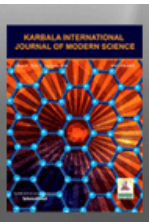

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Molecular and ultrastructure study of tight junction during experimental Entamoeba spp. infection

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Abstract

Entamoeba spp. in particular *E.histolytica* is the main reason of human amoebiasis. The molecular mechanism of its pathogenicity is poorly understood, therefore the aim this study is to investigate these mechanism on both molecular and ultrastructure levels. Tight junction (TJ) genes Claudin-1 (Cldn1) and Occludin (Ocln) were investigated by real time PCR and the pathological changes by scanning electron microscopy (SEM) and transmission electron microscope (TEM), the result showed that the gene expression levels of TJ genes were significantly high in rats infected with *E. histolytica E. dispar* and *E. moshkovskii* after 28, 14, 7 days in compare with healthy control. The expression of these genes were highest in rats infected with *E. moshkovskii* after 28 days, 14 and 7 day followed by *E. dispar* then *E. histolytica* .Electron microscope identified the pathogenesis of *Entamoeba* spp. which showed the adhesion of trophozoites and its effect on the epithelium, as well as changes in the properties of trophozoite, internal vesicles and filopodia which are very important in the adhesion and pathogenesis. Moreover, cytolysis was observed and compared among the three types.

Keywords

amoebiasis; Claudin-1; Electron microscope; Entamoeba spp.; Occludin; tight junction gene

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1. Introduction

According to Roure et al. [1], the main causative agent of human amoebiasis is *Entamoeba histolytica,* which can be defined as a single-celled parasite that affects 40−50 million people around ithe world. In other words, Invasive amoebiasis can effect only 10% of the total infected humans. The symptoms of invasive amoebiasis include amoebic colitis and extra-intestinal manifestation represented by a potentially fatal liver abscesses. Amoebiasis has a clinical variation that is associated with the microbiota in the intestine. This association may increase resistance to infection via decreasing the parasites virulence factors and modulate the systemic immune response against the parasites [2].

Normally, *E. histolytica* colonize in colon epithelium and its damage may result from many activities, such as adhesion, lysis and phagocytosis of host cells. These activities can be carried out by several proteins found in the parasite including cysteine proteases [3], the Gal/GalNAc lectin [4] and amoeba pores, which are important in disrupting and invading the intestine. Furthermore, the adherence of *E. histolytica* trophozoite can result in death of the host cell by apoptosis and inflammatory responses [5].

In the intestine epithelia, tight junction (TJ) serve as intercellular cell seal preventing the pathogen from penetrating the epithelia. These TJs consist of a belt like region between the epithelial cells that separate the apical from lateral plasma membrane and regulate ions passage as well as other molecules through the adjacent cells [6]. Also, the TJs consist of integral protein i.e., occludin that has been the best studied member of TJ proteins. In addition, it has more than 20 members of another TJ protein (claudin) that interacts with the actin cytoskeleton via TJ adaptor proteins like zonula occludens [7]. Many studies stated that the parasite has the ability to alter gene expression of the host cells regarding of the parasite [8,9].

The electron microscope (EM) provides a great understanding of the parasite pathology in providing 50 nm resolution combined with great depth of focus. Moreover, the scan EM allows researchers to detect, localize the parasite and expose specific surface molecule [10]. The EM has been used to confirm *E. histolytica* invasion into the intestine [11].

The aim of this study was to investigate *Entamoeba* spp. infection, effect of TJ protein (occludin and claudin-1) in a molecular level and in combination with ultrastructural study in experimental rats.

2. Material and methods

2.1. Experimental infection

Thirty six male experimental rats of *Rattus norvegicus* were used, aged 8−10 weeks, weight 110−120 g, divided into four groups, three groups injected orally with *Entamoeba* spp.(*E. histolytica, Entamoeba dispar and Entamoeba moshkovskii*) (10^4 cyst/ml) and intra rectally (100 trophozoite/ml) for each species. The *Entamoeba* spp. were already identified using specific primer for each species and amplified by PCR, one group (control) was injected with oral and intra rectal inoculation using normal saline. The animals were sacrificed at 7, 14 and 28 days of infection and part of the large intestine (cecum) was taken to be prepared with paraffin embedding in order to study gene expression for the TJ gene by molecular analysis method. Histological preparation was performed according to Ref. [12].

2.2. Total RNA extraction

Total RNAs were extracted from paraffin embedded samples of rats' cecum according to the procedure of ReliaPrep^{TM}FFPE Total RNA Miniprep System (Promega, USA).

2.3. Revers transcription of RNA

A total of 400 ng of RNA from each sample was used to generate cDNA by GoTaq®2-Step RT-qPCR System (Promega, USA). Thermal cycler condition for cDNA synthesis was represented in annealing temperature 25 °C for 5 min, incubation at 42 °C for 1 h, inactivate the reverse transcriptase at 70 °C for 15 min with 1 cycle.

2.4. Quantitative real −time PCR (qRT-PCR)

The cDNA served as templates for **qRT-PCR**, which was performed using SYBR-Green PCR core reagents. A total of 120 ng of cDNA from each sample was used to measure the gene expression of Claudin-1(Cldn1), Occludin (Ocln) and GAPDH (Gapdh) as a housekeeping gene (HK) Primers used for Cldn1:

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forward primer (FP) 5′-TGTCCACCATTGGCATG AAG-3′ and reverse primer (RP) 5′GCCACTAATGT CG CCAGACC-3'; Ocln: FP 5′CTACTCCTCCAACG GCAAAG3′ and RP 5′-AGTCATCCACGGA CAAGGTC-3'; Gapdh: FP 5′TGGAGTCTACTGGCG TCTTC-3′ and RP 5′-TCCACACCCATCACAAA CATG-3′, [13]. The volumes of each single reaction were GoTaq® qPCR Master (10 µl), FP (1 µl), RP (1 µl), cDNA template (6 µl), Nuclease-Free Water (2 µl). DNA amplifications were performed under the following reaction conditions: an initial heating cycle of 95 °C for 2 min; 40 cycles alternating between denaturation at 95 °C for 15 s, primer annealing and extension at 60 °C for 15 min. The data were analyzed by calculating the expression level of the genes of interest using cycle threshold number (CT) value. Relative quantification 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 [14] as follow:

$$\Delta CT_{infection} = CT_{(target\ gene)} - CT_{(HKgene)}$$

$$\Delta CT_{control} = CT_{(target\ gene)} - CT_{(HKgene)}$$

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

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

- $\Delta CT_{infection}$ = 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)

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

2.5. Electron microscope study

Cecum samples after 28 days of amoebiasis infection were cut to small pieces (1 cm) and prepared for ultrastructural study by (TEM and SEM) according to Ref. [15].

2.6. Statistical analysis

Two-way ANOVA were performed to evaluate the difference in gene expression levels among Entamoeba spp. (*E. histolytica, E. dispar and E. moshkovskii*) for three periods for the results which showed that the expression level of Cldn1 and Ocln were significant using SPSS under $P \leq 0.05$.

3. Results and discussion

3.1. Expression of tight junction genes

The gene expression levels of TJs genes represented by Cldn1 and Ocln showed a significant high levels in rats infected with *E. histolytica, E. dispar* and *E. moshkovskii* after 7, 14, 28 days compared with healthy control. One peak melting curves for Cldn1 and Ocln genes was obtained to illustrate the specific binding of primers with the target gene (Fig. 1).

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. 2).

Along with Betanzos et al. [16], the changes produced by *E. histolytica* are well known, the interaction between trophozoite and host cell begins with the attachment of the parasite to the intestinal epithelium followed by sequent invasion. Morphological changes in the epithelial cells can be described as widening of intracellular spaces resulted from the loss cells that are affected by the parasite causing disruption in the epithelial barrier. On the other hand, the molecular mechanisms of invasion for the trophozoite and alteration in the intestinal barrier especially the TJ protein that participates in maintaining cell contact is still not fully understood. The TJ proteins play an essential role in preventing a parasite invasion into the intestine [17,6].

Therefore, the TJ genes Cldn1 that affects amoebiasis was one of the genes of interest in our study. The Cldn1 is integral TJ protein regulating the size selectivity of the barrier [18]. Our results revealed a

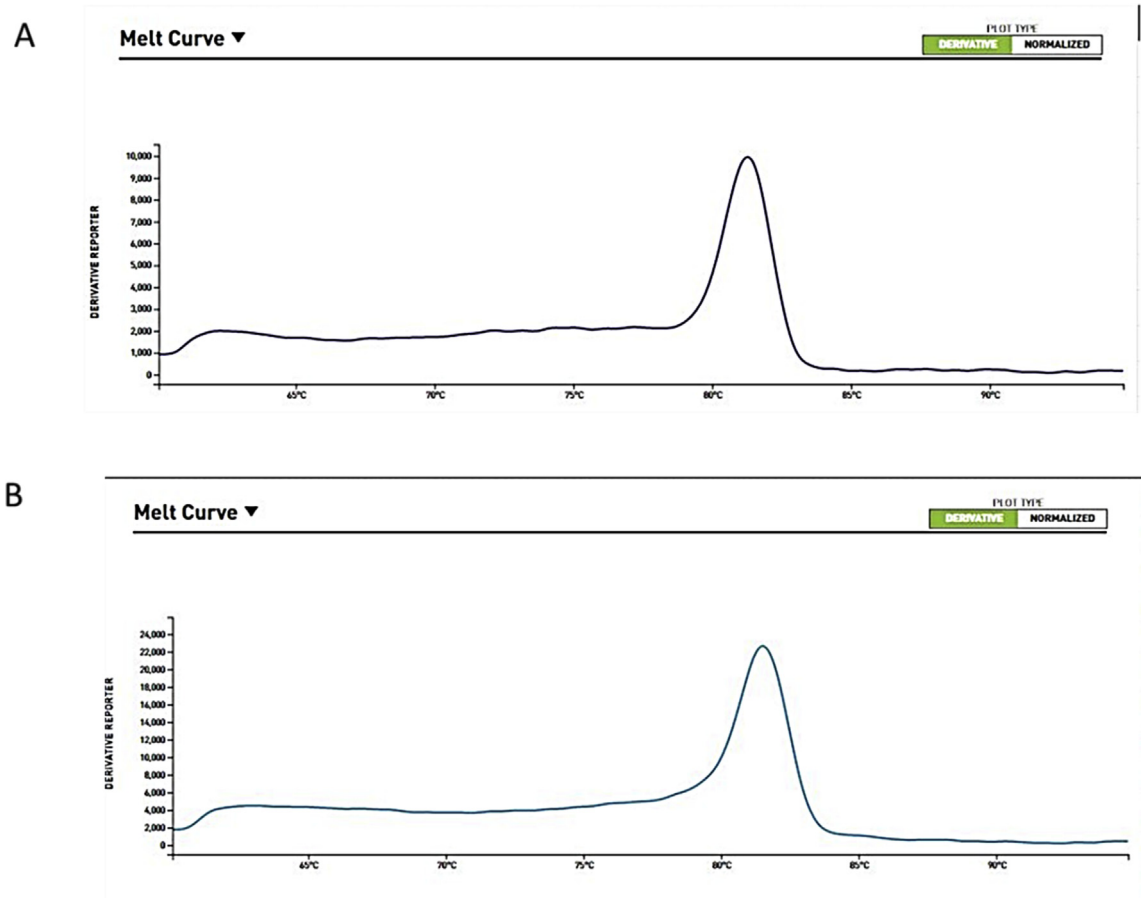

Fig. 1. Melting curves of real time – PCR products. A single peak representing the specific binding of SYBER green dye for the genes of interest. Cldn1 gene **(A)** and Ocln gene (**B)** in rats infected with *E. moshkovskii.*

significant high expression of Cldn1 infected rats suggesting that *E. moshkovskii* has a strong effect on Cldn1.

The Ocln is another TJ gene that is important for the primary intercellular seal between epithelial cells [13]. Our results showed a high levels of expression for this gene, especially *E. moshkovskii.* Moreover, it was higher than the other TJ gene (Cldn1). The reason of high expression of Ocln may result from the nature of gene function as a seal between epithelial cells. Furthermore, studies suggest that Ocln is an important in the maintenance and assembly of TJ proteins [19]. In addition, when we compared the expression levels of Cldn1 and Ocln among *Entamoeba* spp., the lowest expression for both genes was found in rats infected with *E. histolytica*. This may be resulted from the invasive nature of *E. histolytica* [20], which destroyed the cell of large intestine by secreting cysteine protease leading to a loss and destroyed cells [16]. While, *E. moshkovskii* and *E. dispar* secrete these cysteine protease in low levels [21,22].

3.2. Electron microscope study

3.2.1. Transmission electron microscopy

Cecum sections of rats infected with *E. dispar* and *E. moshkovskii* at 28 days post-infection that were examined with transmission EM showed that some of the microvilli were preserved in normal appearance and normal free surface, in addition to the presence of dilated and elliptic mitochondria (Plate1A & B). While, cecum sections of rats infected with *E. histolytica* showed irregular microvilli (Plate 1 C) compared with control sections that showed the apical region of two adjacent intestinal epithelial cells. The junction complex consist of zonula adherence, desmosome,

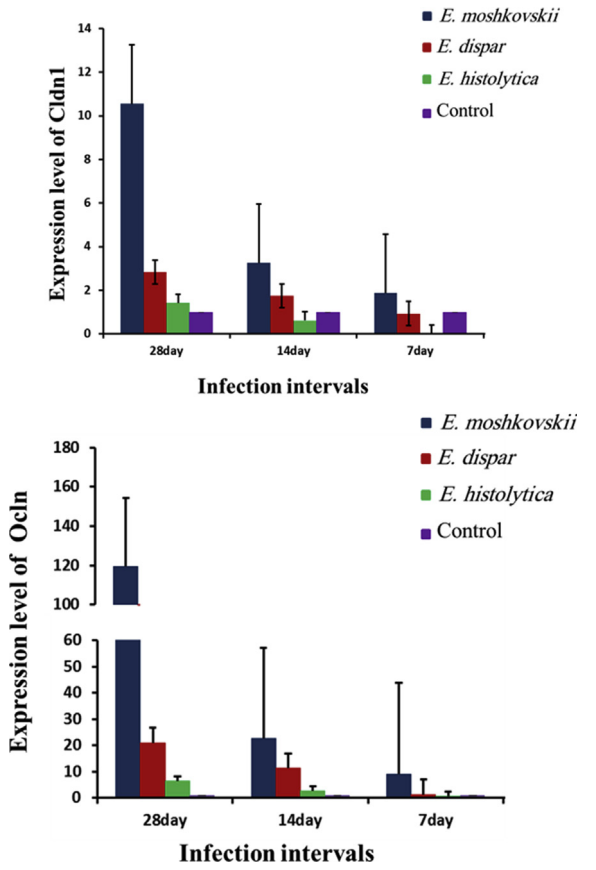

Fig. 2. The gene expression levels of TJ genes Cldn1 and Ocln in *Entamoeba* spp. experimental infection after 28, 14, and 7 days. **A**. Cldn1 gene expression in the large intestine (cecum) of rats infected with *Entamoeba* spp. (*E. histolytica, E. dispar, E. moshkovskii*). **B**. Ocln gene expression in the large intestine (cecum) of rats infected with *Entamoeba* spp. (*E.histolytica, E. dispar, E. moshkovskii*).

electron dense cytoplasm and normal nucleus (Plate 2A).

3.2.2. Scanning electron microscopy (SEM)

The results of SEM showed the changes of infected cecum with amoebiasis at 28 days post-infection as variable numbers of trophozoites were attached to the mucosal epithelial surface according to the parasite type. It was heavy association to the epithelial cell near interglandular in cecum infected rats with *E. histolytica* (Plate 3 A) compared to the adherence of *E. dispar* and *E. moshkovskii* trophozoites in mucosal layer with enterocyte, in others section the trophozoites were distributed freely in lumen (Plate 3 B & C).

The most severe intestinal damage was found in rats infected with *E. histolytica*, the damage represented in extensive lysis enterocyte, degraded mucus and absence of microvilli in some regions as well as irregular crypts opening and intercellular fibers (Plate 4). While, changes in the cecum section of infected rats with *E. dispar* and *E. moshkovskii* showed a mild degraded mucus layer after adherence of trophozoites by filopodia, in addition to a regular mucosal structure and normal microvilli of the cecum epithelium (Plate 5A1, A2 & B), compared with control sections that showed the mucosa surface composed of normal microvilli, apical surface of microvilli and regular opening of interglandular crypts (Plate 2B).

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 confirmed these changes as the trophozoite found in close contact with intestinal epithelium [23,24,8,25,and5]]. Furthermore, the displacement of intracellular spaces, the penetration of cells and delocalization of occludin and claudin-1 [20].

Several virulence factor, such as Gal/GalNAc lectin, amoeba pores, cysteine, serine proteases and prostaglandin E2 (PGE2) participate in increasing the permeability of the intestine by altering claudin-4 [26]. The cysteine proteases can affect claudin-1 and occludin, damage the adherence junctions (AJ) and desmosomes (DSM) [27]. Our results showed the most severe intestinal damage found in rats infected with *E. histolytica*, the damage represented in more irregular distribution of microvilli. Cysteine proteases have an important role in damaging the integrity of microvilli in vivo, the parasite uses the proteinases to overcome microvilli and the barriers of TJ when it invades the intestine [21].

Our results in all sections of infected intestine showed that there were intercellular connection (spaces) separated among the enterocytes bases, these spaces were more dilated. Also, they showed irregularity in sections infected with *E. histolytica* and desmosomes that identify TJ that were irregular.

Another study showed different pathophysiological changes represented in the induction of actin filaments contraction attached to the TJ contributing to their opening [28].

Our results showed fibers deposition, amorphous material, fine fibrils substance and cells remnants, in addition to fat droplets were noticed at a variable quantity in the lumen.

After the attachment of trophozoite to the epithelium, the permeability of cells were increased, suggesting that the TJ proteins are disrupted. This

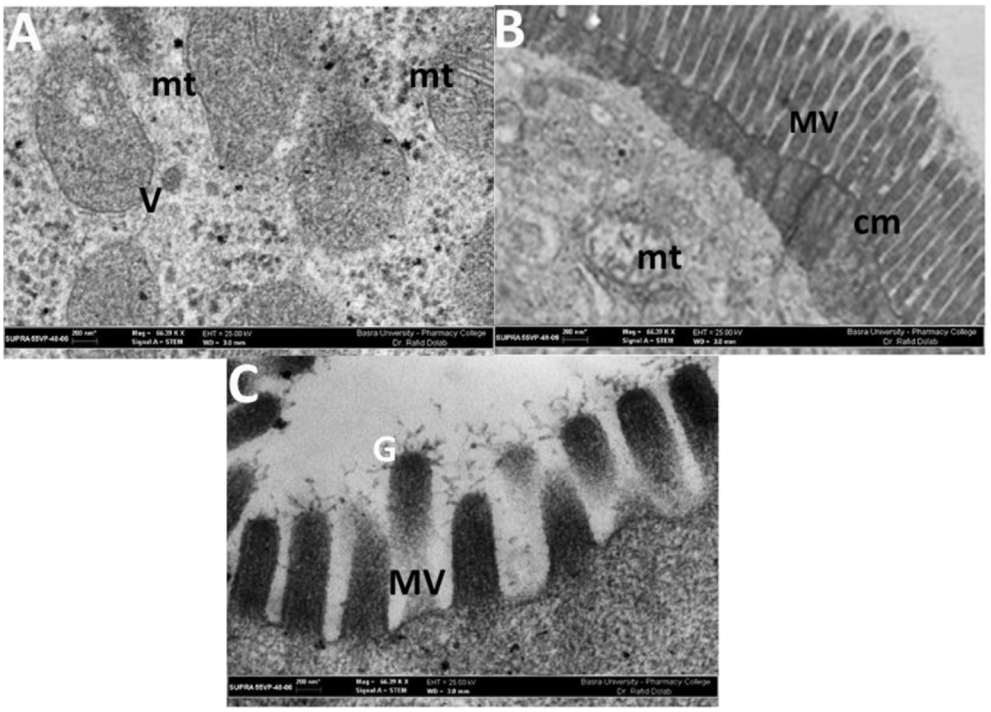

Plate 1. **Micrograph with TEM on rat cecum infected with *Entamoeba spp.* A. Infected with *E. dispar.*** Showed dilated mitochondria (**mt**) multivesicular bodies (**V**).**B. Infected with *E. moshkovskii.*** Showed normal microvilli (**MV**), normal free surface (**cm**) dilated, elliptic mitochondria (**mt**).**C**. **infected with *E. histolytica.*** Showed irregular microvilli (**MV**), glycocalyx (**G**).

disturbance is resulting from the actin cytoskeleton disruption, in addition to 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 [26]. Moreover, reproduction activities of the parasite can cause more adherence of the trophozoite to epithelium as well as more tissue damages due to the amoeba secretion including amoeba pore that cause pores allowing a massive influx of extracellular Ca^{2+} across the plasma membrane. Also, it results in irreversible rise of $Ca^{2}+$ in the target cell, increase of the $Ca^{2}+$ is caused by activities of many enzymes, such as ATPase, Phospholipase, endonuclease and Proteases, which break down the proteins that found in both membrane and cytoskeleton [21].

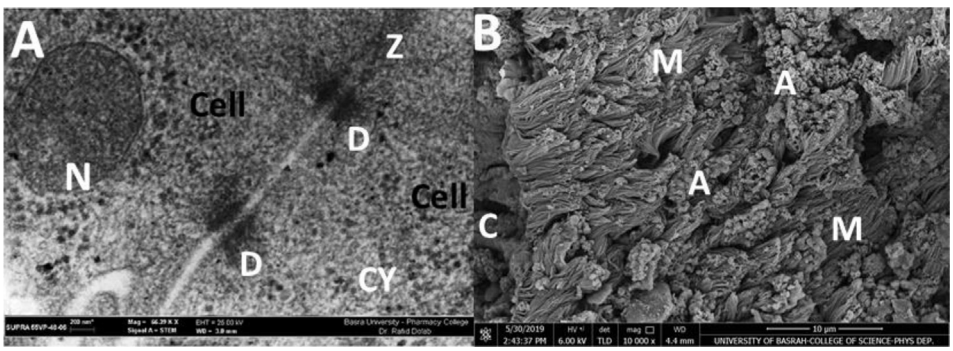

Plate 2. **Ultrastructure of control rat cecum (normal structure). A. TEM micrograph** showed the apical region of two adjacent intestinal epithelial cells (**Cell**), the junction complex consist of zonula adherence (**Z**) and desmosome (**D**), electron dense cytoplasm (**CY**), normal nucleus (**N**). **B**. **SEM micrograph** showed the mucosa surface composed of normal microvilli (**M**), apical surface of microvilli (A) opening of interglandular crypts (**C**).

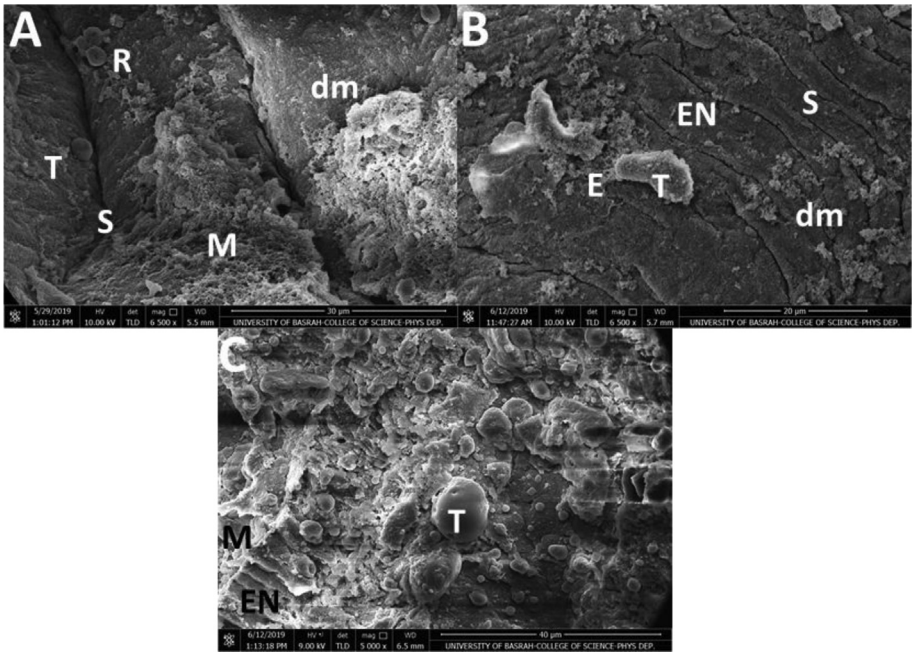

Plate 3. **SEM micrograph on cecum section infected with *Entamoeba spp.* A. Infected with *E. histolytica,*** showed the trophozoite (**T**)near the interglandular epithelial cell (**S**), degraded mucus (d**m**) mucus in some region (**M**) red blood cells (**R**).**B. infected with *E. dispar,*** showed the trophozoite (**T**) start to form elongated extend (**E**) to bind with enterocytes (**EN**) normal intercellular space (**s**) and mild degraded mucus (dm).**C. cecum infected with *E. moshkovskii*,** showed the trophozoite (**T**) distributed freely within the mucosal surface, some normal enterocytes (**EN**) appeared coated with mucus (**M**).

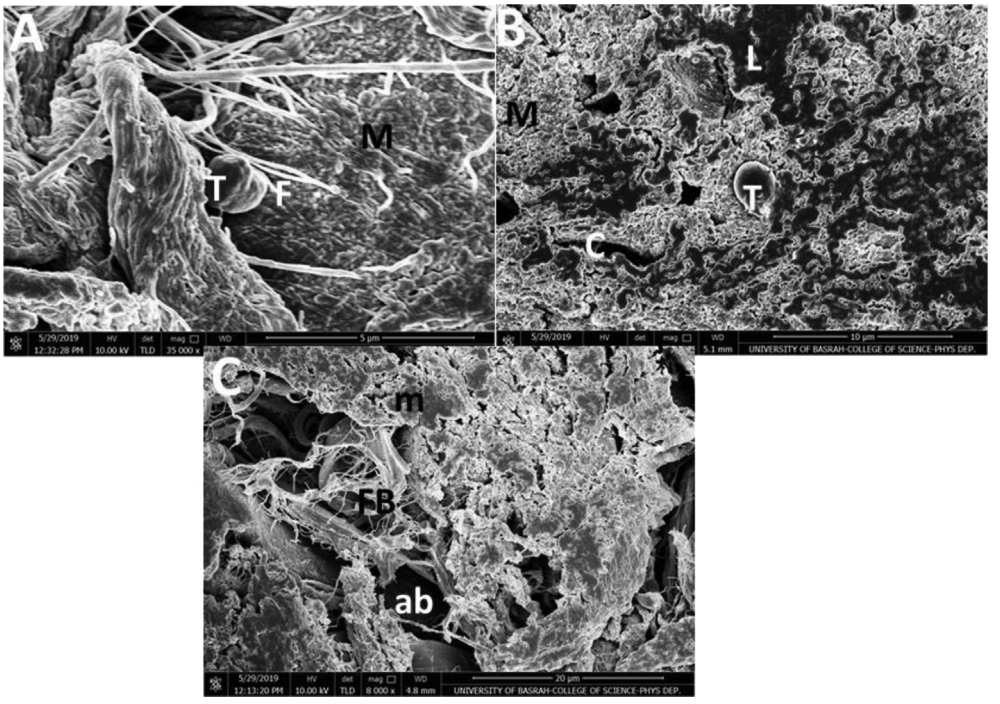

Plate 4. **SEM micrograph on cecum mucosa infected with *E. histolytica.* A.** Showed adhesion of trophozoite (**T**) to enterocytes by filopodia (**F**) area with preserved microvilli (**M**).**B**. Showed trophozoite (**T**) attached to the enterocyte surface, extensive lysis (**L**) microvilli (**M**) observed and irregular crypts opening (**C**) .**C.** Showed degraded mucus (m) absence of microvilli in some region (**ab**) intercellular fibers (**FB**).

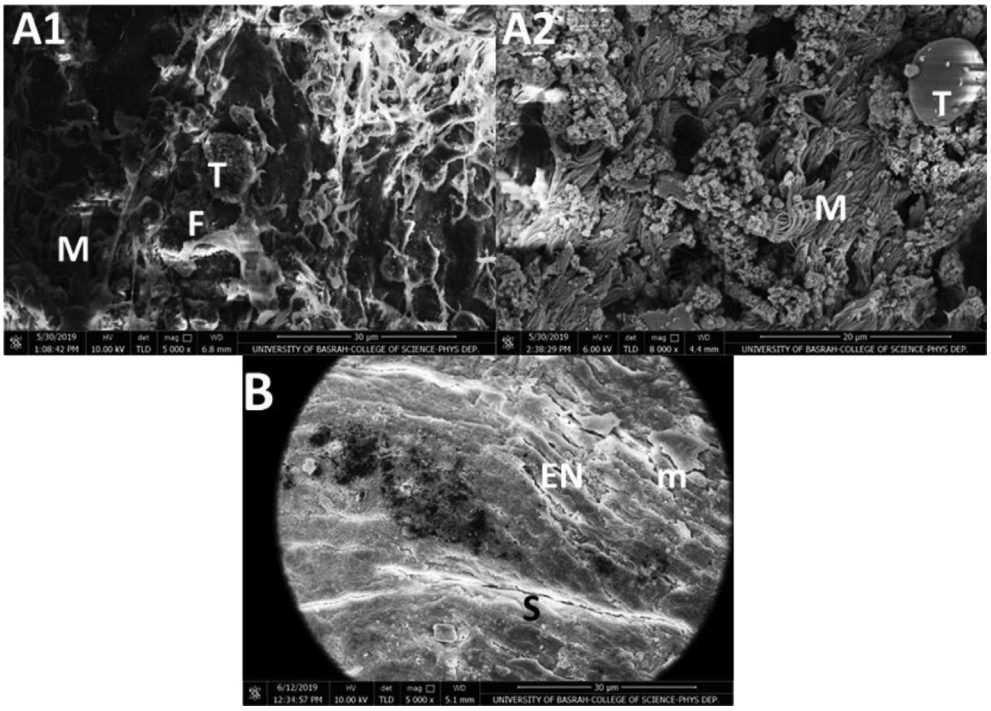

Plate 5. **SEM micrograph on rat cecum infected with *Entamoeba spp.* A. Infected with *E. moshkovskii*. 1.** Showed the trophozoite attached to the mucosa (T) filopodia (**F**) and mucus (M). **2.** Showed trophozoite (**T**) normal microvilli (M).**B**. **Infected with *E. dispar.*** Showed regular enterocytes (EN) separated with normal intercellular space (**S**) and thick mucus (**m**) covered the mucosa surface.

4. Conclusion

The rats infected with *E. moshkovskii* showed significant high expression levels in comparison with other Entamoeba spp. The electron microscopy confirmed the alteration in the intestinal TJs and other pathological changes including the attachment of trophozoite and tissue damages.

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