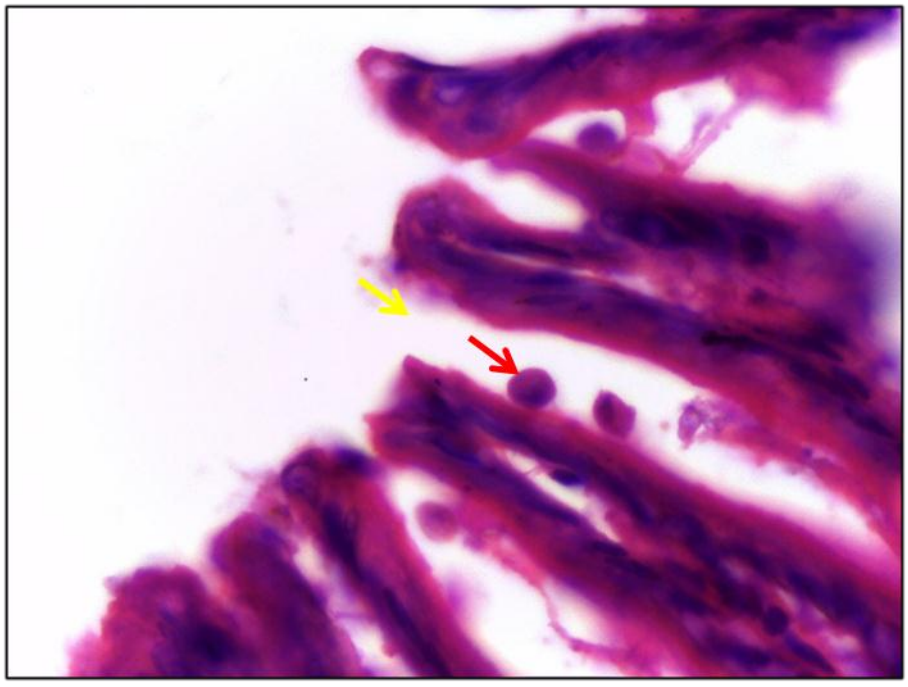


Fig.4.36 Cecum section of infected rat with *E.dispar* 14 days post-infection . Showed the attachment of trophozoite(➔) mucosa modified to longitudinal folds(➔). H&E stain(948x).

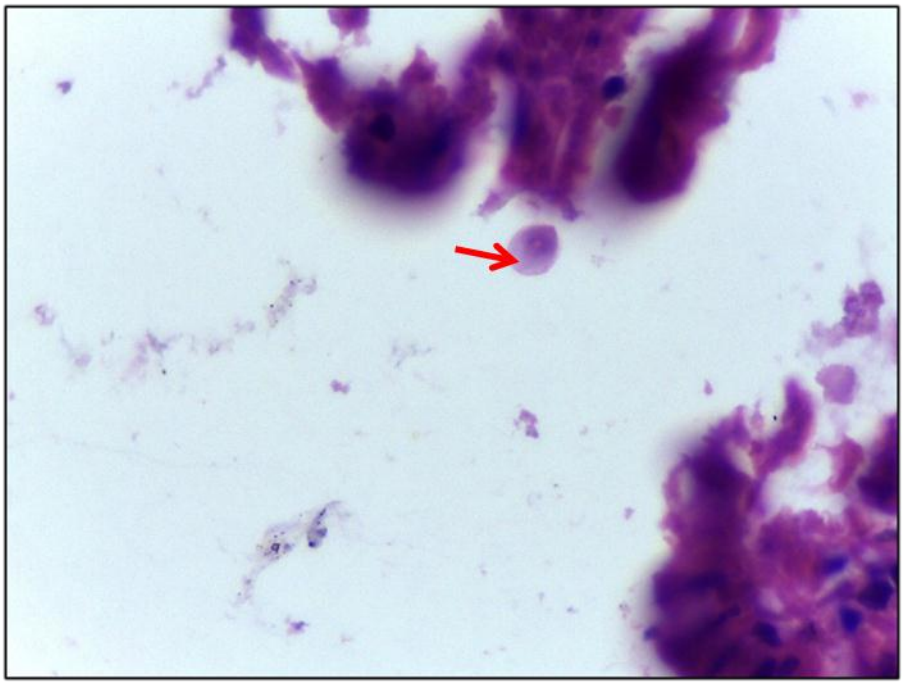


Fig.4.37 Cecum section of infected rat with *E.dispar* 14 days post-infection .Showed the trophozoite within lumen (➔)H&E stain(948x).

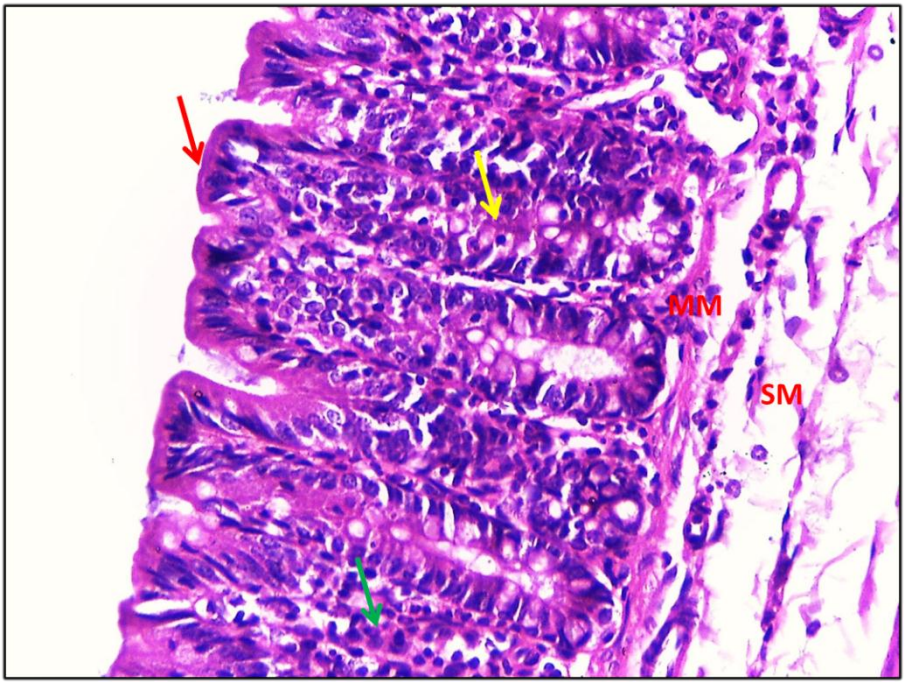


Fig.4.38 Transverse cecum section of infected rat with *E.dispar* 14 days post-infection. Showed flat mucosa surface (➔)elongated-deep crypts (➔)moderate inflammation in lamina propria (➔) regular muscularis mucosa (**MM**), submucosa with blood vessels (**SM**). H&E stain (379x).

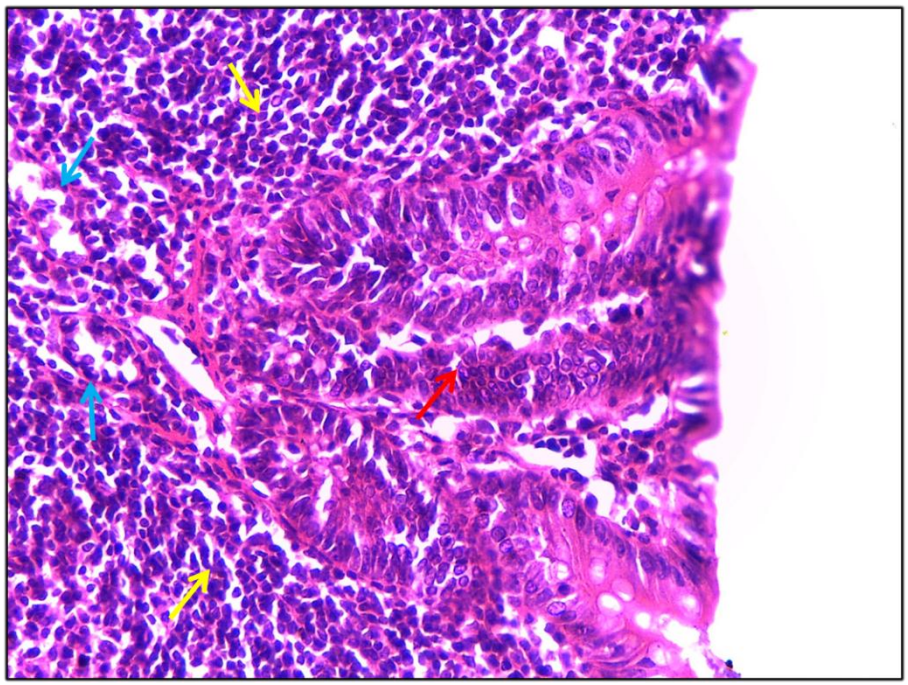


Fig.4.39 Transverse cecum section of infected rat with *E.dispar* 14 days post -infection .Showed deep crypts (➔) the lymph nodule with active germinal center (➔) heavy infiltration of inflammatory cells (➔) H&E stain(379x).

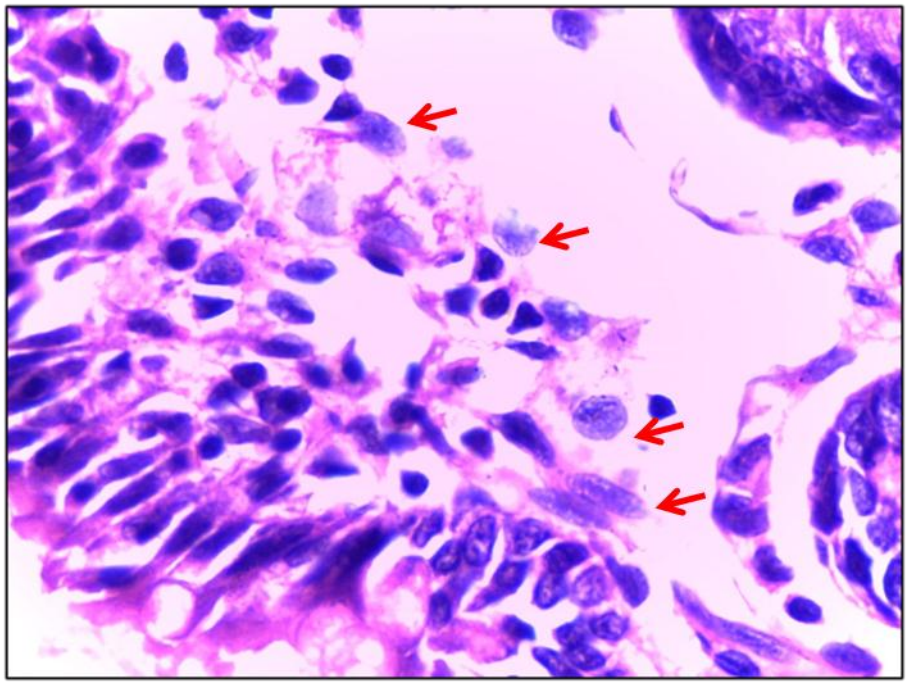


Fig. 4.40 Cecum section of infected rat with *E.dispar* 28 days post-infection . Showed the large number of trophozoite attached on the surface layer with typical shape (➔)H&E stain(948x).

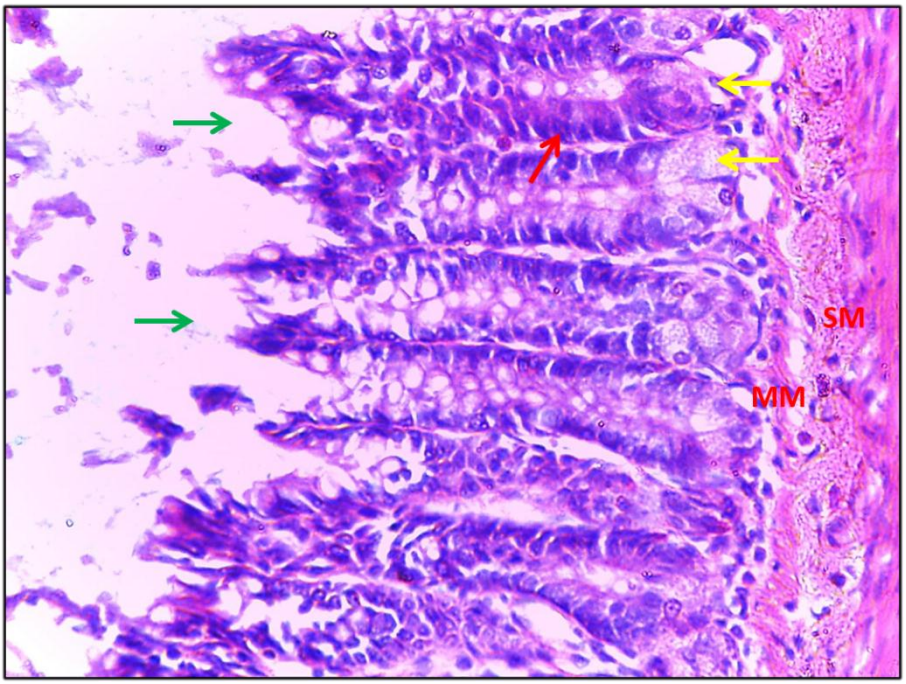


Fig.4.41 Transverse cecum section of infected rat with *E.dispar* 28 days post- infection. Showed unclear mucosal layer (➔)deep crypts (➔)mild degeneration of some crypts base (➔)regular muscular mucosa(**MM**) and submucosa (**SM**) H&E stain(379x).

2.Rectum

Histological changes in rectum sections of infected rats at 7 days post-infection with *E. dispar* revealed that the mucosa was covered with thick mucus and the regular columnar cells with elongated nuclei. Furthermore, the lamina propria infiltrated with high number of inflammatory cells and degeneration in other regions (Fig.4.42&4.43).

At 14 days post-infection of *E. dispar* trophozoites appeared attached to the epithelial layer and embedded within the mucus, degeneration in the mucosa layer , in addition, to inflammatory cells infiltrates in the varied regions. The muscularis mucosa extend at the base of crypts as well as the submucosa with large blood vessels can be seen (Fig.4.44,4.45&4.46).

The findings in rectum of infected rats at 28 days post- infection showed degeneration in the mucosa layer and elongated crypts which it where lined hyperplasia goblet cells, in addition to inflammatory cells diffused within lamina propria around the crypts (Fig.4.47).

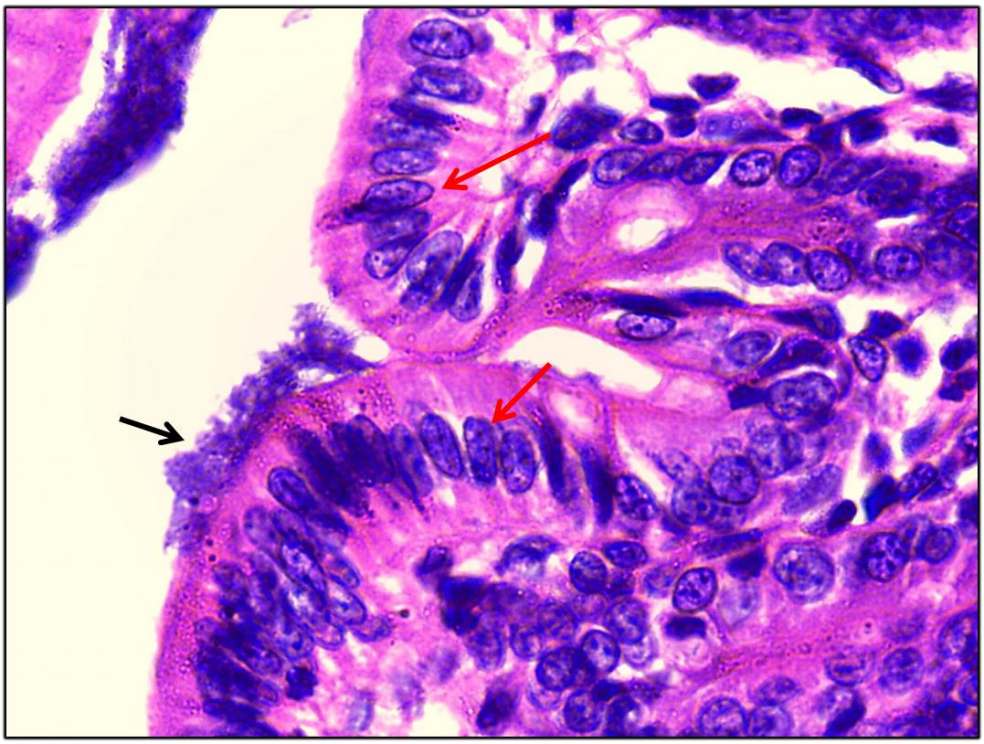


Fig.4.42 Transverse rectum section of infected rat with *E.dispar* 7 days post- infection. Showed the mucosa was covered with thick mucus (➔) columnar cells with normal nuclei (➔) H&E stain(379x).

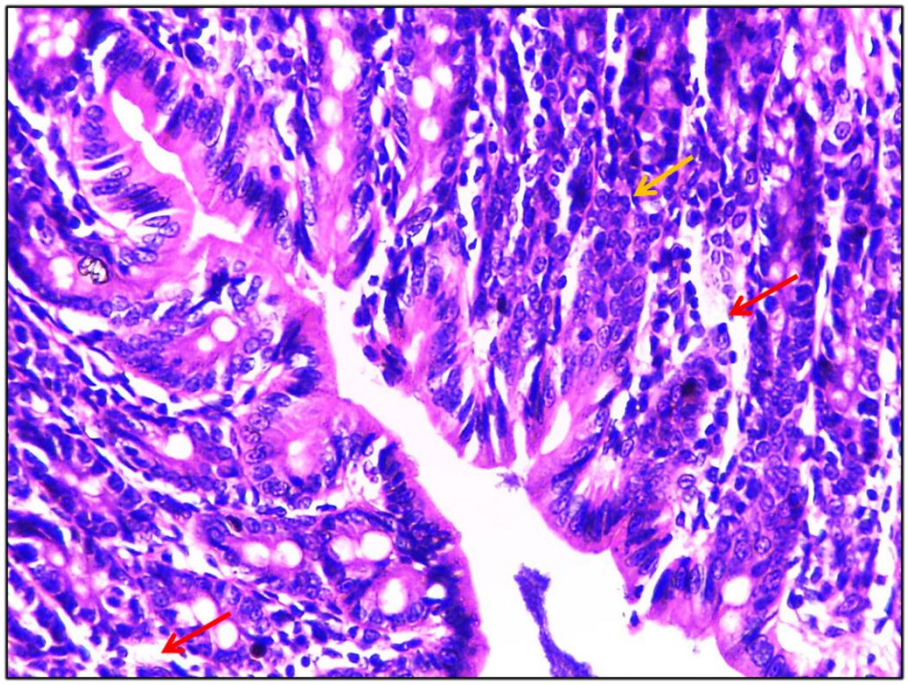


Fig.4.43 Transverse rectum section of infected rat with *E.dispar* 7 days post- infection. Showed degeneration in the lamina propria (➔) heavy infiltration of inflammatory cells (➔) . H&E stain(379x).

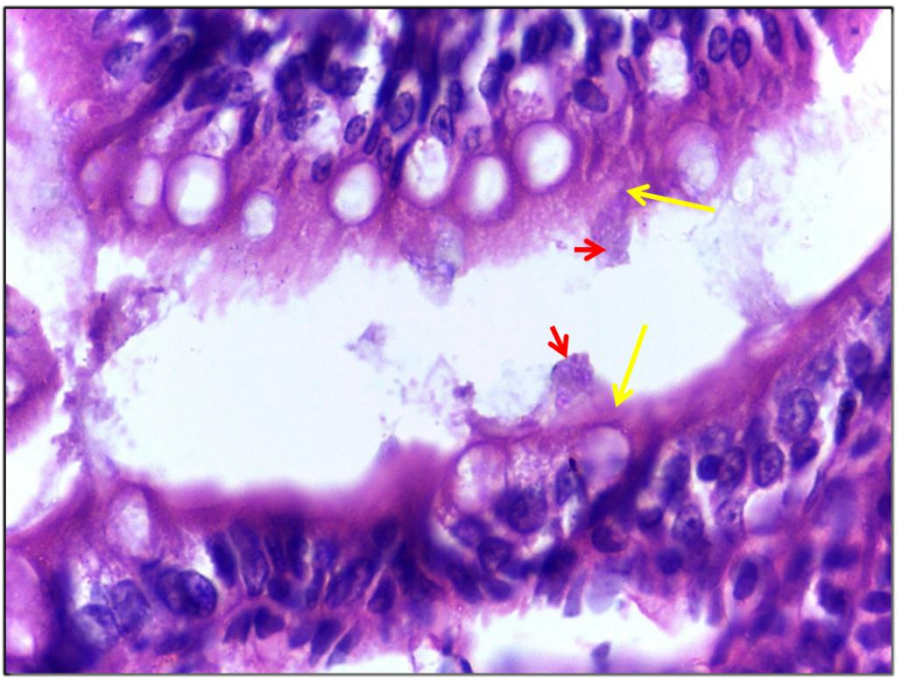


Fig. 4.44 Rectum section of infected rat with *E.dispar* 14 days post-infection . Showed the trophozoite (➔)near the epithelial layer and embedded with the mucus (➔).H&E stain(948x).

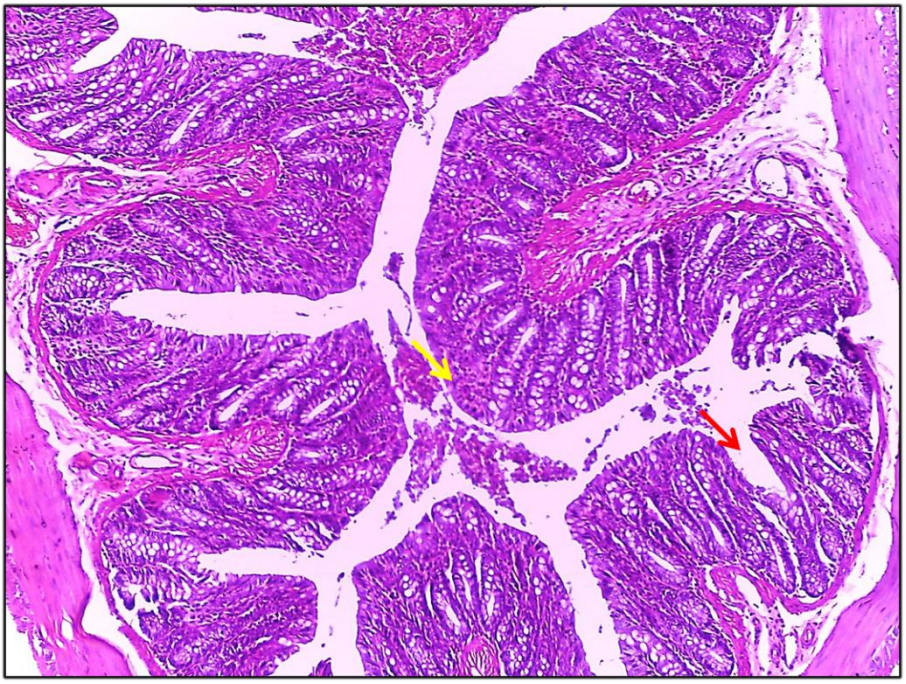


Fig. 4.45 Transverse rectum section of infected rat with *E.dispar* 14 days post – infection. Showed degeneration in the mucosa layer (➔)mild infiltration of inflammatory cells (➔)H&E stain(98x).

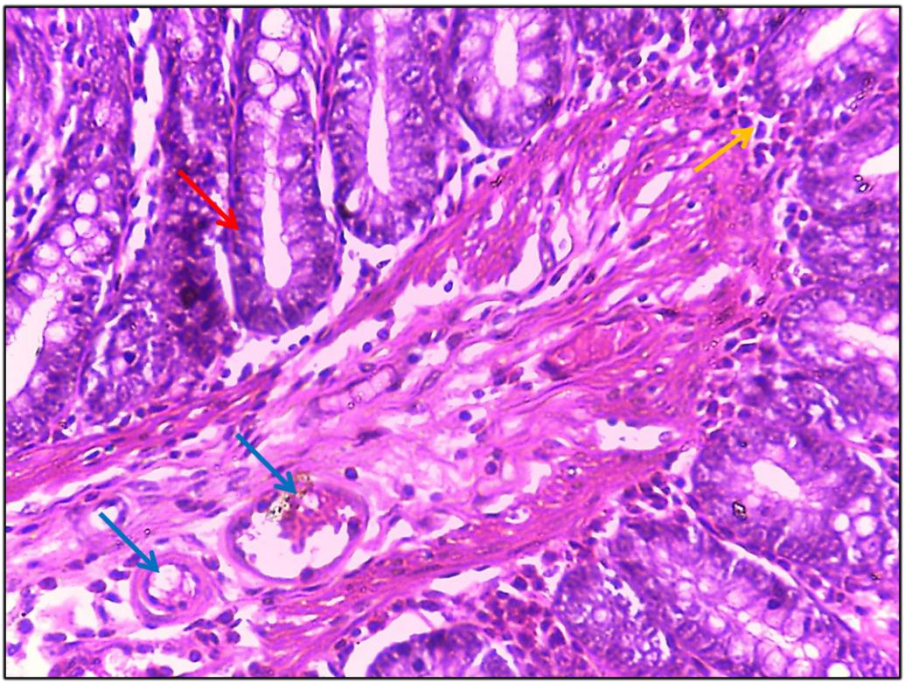


Fig.4.46 Transverse rectum section of infected rat with *E.dispar* 14 days post- infection. Showed elongated crypts (➔) inflammatory cells at the base of the crypts(➔)submucosa with blood vessels(➔)H&E stain(379x).

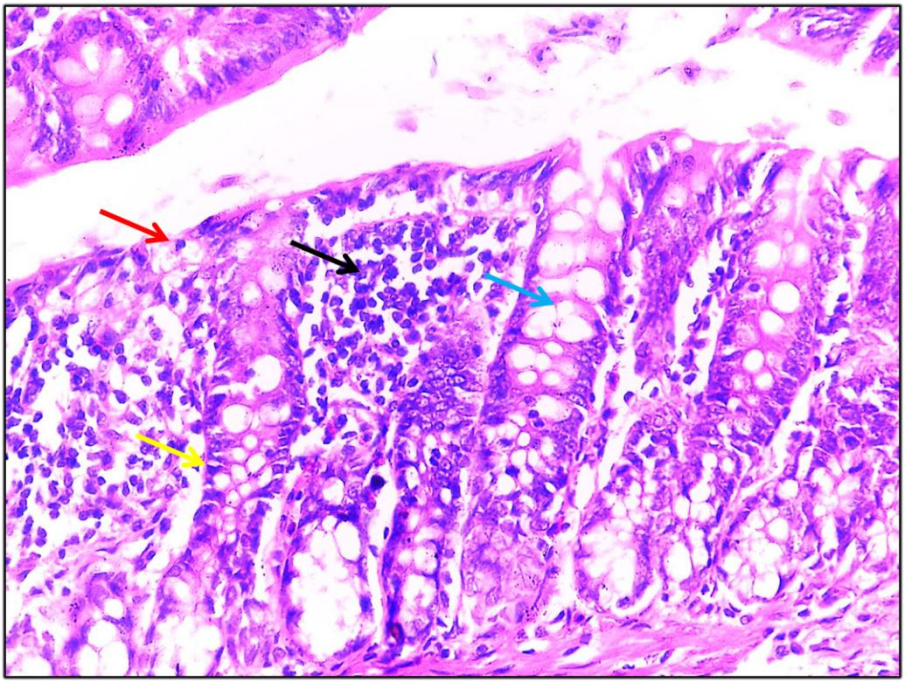


Fig. 4.47 Transverse rectum section of infected rat with *E.dispar* 28 days post- infection .Showed degeneration in the mucosa layer(➔) elongated crypts (➔)goblet cells hyperplasia (➔) moderate inflammatory cells within lamina propria (➔)H&E stain(379x).

4.4.1.3 Histological changes in *E. moshkovskii* experimental infection

1.Cecum

The histological observations in cecum of infected rats *E. moshkovskii* at 7 days post-infection showed dissociation and degeneration in the mucosa layer and heavy infiltration of inflammatory cells can be seen (Fig.4.48).

Figures on sections from cecum related to infected rats with *E. moshkovskii* at 14 days post- infection showed moderate alteration included disorganized mucosa and mild inflammatory cells (Fig.4.49).

Observation of light microscopy on cecum sections from infected rats with *E. moshkovskii* at 28 days post-infection appeared that the trophozoites with ovale shape were noticed at the apical surface of crypts and normal mucosa with deep crypts . The presence of mild inflammatory cells (lymphocytes) . Moreover, other lymphocytes were aggregated and formed what is known as the gut associated lymphoid tissue (GALT) within submucosa ,also heavy infiltration of inflammatory cells in the lamina propria with dilated blood vessels can be noticed (Fig.4.50,4.51&4.52).

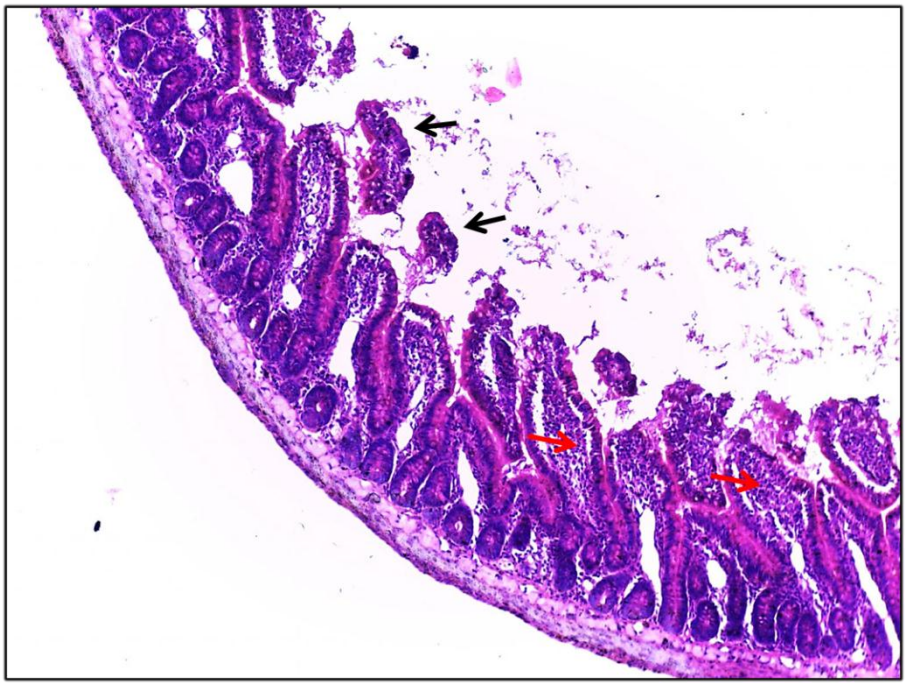


Fig.4.48 Transverse cecum section of infected rat with *E. moshkovskii* 7 days post- infection .Showed dissociation and degeneration in the mucosa layer(➔)heavy infiltration of inflammatory cells(➔)H&E stain(98x)

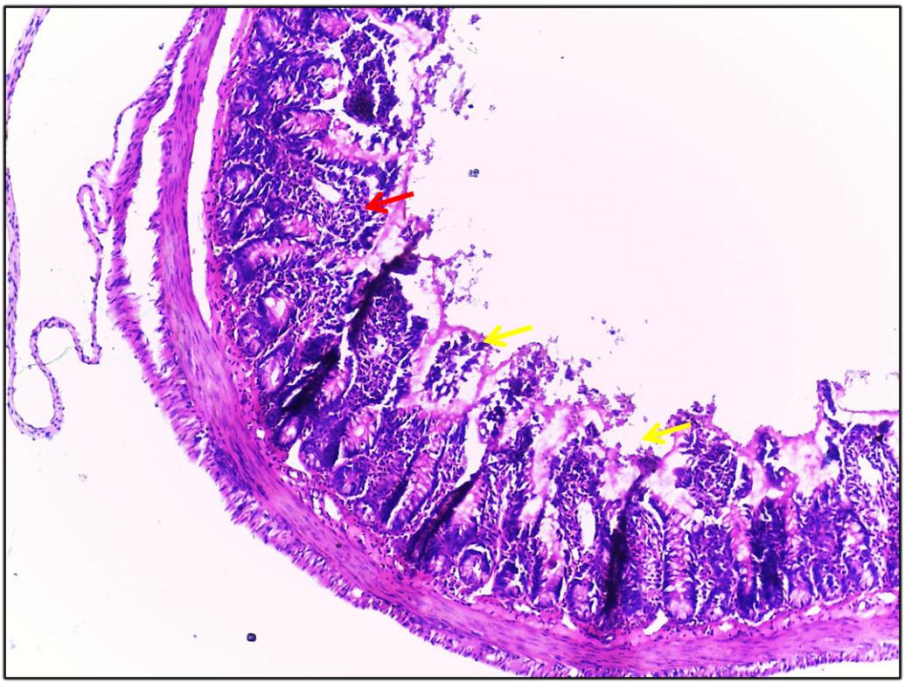


Fig.4.49 Transverse cecum section of infected rat with *E. moshkovskii* 14 days post- infection. Showed mild inflammation(➔) dissociation and degeneration in the mucosa layer (➔) H&E stain(98x)

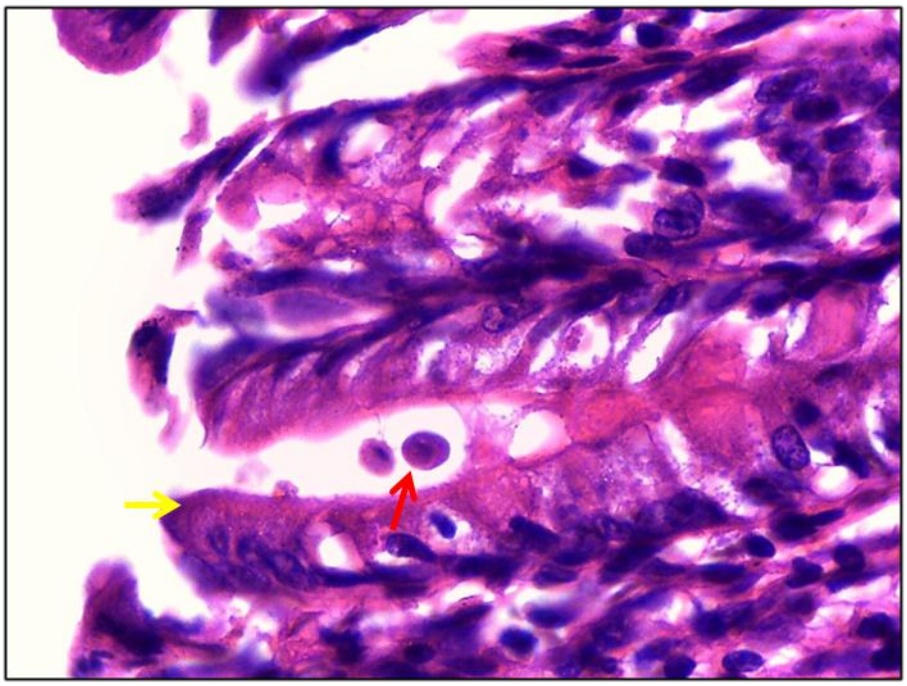


Fig.4.50 Cecum section of infected rat with *E. moshkovskii* 28 days post -infection .Showed the trophozoite (➔)at the apical surface of crypts (➔).H&E stain(948x).

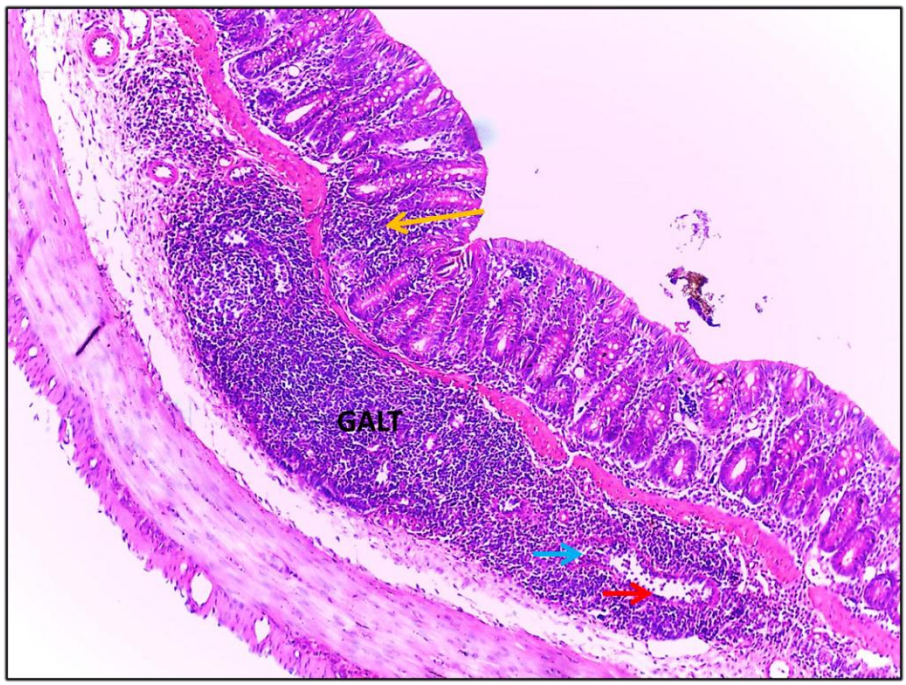


Fig.4.51 Transverse cecum section of infected rat with *E. moshkovskii* 28 days post- infection .Showed gut associated lymphoid tissue (**GALT**) within submucosa, heavy infiltration of inflammatory cells in the lamina propria(➔)dilated blood vessel(➔) lymphocyte (➔)H&E stain(98x) .

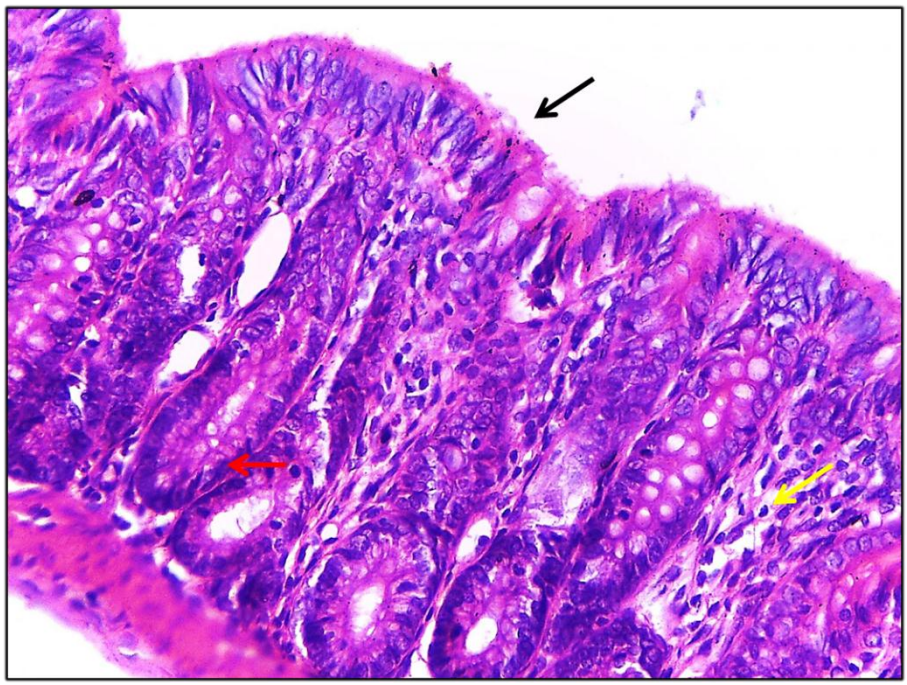


Fig.4.52 Transverse cecum section of infected rat with *E.moshkovskii* 28 days post- infection .Showed normal mucosa (➔)deep crypts (➔)mild inflammatory cells (➔) . H&E stain(379x).

2.Rectum

Finding from rectal histological sections of infected rats with *E. moshkovskii* at 7 days post-infection clarified heavy infiltration of inflammatory cells of mucosa layer also dilated blood vessel and edema in submucosa, Furthermore, debris and amorphous material in lumen (Fig.4.53&4.54).

The trophozoites were observed attached to the epithelial layer at 14 days post-infection with *E. moshkovskii* causing moderate changes in rectum structure and crypts were lined by large number goblet cells, congested blood vessels within submucosa, in addition to the presence of inflammation and red blood cell in lumen (Fig.4.55,4.56&4.57).

The results at 28 days post-infection showed that the lymph nodule in submucosa extended to lamina propria, inflammatory cells aggregation can also be seen (Fig.4.58).

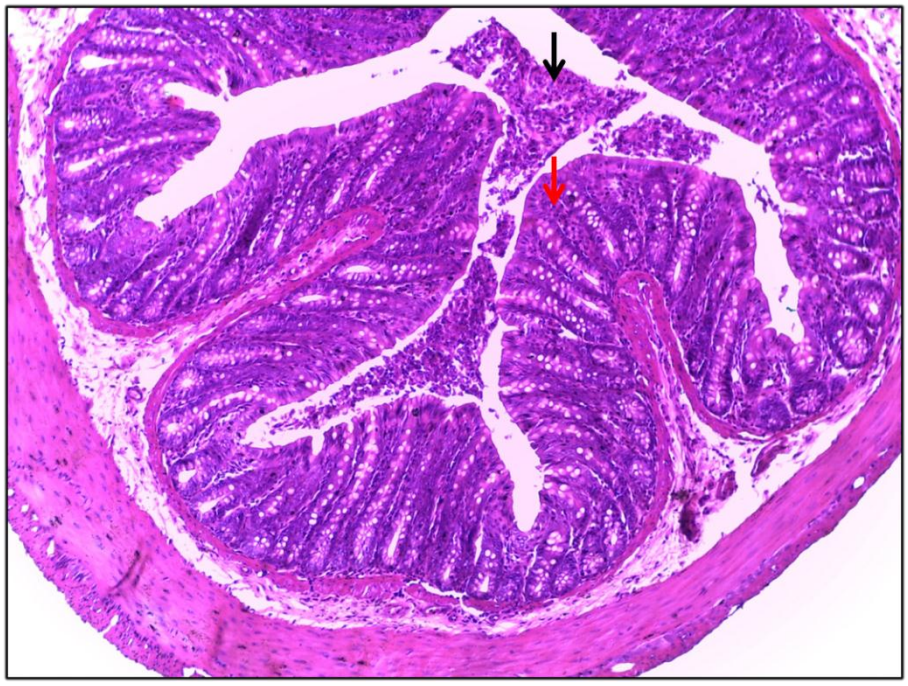


Fig.4.53 Transverse rectum section of infected rat with *E. moshkovskii* 7 days post –infection. Showed mild inflammatory cells(➔)debris in lumen (➔).H&E stain(98x)

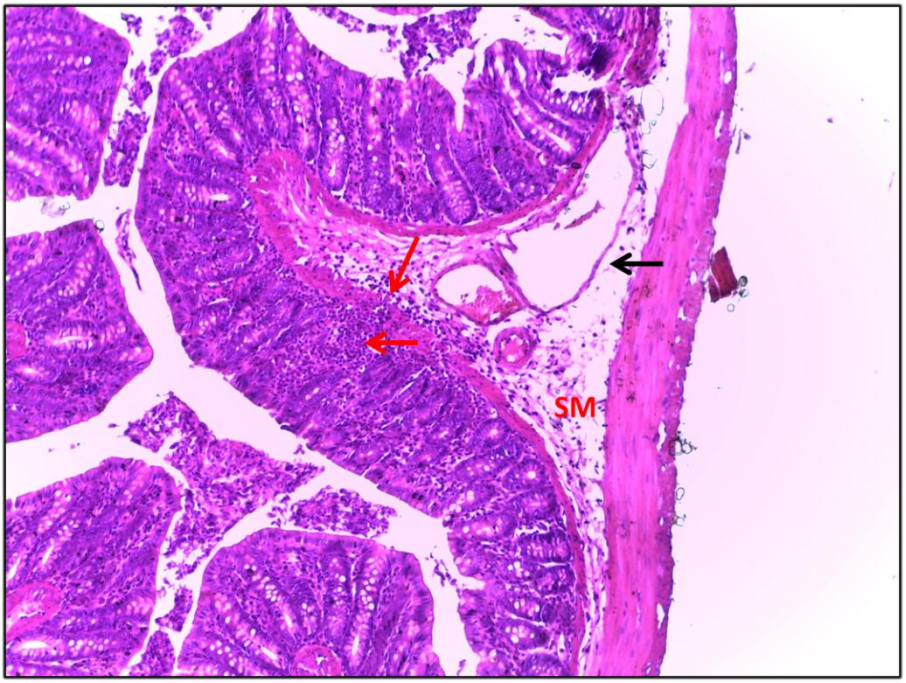


Fig.4.54 Transverse rectum section of infected rat with *E. moshkovskii* 7 days post- infection. showed dilated blood vessel(➔) of submucosa ,edema in submucosa (**SM**)heavy infiltration of inflammatory cells (➔). H&E stain(379x).

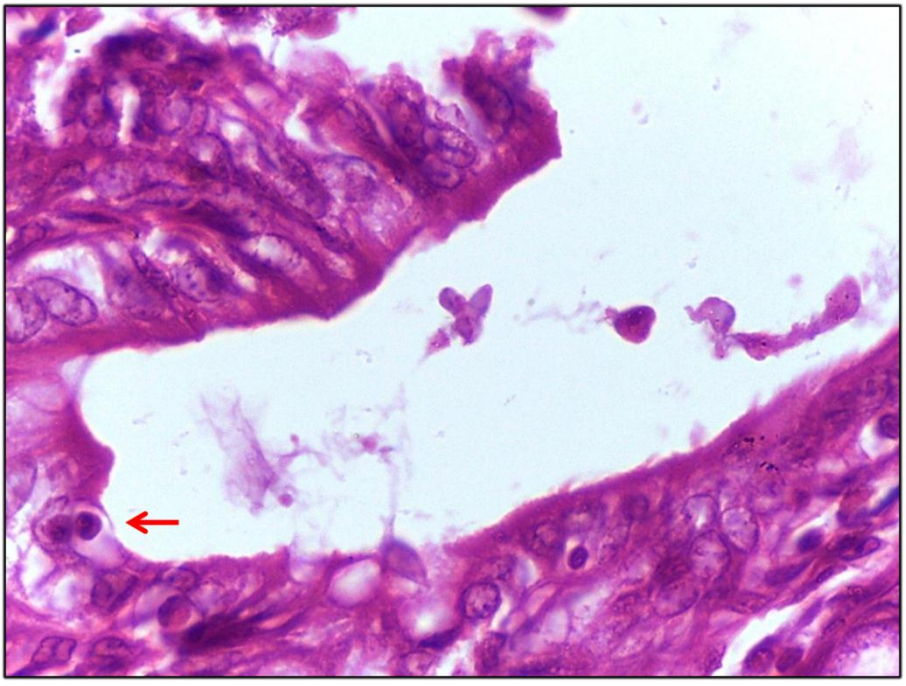


Fig.4.55 Rectum section of infected rat with *E. moshkovskii* 14 days post -infection . Showed the trophozoite(➔)within mucosa layer .H&E stain(948x).

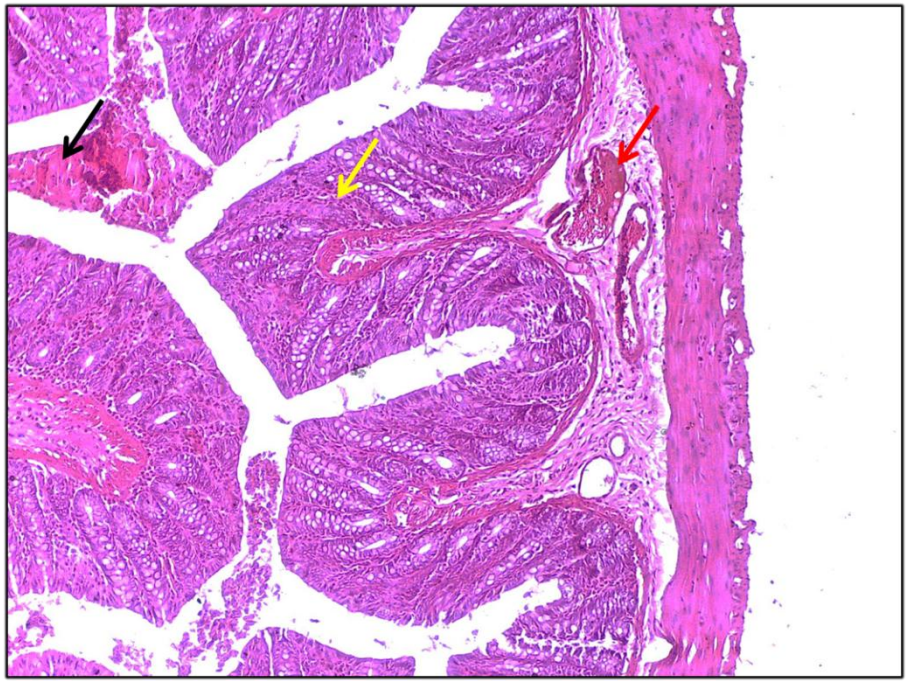


Fig.4.56 Transverse rectum section of infected rat with *E. moshkovskii* 14 days post- infection. showed congested blood vessels(➔) of submucosa , inflammatory cells (➔) red blood cell in lumen(➔)H&E stain(98x).

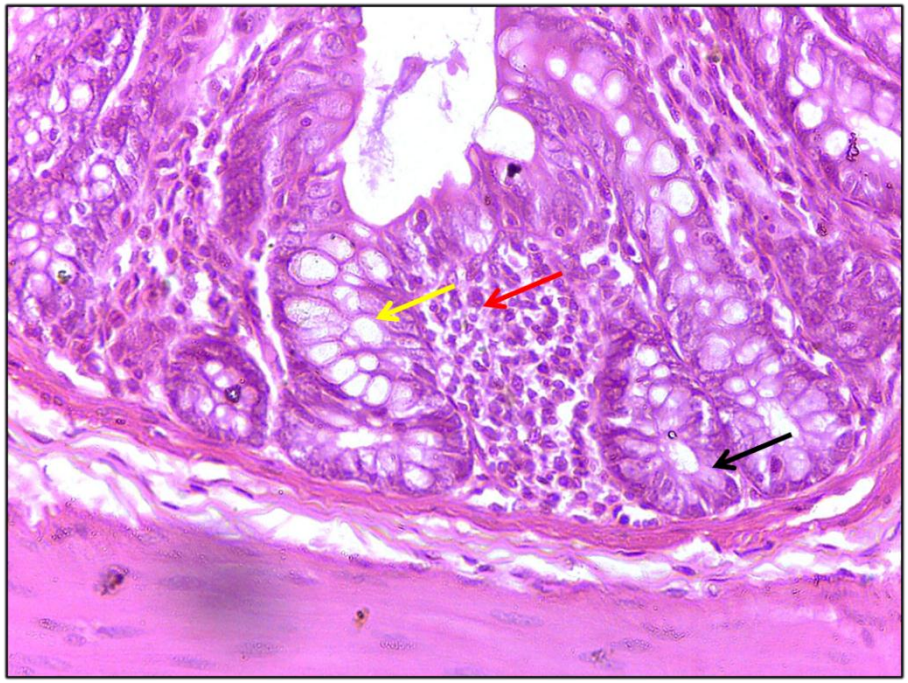


Fig.4.57 Transverse rectum section of infected rat with *E. moshkovskii* 14 days post- infection. showed inflammation (➔)deep crypts (➔)goblet cells hyperplasia (➔) H&E stain(379x).

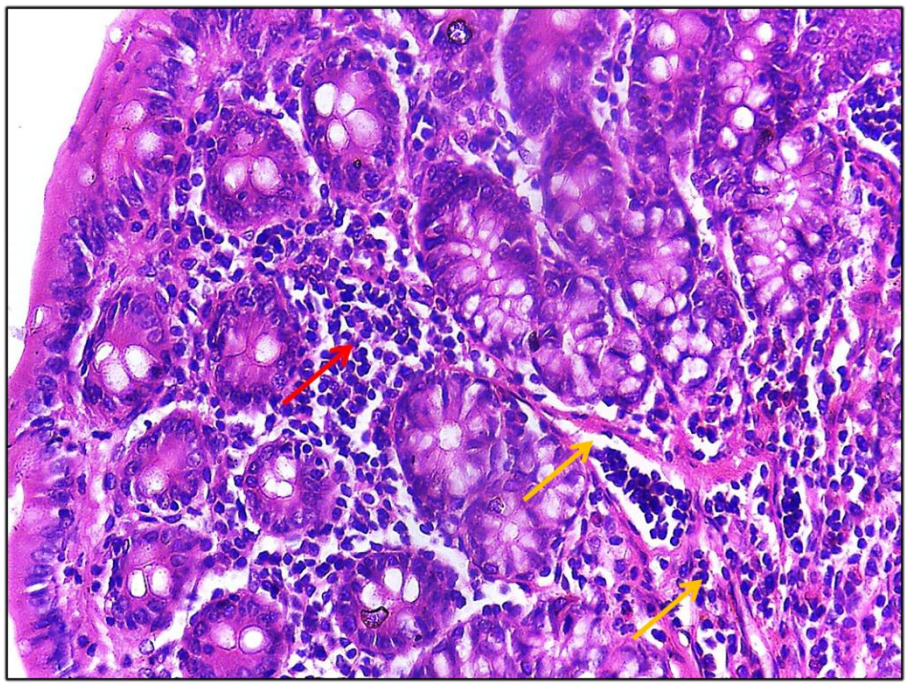


Fig.4.58 Transverse rectum section of infected rat with *E. moshkovskii* 28 days post- infection. showed lymph nodule in submucosa extend to lamina propria (➢) aggregation inflammatory cells(➢)H&E stain(379x).

4.4.2 Electron Microscope study

1.Transmission Electron Microscopy

Cecum sections from large intestine of control rats which were examined with TEM showed the laterial region of two adjacent intestinal epithelial cells , the junction complex consist of zonula adherence and desmosome ,electron dense cytoplasm ,normal nucleus (Fig. 4.59).

The results clarified the changes of infected cecum at 28 days post-infection with *E. histolytica* showed irregular microvilli , glycocalyx, and multivesicular bodies can be seen (Fig.4.60 & 4.61).

Sections of rats infected with *E. dispar* and *E.moshkovskii* showed that the presence of dilated ,elliptic mitochondria and multivesicular bodies in *E. dispar* while microvilli were preserved in normal appearance and normal free surface in *E.moshkovskii* (Fig. 4.62 & 4.63)

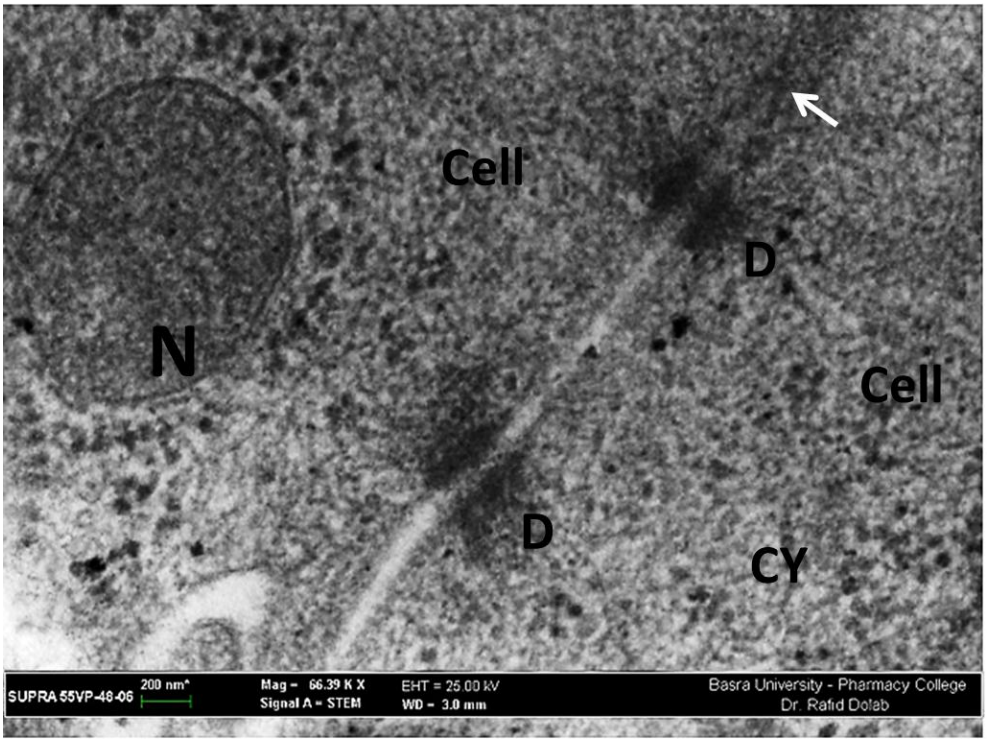


Fig.4.59 TEM micrograph of control rat cecum (normal structure).showed the laterial region of two adjacent intestinal epithelial cells (**Cell**) , the junctional complex consist of zonula adherens (➔) and desmosome (**D**),electron dense cytoplasm (**CY**),normal nucleus (**N**).

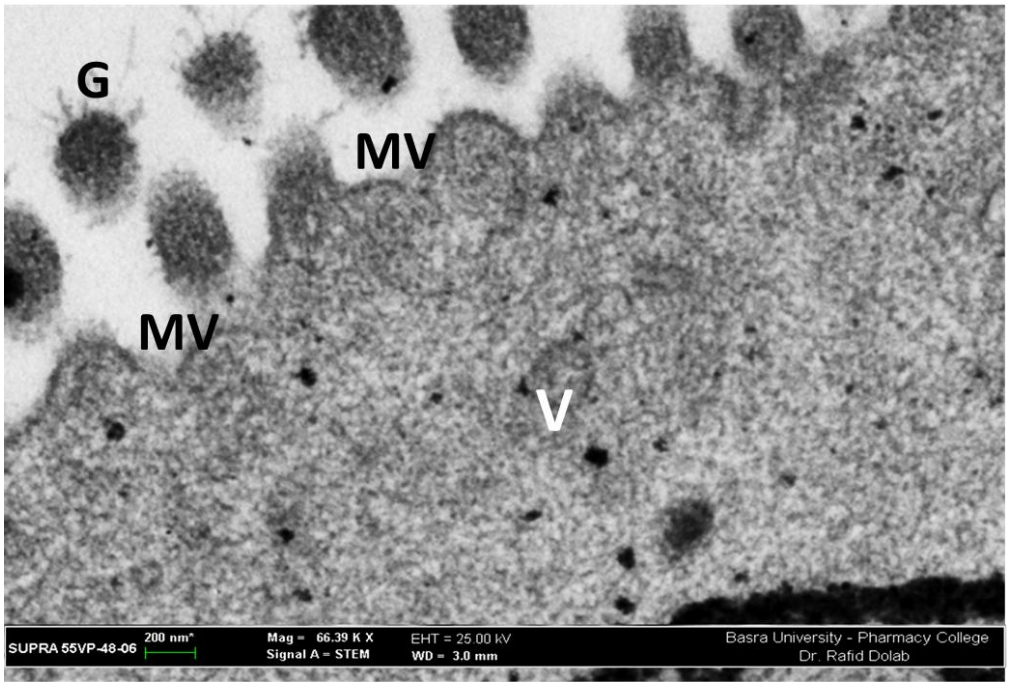


Fig.4.60 micrograph with TEM on rat cecum infected with *E.histolytica.* Showed irregular microvilli (**MV**),glycocalyx (**G**) multivesicular bodies (**V**)

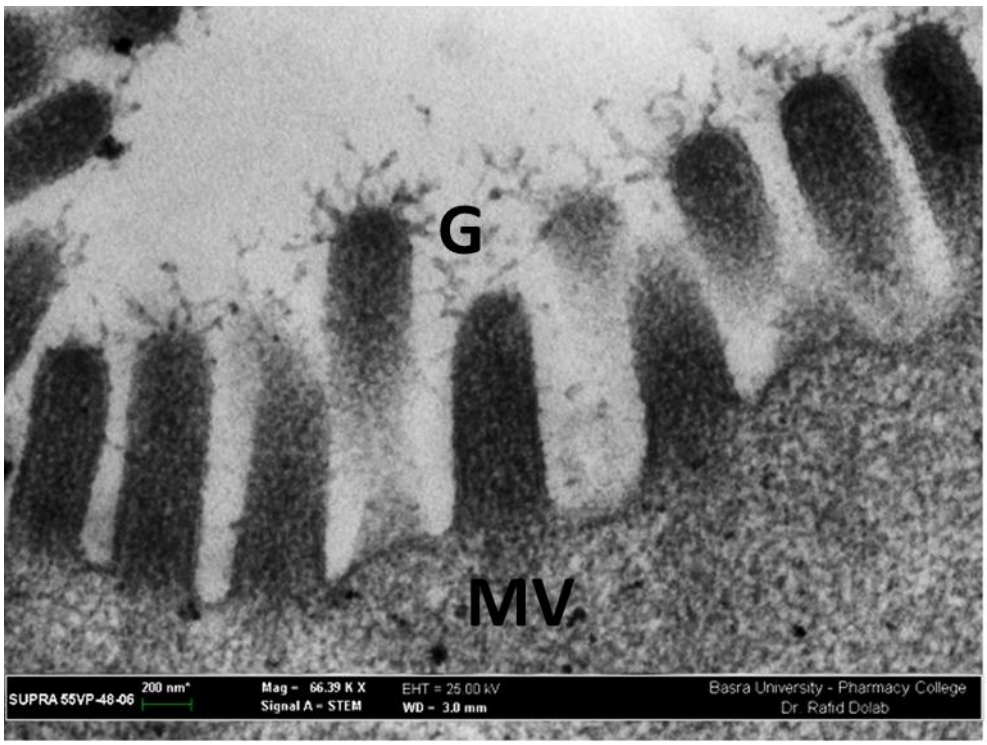


Fig.4.61 micrograph with TEM on rat cecum infected with *E.histolytica.* Showed irregular microvilli (**MV**),glycocalyx (G)

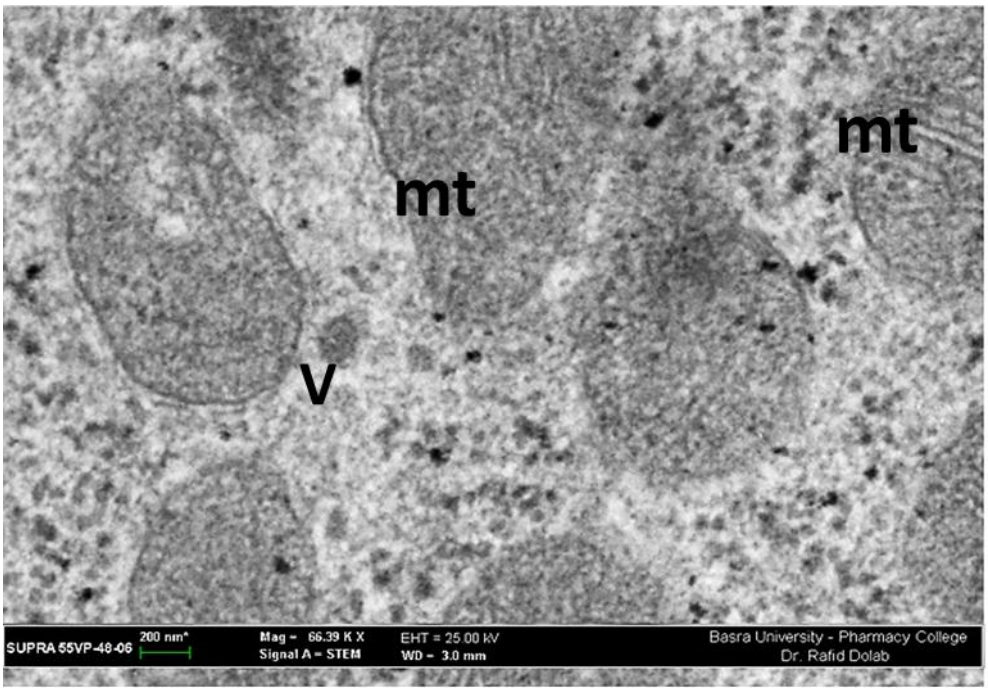


Fig. 4.62 micrograph with TEM on rat cecum infected with *E.dispar* . Showed dilated mitochondria (**mt**) multivesicular bodies (**V**)

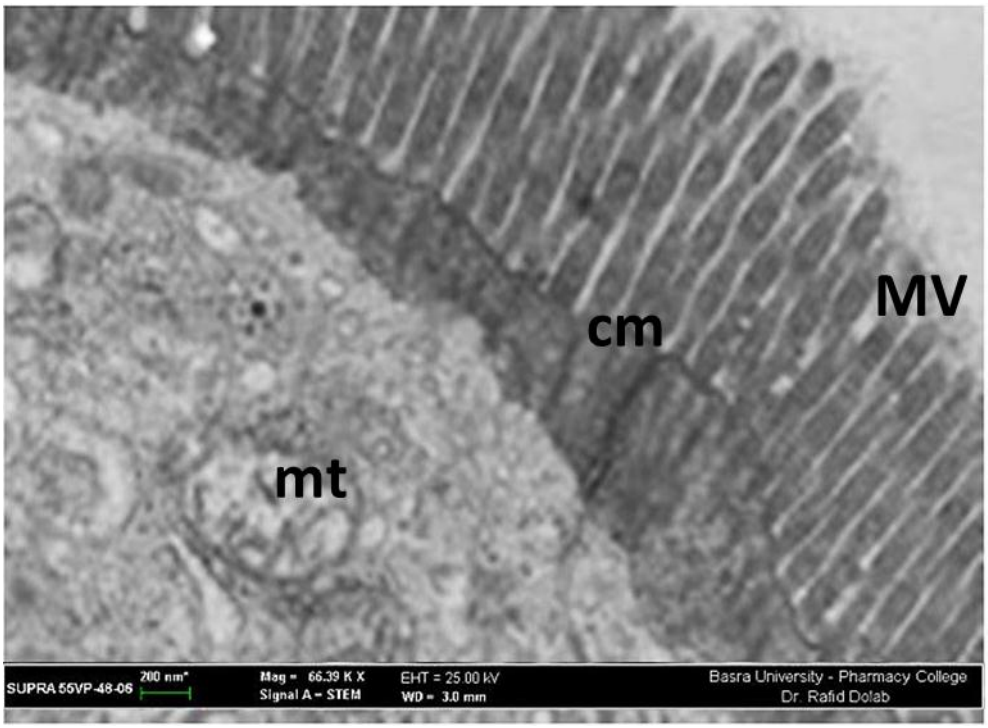


Fig.4.63 micrograph with TEM on rat cecum infected with *E. moshkovskii.* Showed normal microvilli (**MV**),normal free surface (**cm**) dilated, elliptic mitochondria (**mt**)

2. Scanning Electron Microscopy

The results of SEM showed the changes of infected cecum with amoebiasis at 28 days post-infection that clarified the adhesion between the parasite and target cells .It revealed that trophozoites were attached to the mucosal epithelial surface, it was associated to an epithelial cell near interglandular and degraded mucus, mucus in some region as well as red blood cells can be seen in cecum infected of the rats with *E. histolytica* (Fig. 4.64). Compared to the adherence of *E. dispar* showed the trophozoite start to form elongated to bind with enterocytes, normal intercellular space and mild degraded mucus (Fig. 4.65). while the changes with *E.moshkovskii* showed trophozoite in mucosa layer with enterocyte, in others section the trophozoites were distributed freely in lumen with large number of lipid droplets (Fig. 4.66 & 4.67).

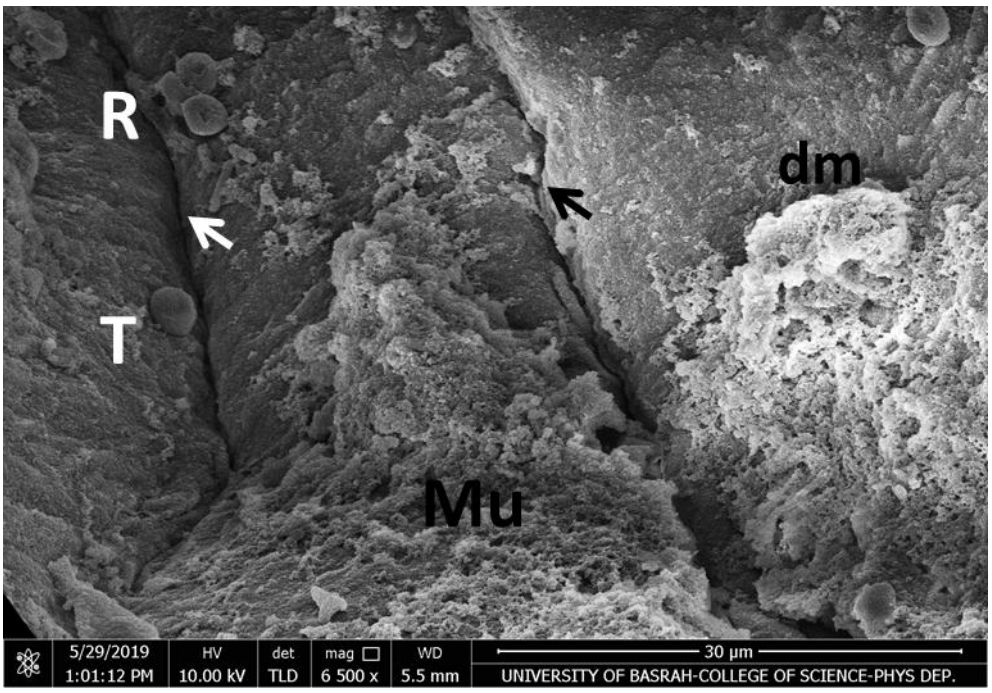


Fig.4.64 SEM micrograph on cecum section infected with *E.histolytica*. showed the trophozoite (**T**)near the interglandular epithelial cell (➔), degenerated mucus (d**m**) mucus in some region (**Mu**) red blood cells (**R**).

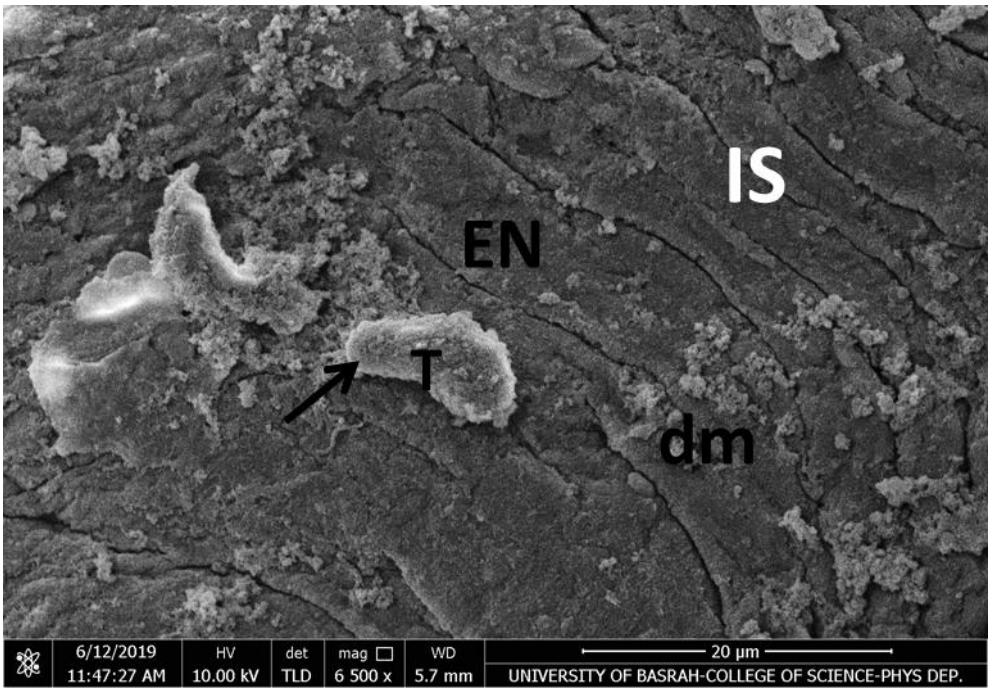


Fig.4.65 SEM micrograph on ileocecal region infected with *E.dispar*. showed the trophozoite (**T**) start to form elongated extend (➔)to bind with enterocytes (**EN**) normal intercellular space (**IS**) and mild degenerated mucus (**dm**).

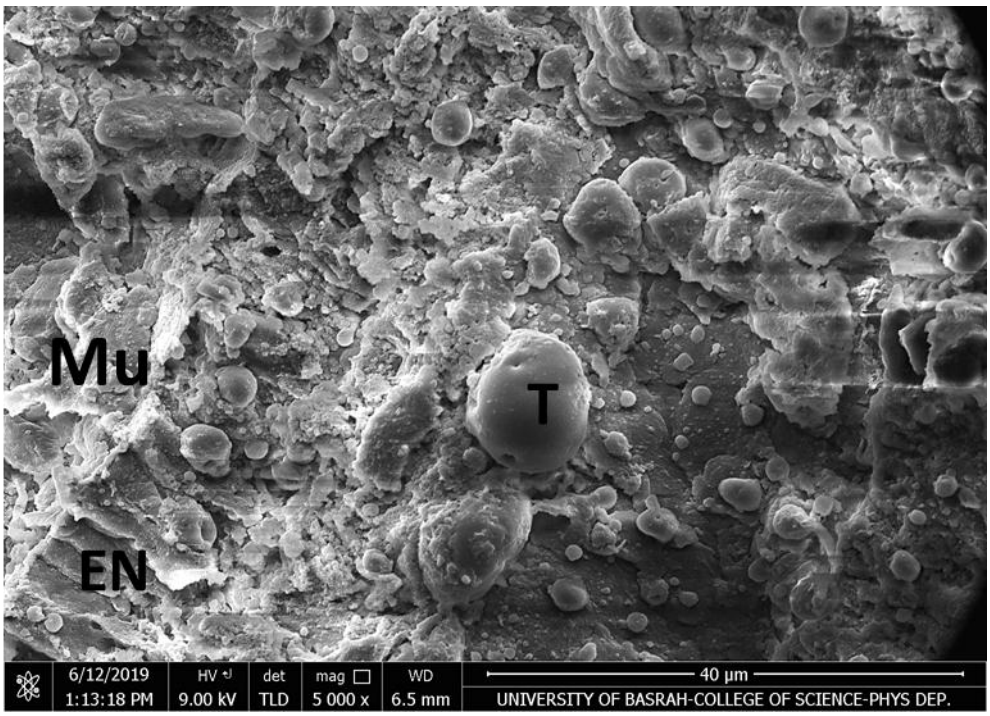


Fig.4.66 SEM micrograph on rat cecum infected with *E.moshkovskii* . showed the trophozoite (**T**) distributed freely within the mucosal surface , some normal enterocytes (**EN)**appeared coated with mucus (**Mu**).

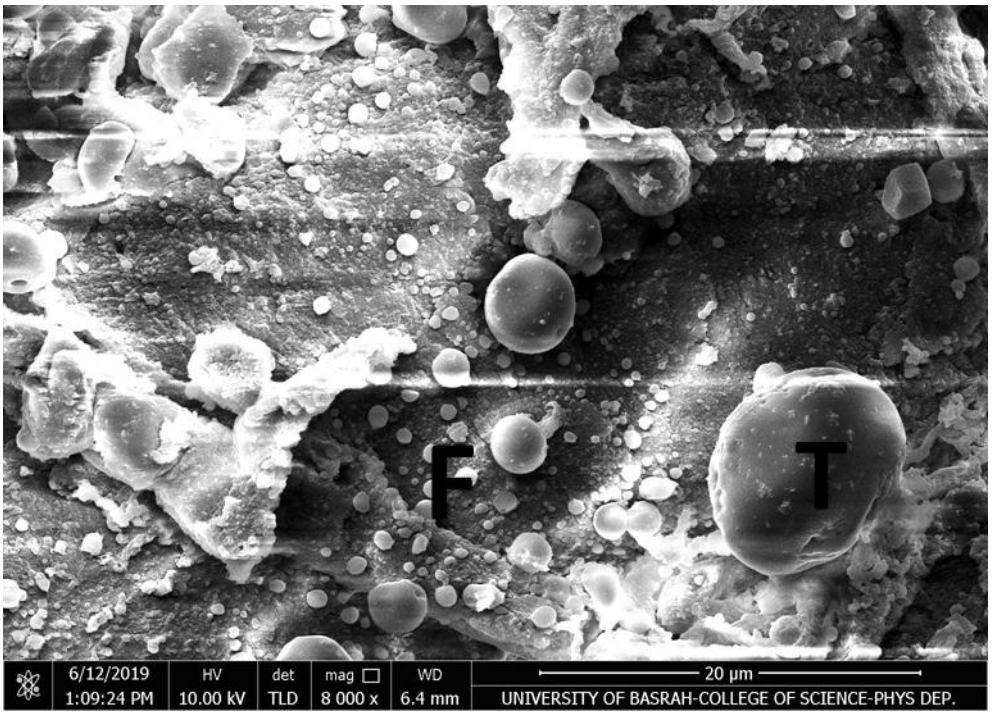


Fig.4.67 SEM micrograph on rat cecum infected with *E.moshkovskii* . showed the trophozoite (**T**) free within lumen , large number of lipid droplets (**F)**.

The most severe of intestinal damage found in rats infected with *E. histolytica* showed adhesion of trophozoite to enterocytes by filopodia, the damage represented by extensive lysis enterocyte, degenerated mucus and absence of microvilli in some region as well as irregular crypts opening and intercellular fibers can be observed (Fig. 4.68,4.69 & 4.70) while changes in the cecum section of infected rats with *E.moshkovskii* and *E. dispar* showed a mild degenerated mucus layer after adherence of trophozoites by filopodia and normal microvilli in *E.moshkovskii*, in addition to regular enterocytes, normal intercellular space and thick mucus in *E. dispar* (Fig. 4.71,4.72 & 4.73) compared with sections control that showed the mucosa surface composed of normal microvilli , apical surface of microvilli can be seen as well as regular opening of interglandular crypts (Fig.4.74).

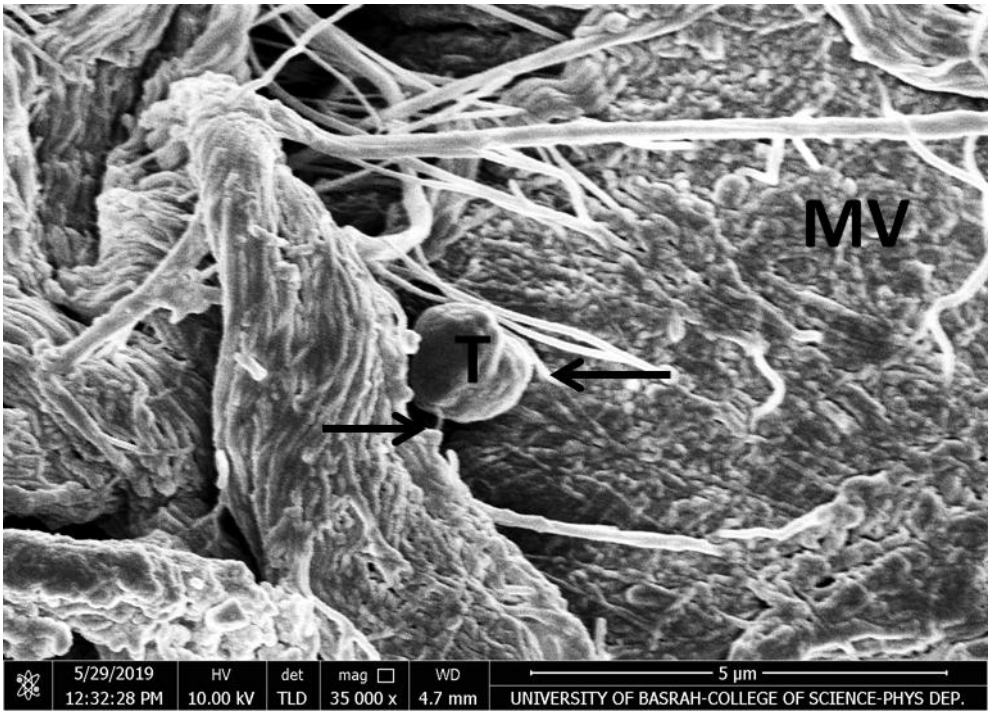


Fig.4.68 SEM micrograph on cecum mucosa infected with *E.histolytica*. Showed adhesion of trophozoite (**T**) to enterocytes by filopodia (→) area with preserved microvilli (**MV**).

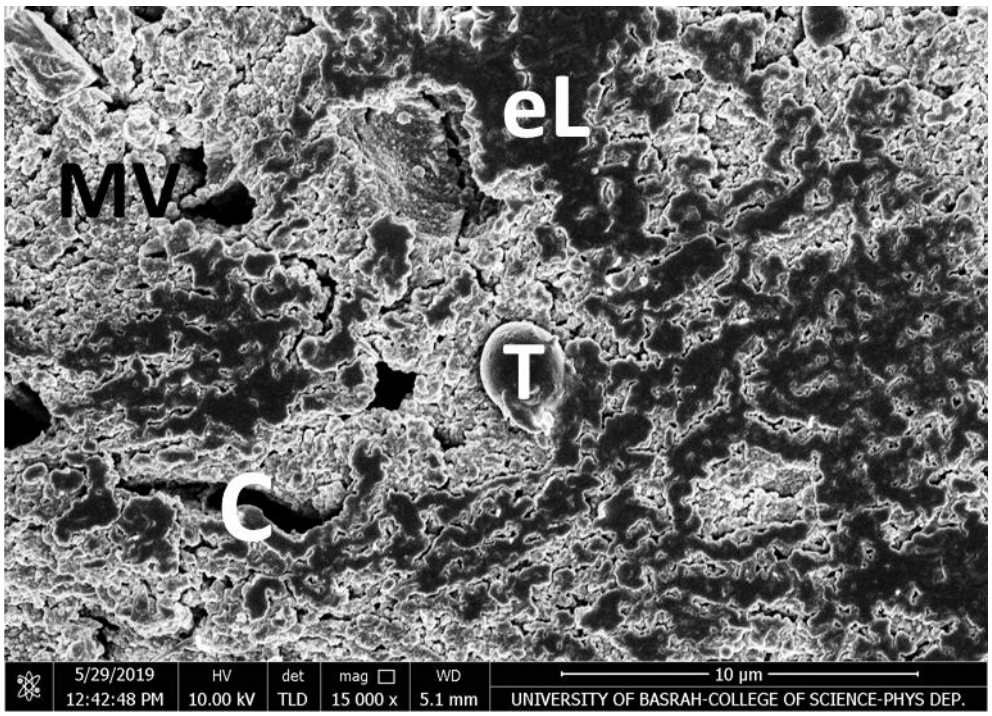


Fig. 4.69 SEM micrograph on rat cecum infected with *E.histolytica* . Showed trophozoite (**T**) attached to the enterocyte surface , extensive lysis (e**L**) microvilli (**M**V) observed and irregular crypts opening (**C**) .

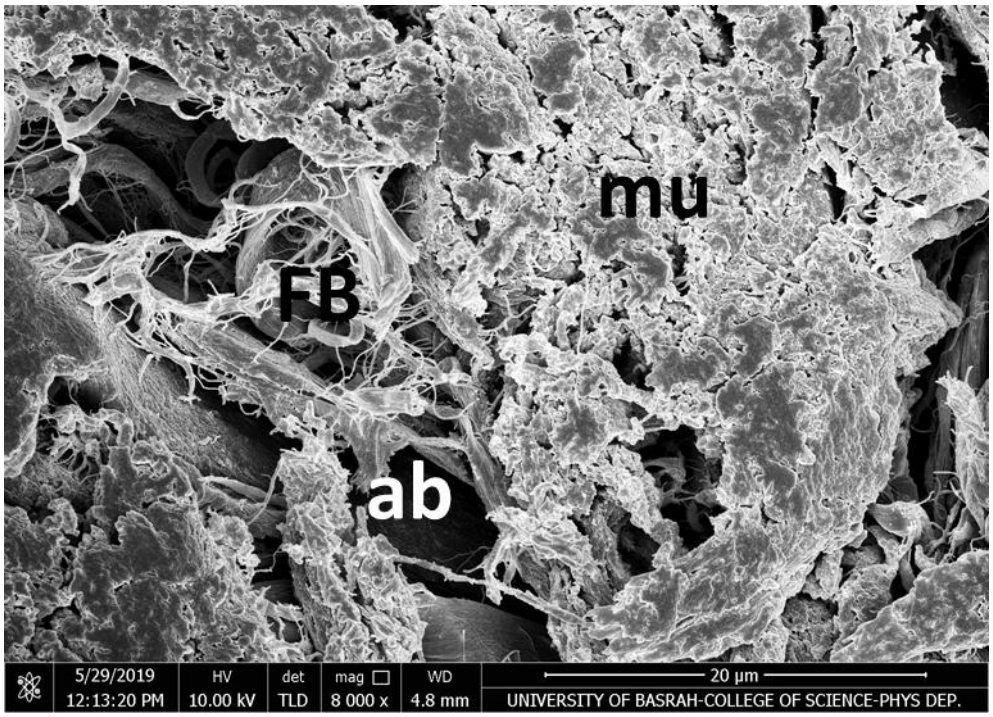


Fig.4.70 SEM micrograph on rat cecum lumen infected with *E.histolytica* . Showed degenerated mucus (**mu**) absence of microvilli in some region(**ab**) intercellular fibers (**FB**)

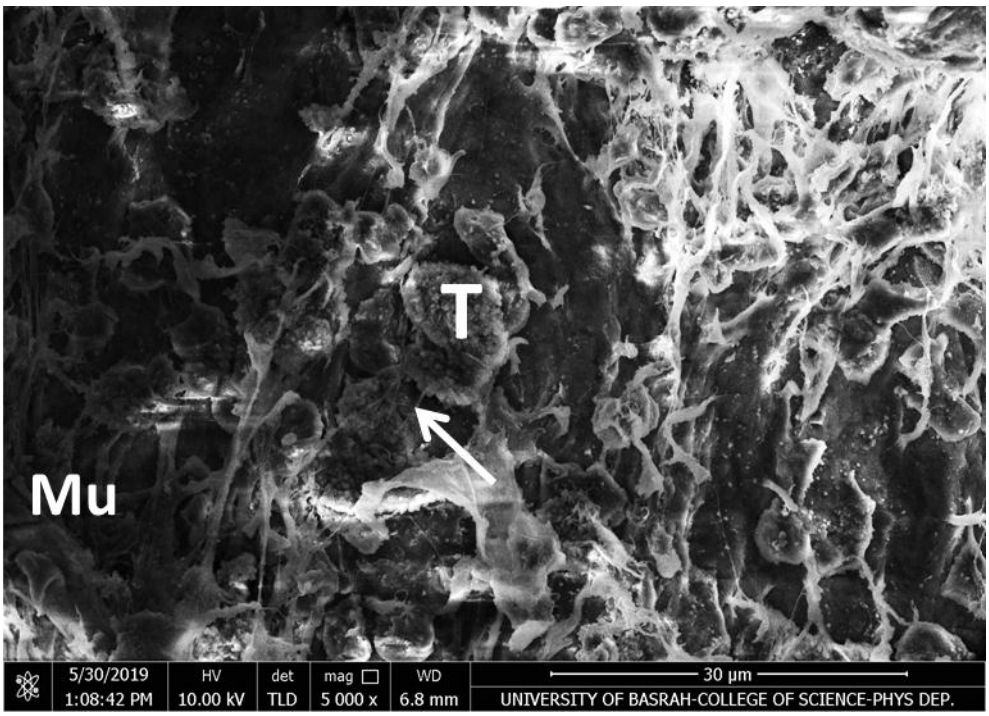


Fig.4.71 SEM micrograph on rat cecum infected with *E.moshkovskii* .
Showed the trophozoite attached to the mucosa (**T**) filopodia (➔) and mucus(**Mu**).

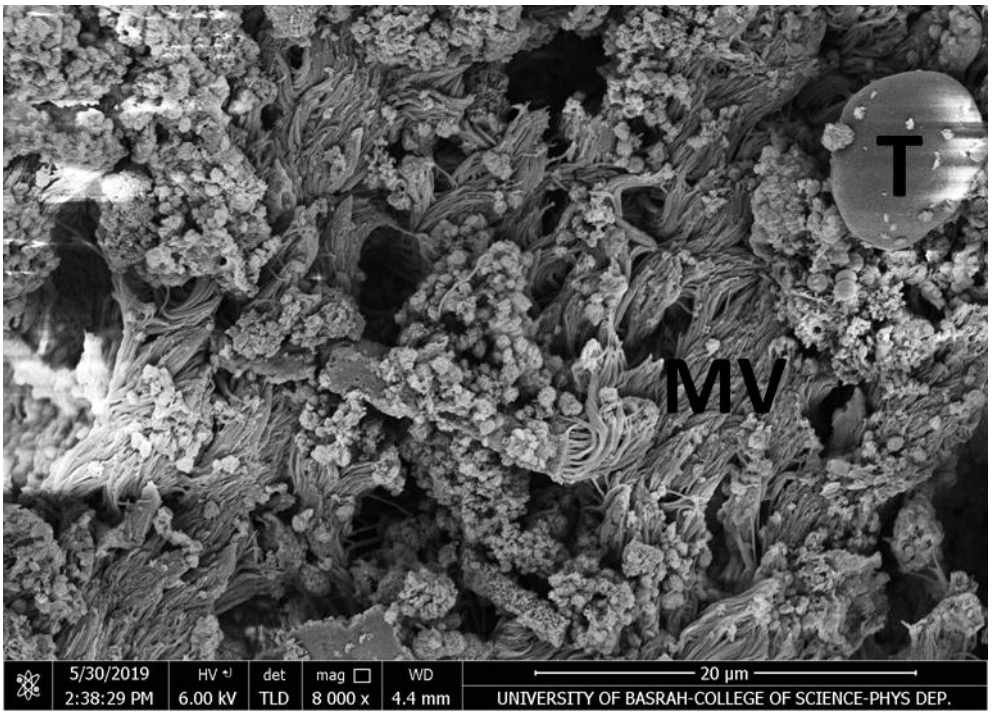


Fig.4.72 SEM micrograph on rat cecum infected with *E.moshkovskii* .
Showed trophozoite (**T**) normal microvilli (**MV**).

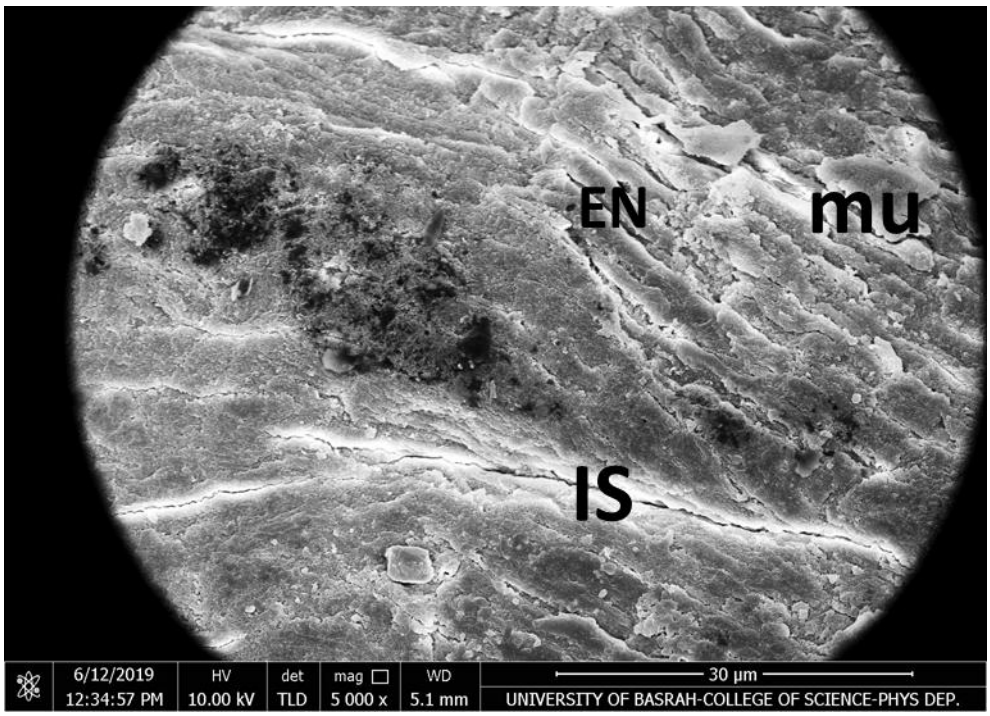


Fig.4.73 SEM micrograph on rat cecum infected with *E.dispar* . Showed regular enterocytes (**EN**) separated with normal intercellular space (I**S**) and thick mucus (**mu**) covered the mucosa surface.

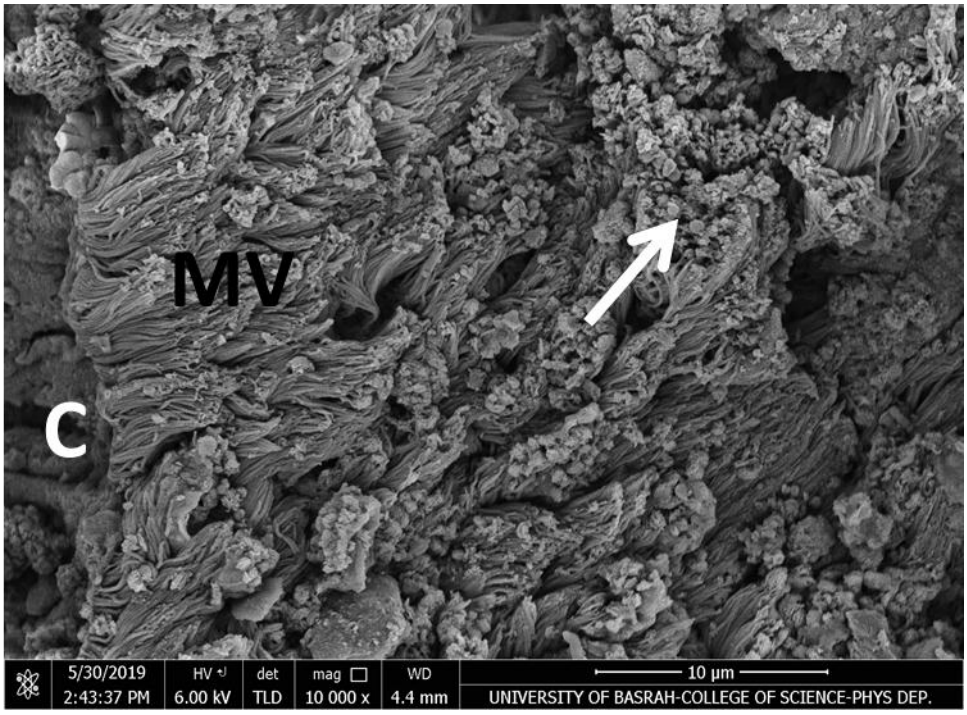


Fig.4.74 SEM micrograph of control rat cecum (normal structure).showed the mucosa surface composed of normal microvilli (**MV**), apical surface of microvilli (➔)opening of interglandular crypts (**C**) .

4.5 Quantitative Real –Time PCR(qRT-PCR)

4.5.1 Expression of tight junction genes

The gene expression levels of tight junction proteins represented by Cldn1 and Ocln showed a significantly high levels in rats infected with *E. histolytica E. dispar* and *E. moshkovskii* after 7, 14 ,28 days in compare with healthy control. One peak melting curves for Cldn1and Ocln genes obtained to illustrate the specific binding of the primers with the target gene (Fig. 4.75).

The highest expression of Cldn1 was found in rats infected with *E. moshkovskii* after 28 days, 14 and 7 day (10.55±1.63 , 3.24±1.63 and 1.86± 0.81 respectively). The same pattern was seen in *E. dispar* but with less expression levels from *E. moshkovskii* (2.82±1.63 , 1.74±0.163 and 0.93±0.163 respectively) followed by *E. histolytica* (1.41± 0.81 , 0.61± 0.163 and 0.01± 0 respectively). All samples were also compared with healthy control. In Ocln the highest expression was found in rats infected with *E. moshkovskii* after 28 days, 14 and 7 day (119.42± 1.63, 22.62±1.63 and 9.18±1.63 respectively). The same pattern was seen in *E. dispar* but with less expression levels from *E. moshkovskii* (21.11±1.63 , 11.31±1.63 and 1.23±0.81 respectively) followed by *E. histolytica* (6.49±1.63 , 2.82±1.63 and 0.65±0.163, respectively). All samples were also compared with healthy control (Fig. 4.76) .

The fold changes of these genes in *E. histolytica*, *E. dispar* and *E. moshkovskii* in compare with healthy control found in Table 4.5 .

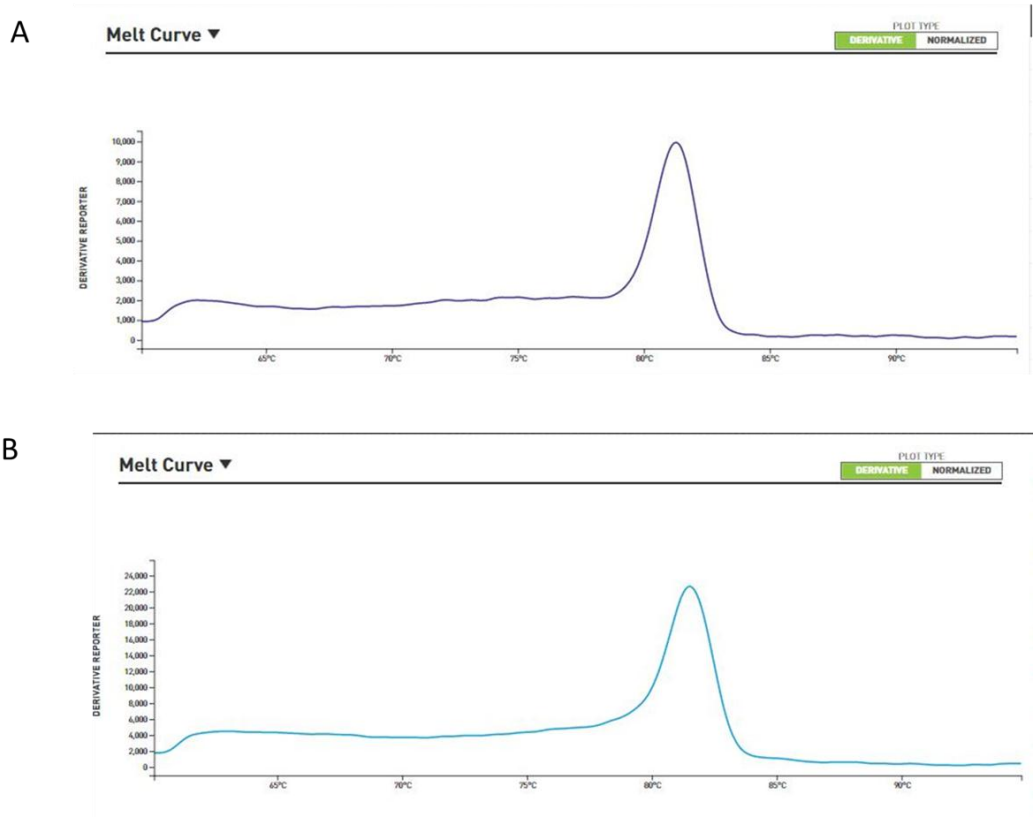


Fig. 4.75 Melting curves of real time – PCR products. A single peak representing the specific binding of SYBER green dye for the genes of interest.Cldn1 gene (A) and Ocln gene (**B)** in rats infected with *E. moshkovskii* .

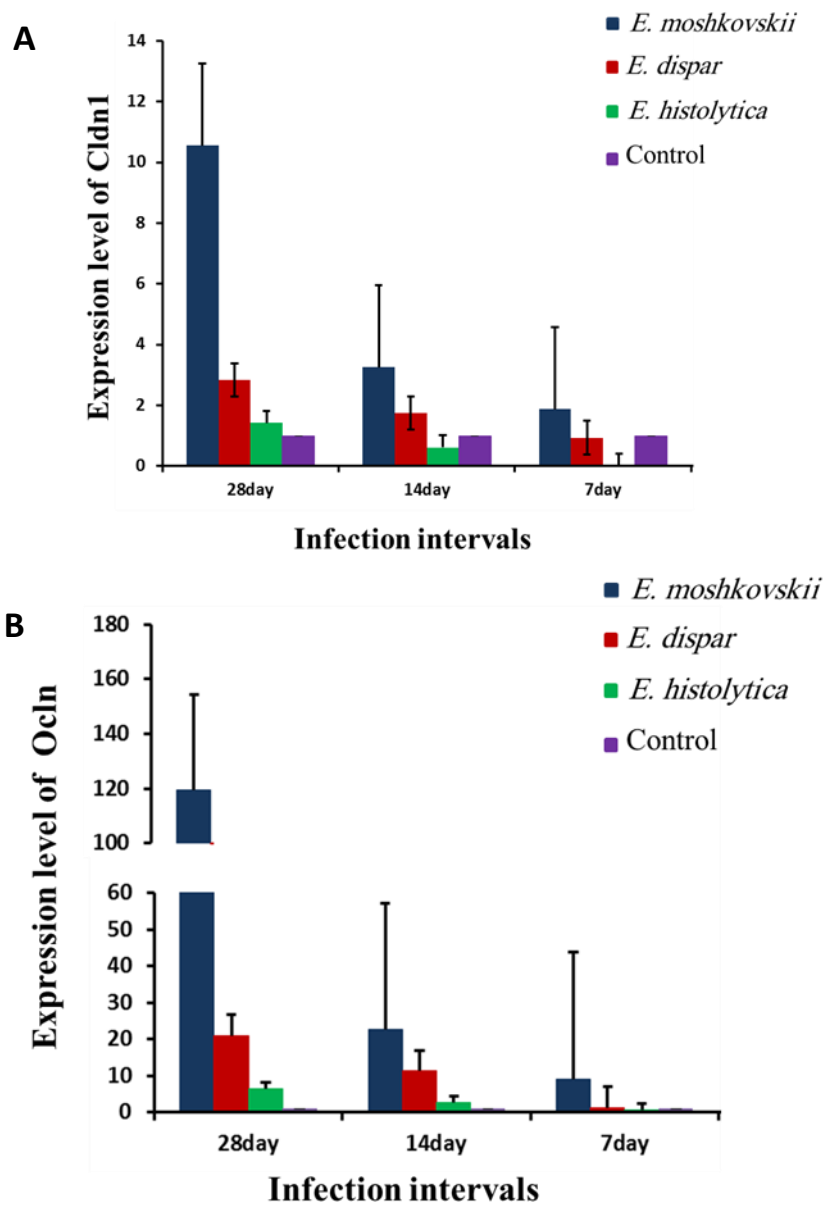


Fig.4.76 The gene expression levels of TJ genes Cldn1 and Ocln in *Entamoeba* spp. experimental infection after 28,14,7 days . **A.** Cldn1 gene expression in the large intestine (cecum) of rats infected with *Entamoeba* spp. (*E. histolytica, E. dispar, E. moshkovskii*).**B**. Ocln gene expression in the large intestine (cecum) of rats infected with *Entamoeba* spp.(*E. histolytica, E. dispar,* and *E. moshkovskii).*

Table 4. 5 The fold changes of Cldn1 and Ocln of *Entamoeba.* spp. in compare with healthy control.

Genes	*E. moshkovskii*			*E. dispar*			*E. histolytica*		
	7days	14days	28days	7days	14days	28days	7days	14days	28days
Cldn1	1.86	3.24	10.55	0.93	1.74	2.82	0.01	0.61	1.41
Ocln	9.18	22.62	119.42	1.23	11.31	21.11	0.65	2.82	6.49

Fig.4.77 The Fluorescent Ct curve for (1= Ocln**,** 2= Cldn1**,** 3= HK) genes for *E. moshkovskii*

5.Discussion

5.1 prevalence of *Entamoeba* spp. infection

Generally, the most common intestinal parasites in Iraq are *Entamoeba* spp. These species are similar to each other in morphology but they are genetically different (Hamzah *et al.* ,2006) .

The present study showed prevalence of *Entamoeba* spp. infection in Basrah patients were 60% examined by direct microscopic examination , similar findings were reported by other researchers in Al-Qadisiyah province as they found that the prevalence of *Entamoeba* spp. was 61.26% (Al-Abodi ,2015) , in addition Al-Kaeebi and Al-Difaie (2016) reported 61.1% in urban areas, finally, 60% was reported in Nineveh province (Ahmed,2017). In another study conducted in Anbar province the infection prevalence was slightly lower than our results 58.3% from 782 stool samples(Salah *et al.* ,2017).

A variation of amoebiasis prevalence found among Iraqi provinces, the highest percentage of 68% reported in a study also was conducted in Al-Qadisiyah (Al-Damerchi and Al-Ebrahimi, 2016) while Kadir *et al* . (2018) reported that the lowest percentage in Salah Al-Din 9.3%. Moreover, many resercheres conducted in Al-Qadisiyah, Diyala, Thi Qar, Al-Anbar, Duhok, and Baghdad have varied percentage within these ranges (Al-Khalidi,2016; Al-Hussuny *et al.*,2016; Hussein *et al.*,2016;Al-jnabee,2017;Haider,2017;Ibraheem,2018).This variability may be due to differences in level of sanitation, personal hygiene, population density, lifestyle and the climatic conditions suitable for the survival of cysts for a long time which increasing the possibility of causing the infection.

In addition, different study parameters including geographical location, duration of study, months of sample collection, age groups in a given study may have an effect on the out coming results.

Results regarding sex showed a higher percentage for males than females, this results were confirmed by Al-Kaeebi and Al-Difaie (2016) study as the infection found in 58.3% males and 41.6% females in Al-Qadisiyah province. furthermore, in Anbar 69.6%,46.3% for males and females respectively (Salah *et al.* ,2017) ,similarly Ahmed (2017) reported 20%males 4% females. The variations in the infection percentage between males and females might be caused by the different social behavior between the two sex as males normally are the working sex in the society which made them in contact with the environment. furthermore, males normally tend to feast in a public restaurant which may increase infection incidence(Fletcher *et al*.,2012).

The increased percentage of infection in age group1(1-10) yrs. and group7(61-70) yrs. , could be explained by the influence of different factors as for group1 the behavior of children might be involved less cautious regarding their personal hygiene ,in addition, they are more exposed to food from different sources which results in increased infection percentage in this group. Many articles supported this explanation (Al-Kaeebi and Al-Difaie, 2016;Ahmed,2017;Salah *et al.*, 2017),the high infection percentage in group 7 (61-70) might be resulting from the fact that elderly people exposed to many infectious agents during their life as their immune system is weak, also use drugs for other diseases may be resistance for pathogenic organism (Ali and petri,1999 ; Nakada and Nozaki ,2016).

Our results reported that summer season associated with increased infection results from the availability of the proper environmental factors for parasites growth as intestinal parasites are dominant at tropical area, moreover, the need of fast consumption of drinking water during summer increase the properly of drinking contaminated water, which contain the infective stage of these parasites (cysts))(Khan *et al*. , 2018;Al-Wahid,2018). Furthermore, Summer normally is suitable for the spreading of insects such as house fly which is consider to be a mechanical vector for parasites cysts. Our results were clarified by researchers emphasized the importance of environmental factors (temperature, wind, humidity) in the spread of intestinal parasites and they also reported the highest percentage of infection during summer mentioning that cold weather decreases parasite infection throw killing the infection stages (cyst) (Heymann,2015).

Clinical diagnosis of the sample revealed to mucoid stool in most of the samples caused by increased secretion of mucus in the intestine during the infection, as it is known that the intestinal mucosa is the first line of defense against intestinal infection, this also suggested by others researchers showed that *Entamoeba* spp. have the ability to secret protease, glycosidase in order to destroy mucin resulting in mucous diarrhea (Serrano *et al*.,2013).

Moreover, the bloody appearance of the samples are lowest in number among our samples, this might be explained by the lowest *E. histolytica* infection that we found in our study confirmed by the genetic diagnosis because most studies pointed that *E. histolytica* normally caused bloody diarrhea as one of their well-known symptom .

5.2. Diagnostic of *Entamoeba* spp.

5.2.1 Direct microscopic examination

Direct examination gave a confirmation of *Entamoeba* spp. existence in both trophozoite and cysts stages but the use of modified trichrome stain in the test tube was efficient in getting good quality stained samples free of contaminants (Bacteria and debris), because of the repeated use of centrifugation in this methods helped the sedimentation the parasites in the bottom of the tube leading to clear isolation of the parasite in a high purity.

5.2.2 Cultivation of *Entamoeba* spp.

Regarding the parasite cultivation method. In generally , the samples used in parasite cultivation are stool, rectal biopsies or hepatic abscesses, the method of parasite cultivation is an important technique to obtain large number of pure parasites in order to study the parasite morphologically, pathologically and genetically . In our study we used stool samples, the results showed that it is less sensitive than the microscopic examination(10%), in addition, there is a high risk of contamination by fungi, bacteria and other protozoa therefore it is not recommended as a routine examination (Clark and Diamond,2002).

On the other hand, the growth of bacteria (contamination) in the media is an important factor to maintain the viability of the parasite. Several studies have confirmed the importance of bacteria in parasite cultivation as a source of nutrition (Hamad *et al*., 2018). Furthermore, many studies reported the growth of the parasite in aerobic conditions, in this case *E. moshkovskii* ,because it is free opportunistic amoebae and can grow in both aerobic and anaerobic conditions.(Upcroft and Upcroft 2001;Shimokawa *et al* ., 2012).

The present study showed that Locke's egg medium (basic media) was the best among the other media , because it contains nutrients that help parasite to secrete proteases to digest proteins which is essential for rapid growth (Tillack *et al*.,2007),these nutrients include eggs that it contains the high protein content necessary for parasite growth and reproduction compared with the presence of peptone that found in Robinson medium which is a simple structured protein. The high protein content allow *Entamoeba* spp. to get nutrients before their consumption by bacteria, in addition to this it is contained vitamins that stimulated *Entamoeba* spp. for metabolic reactions (Long *et al*.,2019 ; Clark and Diamond 2002) confirmed that Locke's egg medium is the best to cultivate *Entamoeba* spp. moreover, it has high accuracy in diagnosing amoebiasis in stool.

Regarding Robinson medium which has benzoic acid as one of it's components, this compound act as antibacterial and used to preserve food (Berne and Komel, 2015) . It has an inhibitory effect on the growth of some microorganisms .

During the study, we used 5 new culture media which were highly efficient in cultivating *Entamoeba* spp. and easy to prepare from different animal sources, it is worth mentioning that all the media contain human serum as one of their components, moreover the growth very obvious and extend to variable days.

The results showed that Brain heart infusion agar was the most efficient medium to give dense growth and increase the number of parasites with a high purity for ten to eleven days period. This efficiency may be due to the high chemical contents of the nutrients necessary for parasite's growth and reproduction because Brain heart infusion agar contains amino acids, glucose, mineral salts, highly lipid complex and cholesterol that many microorganisms cannot easily digest, leaving *Entamoeba* spp. with a large

amount of nutrient that it can digest because it have the necessary enzyme (Tillack *et al*. ,2007 ; Ximenez *et al* .,2017).

Results also reported that Sheep testes agar came in the second place for the efficient the medium in parasite isolation and growth, this might be due to it's contents of proteins and carbonate in addition to the presence of high lipid(Abu-Mejdad and Al-Hilfy, 2016), on the other hand ,the parasite contains phospholipase in the plasma membrane of trophozoite that has a role in the decomposition of the fatty substance of cellular membranes to free fatty acids (Tsukui *et al*. , 2019).

The Buffalo milk agar, which showed a lower growth rate compared with Brain heart infusion agar and Sheep testes agar media may related to the component which it was less suitable for parasite growth , the low percentage of fat and protein contents had an effect on the results (Ahmed *et al*. , 2013; Balthazar *et al*., 2017),the same reason applied for Sheep liver agar and Chicken liver agar media which have less percentages of fat and proteins ,respectively (Cerci *et al*. , 2011 ; Cieslik *et al*., 2011; Adeyemi *et al*.,2016; Zaefarian *et al*.,2019).

The human serum that was added to all media during the culture process, because serum normally from riches media with lipoproteins, which had an important role to growth of parasite in pure culture . (Serrano *et al* ., 2010). The sustaining of the parasite each 72 hours was necessary for preventing contamination (Mori *et al* ., 2015).

5.2.3 Molecular diagnosis of *Entamoeba* spp.

There are many methods used to extract DNA from stool directly including direct, boiling, manual , automated extraction methods and using suitable kits , the current study showed extraction of DNA by using Presto ™Stool DNA Extraction Kit with bead beating tube in order to destroy the wall of cyst, the advantages of this kit was simple, not time consuming and does not need preservers for the sample that may affect DNA of parasite. In order to diagnose parasites, the technique of polymerase chain reaction(PCR) was used as a high sensitive way to differentiate between *Entamoeba spp*. since PCR is more accurate to understand and the epidemiology of infection caused by different species of *Entamoeba* compared with direct microscopy, which does not allow the separation among species .

The results of present study showed high efficiency of differentiation among the three species based on the specific primer of each parasite .

The current study reported different infection percentages for three species. The highest infection was *E. moshkovskii* 15% followed by *E. dispar* 10% and *E. histolytica*, which was 5 %. Al-Abodi (2015) in the province of Al-Qadisiya recorded a different infection percentages for the *Entamoeba* spp. were *E. histolytica* has the highest prevalence (74%) followed by *E. dispar* with (26%) finally, *E. moshkovskii* 7%. . In Al-Qadisiya province another study reported the same order of *Entamoeba* spp. has been recorder as *E. histolytica*, *E. dispar* and *E. moshkovskii* 47%, 31% and 19% respectively (AlKhafaji, 2014). Al-Yaquob (2008) in Basrah recorded different prevalence as *E. dispar* was 42% and mixed infection of both *E. histolytica*, *E. dispar* 30% and did not record infection of *E. moshkovskii*. In Thi Qar, a study showed similar prevalence of *E. histolytica* and *E. dispar*(39.29%) (Al-Abady and Al-Saidy, 2017).

Al-Hamdani *et al* . (2011) recorded 18.6% infection percentages *E. histolytica* while *E. dispar* was 53%.

On other part of Asia , for example , in Iran it was the highest prevalence of *E. dispar* 0.58% while the least prevalence was for *E. moshkovskii* 0.07%. (Bahrami *et al* , 2019). In Bangladesh Shimokawa *et al* . (2012) reported a different prevalence's of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections 4.63%, 0.35% and 2.95% respectively.

Worldwide, Ngobeni *et al* . (2017) in South Africa, *E. histolytica* recorded the highest infection percentages (8.5%) while it did not register infection of *E. moshkovskii*.

The specific primers used in this study were derived from the middle of the small-subunit rRNA gene since it was conserve, found in all organism ,used to identify and classify organism since it has unique character for each species (Hamzah *et al*.,2006).The differences found in PCR results may be caused by different reasons first due to the variations in DNA extraction methods from stool samples or due to differences in the number of parasites in stool sample . In addition , the variation may produce from the different time period in which the samples were obtained ,transport and preservation method used in each study. Finally, the fixative and preservative agents used in the study may interfere with cell components resulting in effecting DNA (Parija *et al* ., 2014 ; Lopez *et al*.,2017) .

The discrimination between the species of *Entamoeba* specially the invasive and non-invasive ones is very important to avoid unnecessary treatment that may patients take in case of miss diagnosing to right species of *Entamoeba* because this misuse of drugs may lead to resistance strain of the parasite.(Santos *et al* ., 2007; Dinoop *et al* .,2016).

5.3 sensitivity diagnosis methods

Regarding the sensitivity of the diagnosis methods, the current study reported the microscopic examination has the highest sensitivity (60%) for detection of parasites trophozoite and cysts in stool samples followed by the molecular diagnosis showed (30%)sensitivity and in the cultivation comes last with (10%).

The negative results using PCR or cultivation methods of some positive samples in microscopic examination may be explained by several reasons. Firstly, in microscopic examination there is a chance of misdiagnosis of the parasite as some times the parasite for example *E. histolytica* is mistaking with WBCs as well as with other stages of *Entamoeba* spp. Such as *E. moshkovskii* and *E. dispar* (Santos *et al* .,2007). Secondly, the presence of some inhibitory substances in the stool such as urea, bile salt and polysaccharides which may bind to DNA polymerase and inhibit its work in replicating the DNA (Schrader *et al* ., 2012; Sidstedt *et al*.,2019).

5.4 Histopathological study

5.4.1 Light microscope study

As it is known amoebiasis can be intestinal and extraintestinal amoebiasis. In cases of intestinal amoebiasis the symptoms ranged from a symptomatic cases to acute colitis and chronic colitis. Normally, less than 10% of patients have symptoms and most of this 10% exhibit symptoms similar to chronic colitis (Ichikawa *et al*.,2016).

Section of the intestinal tract particularly cecum and rectum of the infected rats revealed the adhesion of the *Entamoeba* spp. trophozoites to the surface of the intestine. The mechanism of this attachment can be explained by the filopodia which are cytoplasmic projections of the

trophozoite. These filopodia will attach to the substratum and cause cytolysis of the host cells (Lushbaugh and Pittman ,1979). The adhesion of trophozoite to the intestine wall prevent the transformation of them into cyst but when these trophozoite migrate towards the large intestine they could transform into new cysts by binary fission (Kantor *et al* ., 2018).

Another pathological change caused by *E. histolytica* is the degeneration of the epithelial layer and a heavy infiltration of inflammatory cells in to these layers, the reason of this degeneration is the ability of trophozoite to attach and lyse the epithelial layer of the intestine through the Gal/GalNAc lectin, in addition, certain enzyme might be involved in increasing the risk of parasite's invasion for example sialidase which is N-acetylgalactosamidase and it is essential for removing a poly saccharide from mucin cells(Cornick and Chadee 2017). This removal allows the trophozoite to lyse the protective mucous layer and gradually penetrate the epithelial layer of the colon, the penetration will increase the risk of parasitic metastasis to different organ in the host body. Furthermore, different mechanisms participate in the death of the epithelial layer cells, these mechanisms involved the parasitic virulence factor such as cysteine protease. The cysteine protease target the cells causing the lyse of these cells via apoptosis, in addition to that another virulence factor is involved which is amoeba pores, a serine-rich *E. histolytica* protein known to have such an effect.(Quach *et al* ., 2014).

As mentioned in our result there was a formation of ulcer. The invasive species of *Entamoeba* such as *E. histolytica* cause colonic mucosal ulceration as trophozoites eat their way through the mucosa .In rare cases of long-stand infection it cause masses that are ulcerative, inflammatory, big and similar to carcinomas. Focusing on the pathological changes during *Entamoeba* spp. infections, the amebic colitis represented by thickness in

the mucosa, in addition to inflamed and edematous mucosa (Hardin *et al* .,2007)

Histological changes showed intestinal crypts hyperplasia ,branching crypt, most of crypts appeared with degeneration area, this feature that found in our result can be explained by the degeneration the epithelial layer, at the beginning of the infection a non-specific lesion can be formed as a result of glandular hyperplasia and edema (Espinosa and Martínez ,2000). Normally, the gastrointestinal mucosa has the ability to repair the damage but when the integrity of the mucosa is damage the repairing process will rely on the epithelial cells ability to migrate and proliferate. Moreover, the epithelialization can occur to replace the mucosal defect.

It is important to maintain a balance between cell loss and cell renewal as excessive cell loss might lead to atrophy or ulcer on the other hand excessive proliferation of the cells and long live span might lead to hyperplasia (Jones *et al*.,1999) .

The goblet cells in the intestine synthesize and secrete mucin as a result of stimulation by number of stimuli. Our result showed hyperplasia in these goblet cells which might be explained as adaptation of these cells to mechanical stimulation or injuries caused by the parasite during its colonization trophozoite bind to mucin oligosaccharide and Gal/GalNAc lectin to penetrate the mucosal layer ,during this process the amoeba degrade the mucin barrier and eventually attach to the epithelial cells (Bitar ,2003; Nakada and Nozaki ,2016).

The infiltration of inflammatory cell which formed in the crypt of rats at 14 and 28 days post-infection is already confirmed by other researcher as a result of long term infection (Ghosh *et al*., 2019). Parasite infection for a long time lead to intestinal layer invasion and inflammation in case of no treatment.

Moreover pathological feature was observed in intestinal infected with amoebiasis is edema which results from inflammation that lead to fluid exudation, the exudation is caused by the high permeability of blood vessels result in leakage of blood fluid at the site of infection (Zhen and Zhang (2019).

The results showed the presence of the parasite in the rectum similarly Hardin *et al* .(2007) who studied the invasive species of amoeba and the formation of ameboma rectal mass. The reason why the rectum can be infected with *Entamoeba* spp. is that its considered to be a temporary storage site of feces, in addition to the presents of fluid which allow the trophozoite to attach the surface of the epithelium and invade the tissue (Ooi and Seow, 2003).

Results showed moderate histological changes in most cecum and rectum related to rats infected with *E. dispar* after all periods compare to *E histolytica* due to less virulence factors in former species. For example *E. histolytica* secretes approximately 10 to 1000 times more cysteine proteases than *E. dispar*. Moreover, the amoeba pore secretion is approximately 1/3 of that secreted by the *E. histolytica*. Finally, *E.*dispar showed a reduced rate of adhesion and cytotoxicity than *E.histolytica* (Oliveira *et al*.,2015).

Lack of granuloma in our results are similar to other researcher for instance, Espinosa *et al* . (2007), whom infect hamsters with *E. dispar* via intra hepatic rout found that it formed a focal inflammatory cells infiltrate with no granuloma which might be result from the incapability of *E. dispar* to establish experimental lesion because it considered to be commensal parasite, another study Dolabella *et al*. (2012) reported the establishment of amebic liver abscess by *E. dispar*.

Regarding *E. moshkovskii* which is originally classified as a free living protozoan found in anoxic sediment and environments such as brackish coastal pools, found to infect human in some cases.(Shimokawa *et al* ., 2012). In animal model special murine model *E. moshkovskii* caused diarrhea ,weight loss and colitis. In Bangladesh, cases showed the association between *E. moshkovskii* infection and diarrhea in children which were similar to *E. histolytica* (Hamano *et al* ., 2006).

The experimental infection of *E. moshkovskii* was reported in the ceca of C3H/HeN, C3H/HeJ, and CBA/J mice, but not C57BL/6 or BALB/c mice while *E. dispar* failed to infect in any experimental mice. The ability of *E. moshkovskii* and *E. histolytica* to infect murine model is due to shared virulence factors which not found in *E. dispar* ,this joint feature of *E. moshkovskii* and *E. histolytica* lead to similar symptom including diarrhea and blood stool (Mojarad *et al* .,2010).

5.4.2 Electron Microscope study and Expression of tight junction genes

The changes produced by *E.histolytica* are well known. The interaction between the trophozoite and the host cell begins with the attachment of the parasite to the intestinal epithelium followed by sequent invasion. The morphological changes in the epithelial cells can be described as widening of intracellular spaces that result from the loss cells that are affected by the parasite causing disruption in the epithelial barrier (Betanzos *et al*., 2013).

On the other hand the molecular mechanisms the invasion of the trophozoite and the alteration in the intestinal barrier specially TJ protein which participate in maintain cell contact is still not fully understood . TJ proteins play an essential role in preventing a parasite invasion into the intestine (Di Genova and Tonelli ,2016; Capaldo *et al*.,2017).

The TJ genes Cldn1 that amoebiasis affects was one of the genes of interest in our study . It is integral TJ protein regulating the size selectivity of the barrier (Cuellar *et al.*,2017).Our results revealed a significant high expression of Cldn1 infected rats suggesting that *E. moshkovskii* has a strong effect on Cldn1.

Ocln is another TJ gene that is important for the primary intercellular seal between epithelial cells(Li *et al.*,2015), our results showed a high levels of expression for this gene specially *E. moshkovskii* ,moreover, it was higher than the other TJ gene (Cldn1) ,the reason of the high expression of Ocln result from the nature of gene function as a seal between epithelial cells. Furthermore, studies suggest that Ocln is important in the maintenance and the assembly of TJ proteins(Yu *et al*., 2011) .

In addition, when we compare the expression levels of Cldn1 and Ocln among *Entamoeba* spp. the lowest expression both genes found in rats infected with *E. histolytica* this may be results from the invasive nature of *E. histolytica* (Betanzos *et al*.,2018) which destroy the cell of the large intestine by secreting cysteine protease leading to a losses, destroyed cells(Betanzos *et al*., 2013) while *E. moshkovskii* and *E. dispar* secrete these cysteine protease in low levels (Serrano *et al*.,2013 ; Al-Abodi,2015).

The ultrastructure analysis confirm both the pathological changes and the gene expression alteration during *Entamoeba* spp. infection . Our results showed variable numbers of trophozoites were associated with the mucosal epithelial surface according to the parasite type. Other studies also confirm these changes as the trophozoite found in close contact with intestinal epithelium (Meurens *et al* ., 2009; Preet *et al* ., 2011; Faust *et al*., 2011;Rangel *et al* .,2019; Lopez *et al* .,2019) .Furthermore the displaces of intracellular spaces, the penetration of the cells and delocalization of occludin and claudin-1.(Betanzos *et al*.,2018).

Several virulence factor such as Gal/GalNAc lectin, amoeba pores, cysteine and serine proteases, prostaglandin E2 (PGE2) , participate in increasing the permeability of the intestine by altering claudin-4 (Lejeune *et al*., 2011). The cysteine proteases can affect claudin-1 and occludin , damage the adherence junctions (AJ) and desmosomes (DSM), (Hernandez *et al*., 2017).

Our results showed most severe of intestinal damage found in rats infected with *E.histolytica* ,the damage represented by more irregular distribution of microvilli. Cysteine proteases have an important role in damaging the integrity of microvilli in vivo ,the parasite use the proteases to overcome microvilli and the barriers of tight junction when it invade the intestine (Serrano *et al*.,2013).

The contraction of actin filament adjust to TJs can be affected by many pathophysiological factors including infectious agents (Campos *et al*., 2016).

After the trophozoite were attach to the epithelium ,the permeability of the cells were increased suggesting that the TJ proteins are disrupted, this disturbance is resulting from the actin cytoskeleton disruption, in addition, the interaction between cysteine proteases and TJ proteins have an effect on a cytoskeleton, this effect can be seen shortly after the contact of trophozoite (Lejeune *et al*., 2011).

Moreover the reproduction activities of the parasite cause more adherence of the trophozoite to the epithelium as well as more tissue damage due to the amoeba secretion which include amoeba pore that cause pore that allow a massive influx of extracellular Ca+2 across the plasma membrane ,in addition it result in irreversible rise of $Ca+^{2}$ in the target cell the increase of the $Ca+^{2}$ is cause by the activities of many enzyme such as

ATPase, Phospholipase, endonuclease and Proteases which break down the protein found in both membrane and cytoskeleton (Serrano *et al*.,2013)

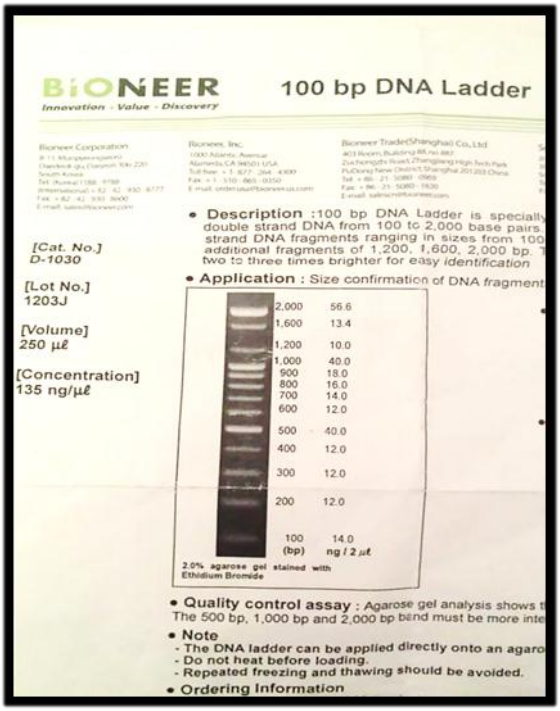
Conclusions

1. Although microscopic examination is a traditional method for the diagnosis of *Entamoeba* spp. the probability of misdiagnosis of these species was high.
2. The molecular diagnosis method considered a precise diagnostic method despite the low sensitivity which is due to the presence of morphological similar species sample .
3. *E. moshkovskii* can be cultivated on different media but the Brain heart infusion agar showed high efficiency and was most suitable in cultivating the parasite
4. *E. dispar* and *E. moshkovskii* were found to be pathogenic species in experimental rats and their presence were associated with diarrhea.
5. The three species showed varied histological changes in rats' cecum and rectum ranged from sever changes during *E. histolytica* infection to moderated changes during *E. dispar* and *E. moshkovskii* infection.
6. This study helped confirm and gave further understanding of the pathogenesis of both *E. dispar* and *E. moshkovskii*.
7. The rats infected with *E. moshkovskii* showed significant high expression levels of TJ genes (claudin-1 and occludin) in comparison with other *Entamoeba* spp.
8. The electron microscopy confirmed the alteration in the intestinal mucosa including variability in microvilli , mechanisms the attachment of trophozoite and changes in the mucus during *Entamoeba* spp. infection.

Recommendations

1. Take more interest in developing Sewage and raise awareness among farmers to limit the use of organic fertilizers (animal waste).
2. Conduct more intensive studies regarding the presence of different virulence factors specially in *E. dispar* and *E. moshkovskii* as these studies are rare in both species.
3. All the positive samples in microscopy examination must undergo molecular testing (PCR) for accurate diagnosis of the species.
4. Liver abscesses also must undergo molecular examination (PCR) for accurate examination.
5. Conducting an ultrastructural study explaining the role of cytoskeleton and intercellular space in cytopathogenesis of amoebiasis

Appendix -I Ladder leaftlet



Appendix-II Basic culture media

1. Locke's egg medium (Von brand *et al.*,1943)

A. Locke's solution

The components below were dissolved in 1000 ml D.W.

Sodium chloride	8.0 g
Calcium chloride	0.2 g
Potassium chloride	0.2 g
Magnesium chloride	0.01g
Sodium phosphate dibasic	2.0 g
Sodium bicarbonate	0.4 g
Potassium phosphate monobasic	0.3 g

The solution was autoclaved for 15 min. at 121◦C,cooled at room temperature followed by filtration.

B. Egg slant

1. The contents of a fresh egg with sterilized surface was empty into a graduated cylinder.

2. Locke's solution 12.5 ml was added to each 45 ml of egg and mixed well.

3.The mixture was filtered using a piece of gauze into a clean flask.

4.The mixture 5 ml was added to each standard culture tube.

5. The culture media tubes were sterilized by autoclave at 100 ˚C for 10 min..

6. Locke's solution 6ml was added to culture media tubes and stored at4˚C.until use

2. Robinson's medium (Robinson,1968)

A. This medium is prepared from:

- Erythromycin
- Phthalate peptone solution
- BRS medium

Each component was prepared as bellow:

1. **Erythromycin 0.5% :**dissolved 0.5 g erythromycin in 100ml distilled water, sterilized by filtration millipore and stored at 4˚C.
2. **Phthalate Peptone Solution:**
   This solution was prepared by mixing 1.25ml of **peptone 20%** with 100 ml of **Phthalate solution 1x .**

- **Peptone 20% :**dissolved 20g peptone in 100 ml distilled water, sterilized by autoclave and stored at 4°C.
- **Phthalate solution 10 x** :

The preparation method was modified using Benzoic acid instead of potassium hydrogen phthalate**.**

Benzoic acid	102 g
Sodium hydroxide 40%	50 ml

Top up to 1000 ml distilled water ,adjusting pH to 6.3 then steriled by autoclave and stored at room temperature. Before use, dilute the solution (1:10) to prepare **Phthalate solution 1x .**

3. BRS medium

Step1. R medium stock 10x

Prepared as follow :

Sodium chloride	25 g
Citric acid	10 g
Potassium phosphate,monobasic	25 g
Ammonium sulfate	5 g
Magnesium sulfate	0.25g
Lactic acide solution 85%	20ml

Top up to 500 ml distilled water ,adjusting pH to 7.3 then steriled by autoclave. The solution was diluted (1:10) to prepare **R medium stock 1x .**

Step2. BR medium

This medium was prepared by inoculating R medium stock 1x with a slandered *Escherichia coli* strain suspension and incubate at 37°C for 48 h and stored at room temperature .

Step3. BRS medium

This medium was prepared by adding an equal volumes of human serum to BR medium and incubate at 37°C for 24 h. Stored at room temperature .

Note: All preparation are stored at 4°C until used.

B. **Agar slant**

Agar	1.5 g
Sodium chloride	0.7 g
D.W.	100ml

The slant were prepared by placing 5 ml of the mixture in a screw tube , autoclaved and put the tubes in a slant position until the agar solution is harden, then it was stored at 4°C.

- In the day of inoculation add **Robinson's medium** (Erythromycin 0.5 ml, Phthalate peptone solution 3ml, BRS medium 1ml) to the agar slant then inoculate the slant with *Entamoeba* spp. suspension

3. Trypticase yeast extract iron serum (TYI-S-33) medium (Diamond *et al*.,1978)

Prepared by dissolving the following components in 600ml distilled water:

Potassium phosphate,dibasic	1g
Potassium phosphate,monobasic	0.6g
Sodium chloride	2g
Casein	20g
Yaest extract	10g
Glucose	10g
L- cysteine	1g
Ascorbic acid	0.2g
Ferric ammonium citrate (22.8mg/ml)	1ml

Bring the final volume to 880 ml distilled water and adjusting PH to 6.8.Ten aliquots of the medium flasks were made 88ml each , sterilized by autoclave for 15 min. afterward flasks were stored at -20◦C until use.

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الخـــلاصـــة

داء الأميبات هو اصابة معوية يسببها عدة انواع من الانتاميبا ومنها المتحوله الحالة للنسيج *E. histolytica* التي تعد النوع الرئيس الذي يسبب أعراض مرضية و ينتشر على نطاق واسع في جميع أنحاء العالم وقد يسبب الوفاة عند حالات الاصابة الشديدة .

شخص في الدراسة الحالية ثلاثة انواع من الانتاميبا من خلال استعمال ثلاث طرائق للتشخيص هي : طريقه الفحص المجهري المباشر ، طريقة المزرعة و تقنية تفاعل البلمرة التسلسلي PCR من أجل الحصول على تشخيص دقيق للاصابة. جمعت 150 عينة غائط تتضمن 50 عينة لاشخاص أصحاء و100 عينه مشخصة سريرياً على انها لمرضى مصابين بداء الاميبات راجعوا مستشفيات مختلفة من محافظة البصرة خلال الفترة من يناير 2018 إلى يناير 2019. فحصت جميع العينات بطريقه الفحص المجهري المباشر وأظهرت النتائج أن 60 ٪ من العينات كانت إيجابية، كما تم زرعت جميع العينات ايضاُ على اوساط زرعيه مختلفة وسجل وسط نقيع القلب والدماغ Brain heart infusion agar كفاءة عالية وكان أكثر ملاءمة في زراعة الطفيلي. وحددت نسبة الإصابة المختلفة للأنواع الثلاثة باستعمال طريقه التشخيص الجزيئي وكانت أعلى نسبة للمتحولة المشكوفسكية *E. moshkovskii* (15%) ، تليها *E. dispar* للمتحولة المتغيرة (10%) كما بلغت نسبة الاصابة (5 %) *E. histolytica* للمتحولة الحالة للنسيج في عينة الدراسة.

شملت الدراسة ايضاً التغيرات الامراضية النسجية الناتجه عن الاصابة بانواع الانتاميبا في جزئين من الامعاء الغليظه (المستقيم - الاعور) في الجرذان التجريبية. وأظهرت النتائج التغيرات النسيجية في اليوم 7 و14 و 28 بعد الإصابة بالأنواع الثلاثة. وكانت التغيرات المرضيه للجرذان المصابه بالمتحوله المشكوفسكيه والمتحوله المتغيره اقل من التغيرات المرضيه للاصابه بالمتحوله الحاله للنسيج وتضمنت هذه التغييرات التصاق الطور المتغذي بالطبقة المخاطية وتحللها مع ارتشاح الخلايا الالتهابية وامتدادها الى الطبقات السفلى .

استعملت في الدراسة تقانة تفاعل البلمرة التسلسلي ذي الوقت الحقيقي (Real-Time PCR) لدراسة التعبير الجيني باستعمال بادئات خاصة لجينات الوصلة الضيقة (Ocln ، Cldn1) لخلايا الامعاء الغليظة (الاعور) للجرذان المصابه بالأنواع الثلاثة كلا على

حده. واظهرت نتائج الدراسة ان جميع العينات المختبره اعطت نتيجة ايجابيه للتعبير الجيني بعد 7 و14 و 28 يوم من الإصابة بالأنواع الثلاثة الا ان المتحولة المشكوفسكيه اعطت اعلى مستوى للتعبير في كل الفترات .

تضمنت الدراسة الحالية استعمال المجهر الالكتروني (الماسح والنافذ) لتحديد التغيرات الامراضية الناتجة عن الإصابة بالانواع الثلاثة بصوره دقيقه. وأظهرت النتائج التصاق الطور المتغذي وتأثيره على الطبقة السطحية للامعاء الغليظه كما بينت التغيرات في خصائص هذا الطور مع وجود الاقدام والحويصلات الداخلية.