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IMMUNOPATHOLOGICAL CHANGES OF THE MICE INTESTINE INFECTED WITH SHIGA TOXIN PRODUCING *E coli* O111

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ABSTRACT

To study the pathogenesis of a diarrheal disease due to shiga toxin producing *Escherichia coli* (STEC), 8 – 10 weeks-old mice were inoculated orally with STEC O111 (3.16 X 10^8 CFU). These mice were examined for clinical signs and histopathological changes as well as immunohistochemistry which was done in order to evaluate the degree of damage in the intestinal epithelial tight junction. Results showed that there were marked damage in the intestinal epithelial tight junction which result in the sloughing of the small intestinal and colonic epithelial cells with marked inflammation, mucinous degeneration and hemorrhage. These changes were started from the 3rd day post bacterial inoculation and continue till the 14th day then progress in to multiple area of ulceration developed in different parts of small and large intestine. Finally these ulcerated sites replaced by fibrosis in the end of experiment at day 28 post inoculation. In conclusion inoculations of the mice with STEC O111 result in sever progressive changes involving both small and large intestine.

INTRODUCTION

E. coli is one of the first species that colonize the intestine during the infancy, and

reach very high density (more than 10^9 CFU per gram of faeces) prior to the expansion of other anaerobic species. *E. coli* can be commensal, existing in a symbiotic state providing resistance against pathogenic organisms [1], or become pathogenic and start to cause diseases in intestine or other sites out of the intestine. the most important pathogenic strains are enterotoxogenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and

diffusely adherent *E. coli* (DAEC) [2]. The variable strains of STEC are characterized by their ability to produce toxins resemble to those produce by *Shigella dysenteriae* type 1.

There are two types STEC Shiga toxins, Shiga toxin 1 and Shiga toxin 2, which is encoded by the *stx*1 and *stx*2 genes respectively. In industrialized countries some of (STEC) strains are important causes of food-borne diseases, their clinical presentation range from mild diarrhea to severe complications, such as hemolytic-uremic syndrome (HUS), which primarily occurs in young children [2]. Animals, particularly cattle, serve as reservoirs for STEC. Ingestion of contaminated food or water, person-to-person contact, direct contact with animals, and exposure to the environment represent the most common modes of transmission for these strains [3]. The STEC were classified according to the serotype importance, in to two major categories, STEC O157 and non-O157 STEC [4].

It has become evident that non-O157 (STEC) strains, particularly the serogroups O26, O45, O103, O111, O121, and O145 (known as the top six non-O157 STEC) cause similar illnesses to those of O157:H7 strain [5], and outbreaks due to non-O157 STEC, particularlly serogroup O111 have been associated with beef or direct contact with cattle [6][7]. Thus, FSIS stated that the top six non-O157 STEC as an adulterant sin beef trim, then FSIS verify testing for these

pathogens in domestic and imported beef manufacturing trimmings which was begin in June 2012 [8]. There are three distinct categories of diarrheagenic O111 *Escherichia coli*, namely, enteropathogenic *E. coli* (EPEC; typical and atypical), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC) [9].

MATERIALS AND METHODS

Bacterial strain: O111 STEC isolate was obtained from human diarrheal cases during a Ph.D. dissertation study in which the isolate was confirm as O111 by PCR for the following genes (*wbdI rfbo111, stx1, stx2, eae and hly*). *rfbo111* is the surface O antigen encoding gene cluster was sequenced and aligned by BLAST to confirm the PCR results and make sure that the isolate is 100% O111 STEC.

Preparation of the mice for the study: One hundred twenty mice aged about 8-10 weeks with average weight 27±5 gm of both sex were divided in to two main groups 60 animals for each, these were:

1- **Infected group:** animals of this group were separated in to six subgroups according to

the intervals of sacrificing each subgroup consist of ten animals, animals in each subgroup were acclimatized with each other for two weeks before starting the experiment and provided with food and water ad libitum.

2- **Control group:** animal of this group were acclimatized by the same way of the infected

group.

All animals had been weighed before starting the experiment and before killing at each interval.

Histopathological preparation: Pathological changes of intestine was studied at 3, 7, 14, 21, and 28 days. Specimens from different parts of small and large intestine were taken, the tissue were fixed in 10% buffer formaline solution immediately after washing with normal saline. After 48 hrs, the specimens were processed routinely with a set of ascending alcoholic concentration from 70% to absolute 100% for 1 hr each, then clearance was done by xylol, the specimens were

infiltrated with liquid paraffin wax at 58-60°C on two stages, 2 hrs each; blocks of specimens were made with paraffin wax and refrigerated finally, they were sectioned by microtome at 4-6 μm, two slides for each specimen was made, one for routine H&E staining and the other was positive charge slides for immunohistochemistry stain of Claudine. Histopathological changes were observed under light microscope.

RESULT

- Grossly: Gross inspection of the intestine of the infected animals showed yellowish watery content along the small and large intestine at the third day post infection, at day 7 post infection there were accumulation of edematous fluid in the small intestine and colon, while there were a hemorrhagic spots in some parts of the intestine and colon at day 14, in which some of them continue to appear even at day 21 Figure (1,2,3).

- Microscopically

Microscopic inspection of the small and large intestine reveal the following changes:-

Section of small intestine from control group reveal that it appears with in normal limits Figure (4,5) immunohistochemistry of Claudine reveals it is completely intact Figure (6, 7)

At the third day there was a variable degrees of epithelial sloughing in the duodenum, jejunum and ilium with some spots of hemorrhage, infiltration of inflammatory cells in the lamina properia and excessive production of mucin in the colon Figures (8, 9, 10 and 11).

Immunohistochemical stain for the Claudine reveal the descohesion between epithelial cells was started at this interval which there were destruction of interepithelial Claudine in both small and large intestine, Figures (12 and 13).

At day 7 and 14 there were marked epithelial sloughing in the different parts of small intestine with accumulation of edematous fluids, infiltration of inflammatory cells in the different layers of small intestine including lamina

properia and sub-mucosa Figure (14).

Also similar changes were seen in different parts of the colon with hemorrhagic sites along the parts of the colon with alternative areas of colonic epithelial necrosis and hyperplasia appear at the day 7 & 14 post infection, all these changes was inconsistent with hemorrhagic enterocolitis Figures (15, 16).

The sloughing of the epithelium along small and large intestinal parts during this period were also revealed by immunohistochemical stain for the Claudine where there were marked colonic epithelial cells descohesion, sloughing with superficial inflammatory infiltrate forming Pseudomembrane indicating pseudomembranous colitis Figures (17 and 18).

Colon revealed variable degrees of submucosal fibrosis particularly in the necrotized sites at day 21, and these changes were also seen at day 28 post infection but with more maturation of fibrosis, Figures (19, 20).

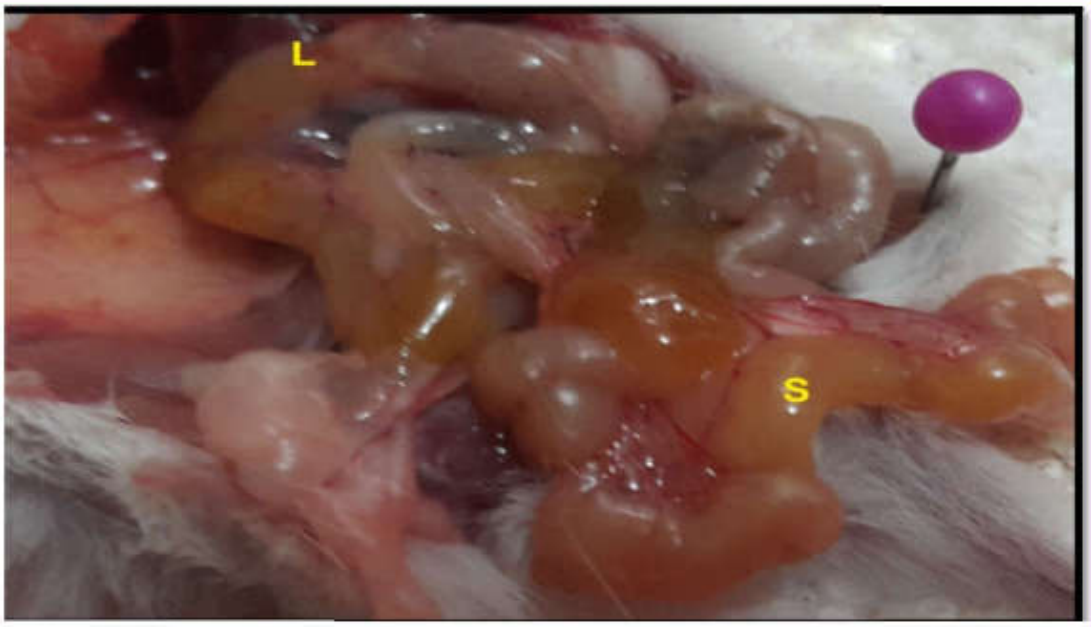

Figure (1) the gross appearance of small (S) and large (L) intestine of infected animal in the third day post infection which is filled with yellowish watery content (diarrhea).

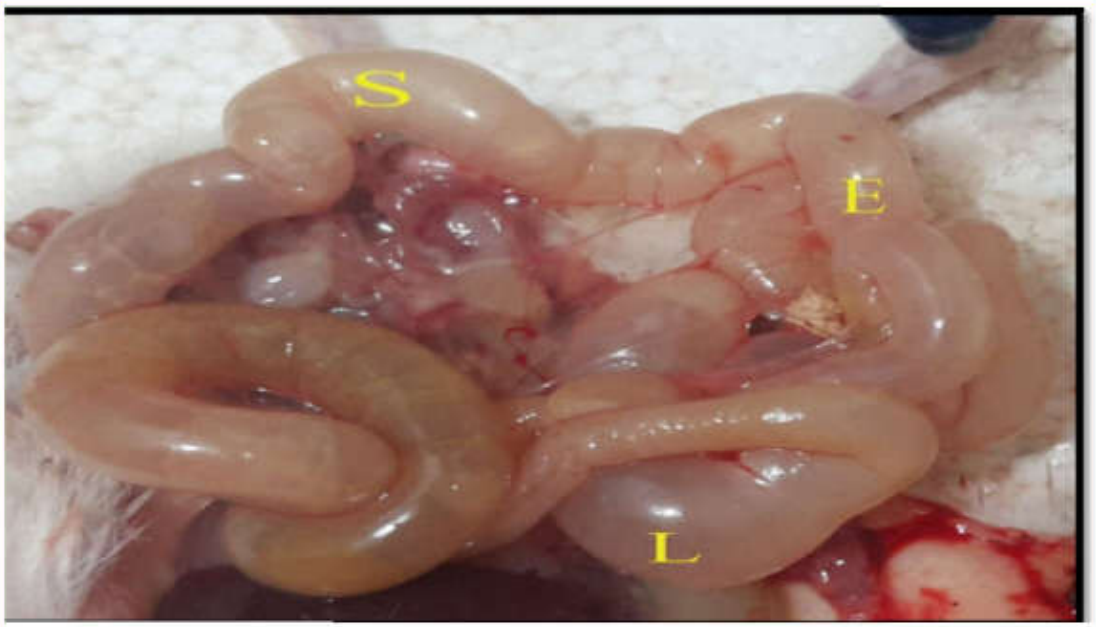

Figure (2) the gross appearance of small (S) and large (L) intestine of infected animal in the seventh day post infection in which there is excessive accumulation of edematous

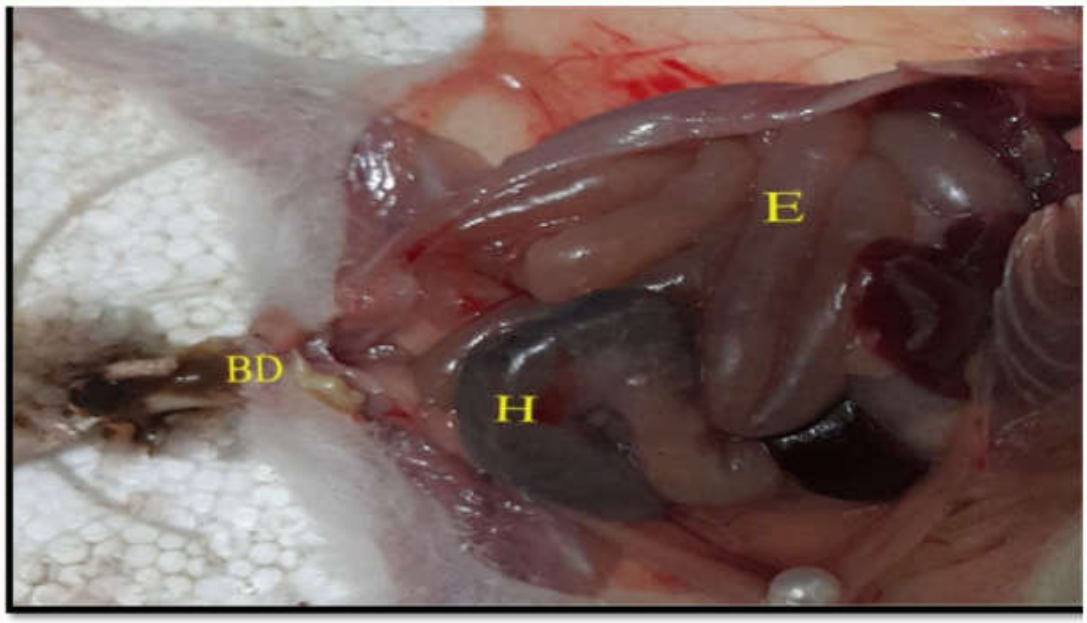

Figure (3) gross appearance of the viscera of infected animal after 21 day of infection, reveal colonic hemorrhage (H), intestinal edema (E) and bloody diarrhea (BD)

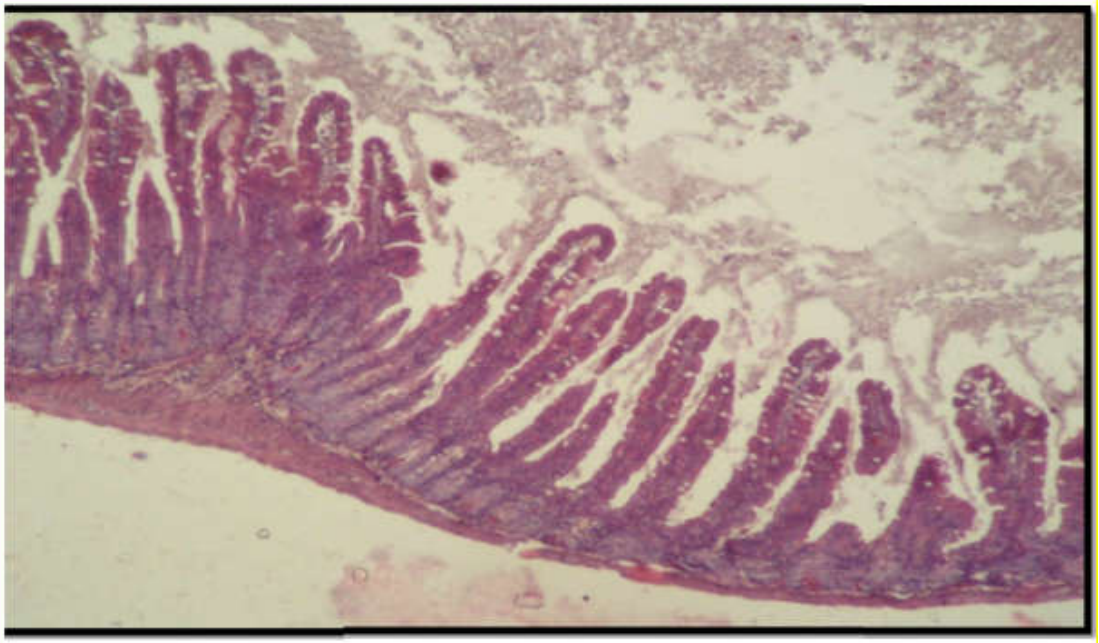

Figure (4) Duodenum of O111 STEC infected animals at 1 day post inoculation reveal intact villi (IV) on low power (125 X) and normal intestinal epithelium with goblet cells (GC) and mild edema in the lamina properia (E) on high power (500X) H&E

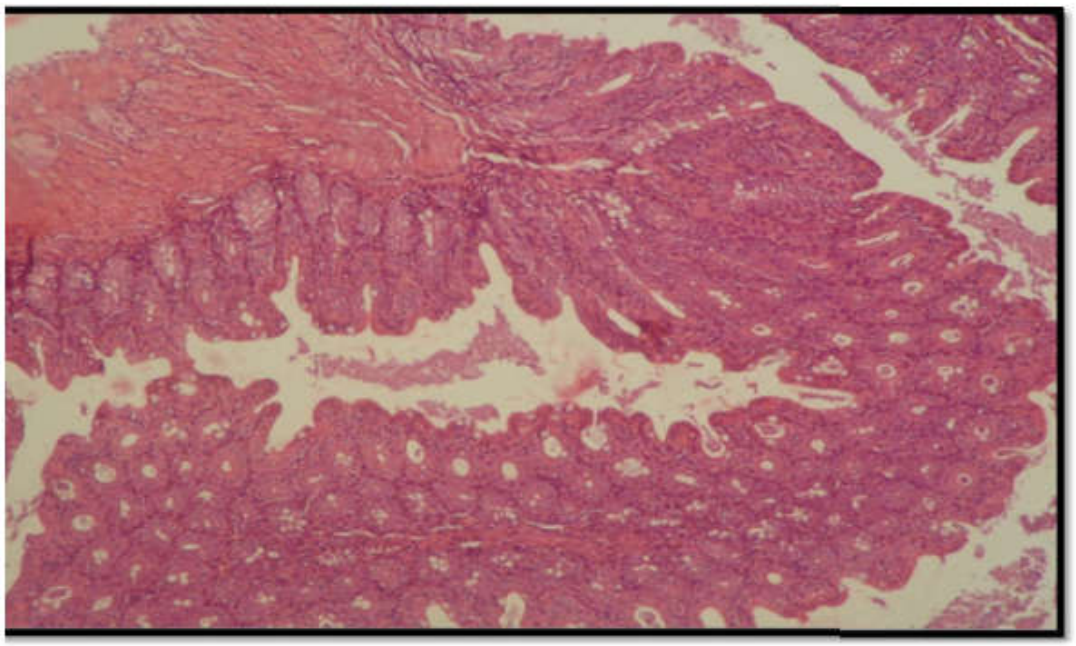

Figure (5) Normal histological structure of the colon of the control animals. H&E A) 125X B) 500X

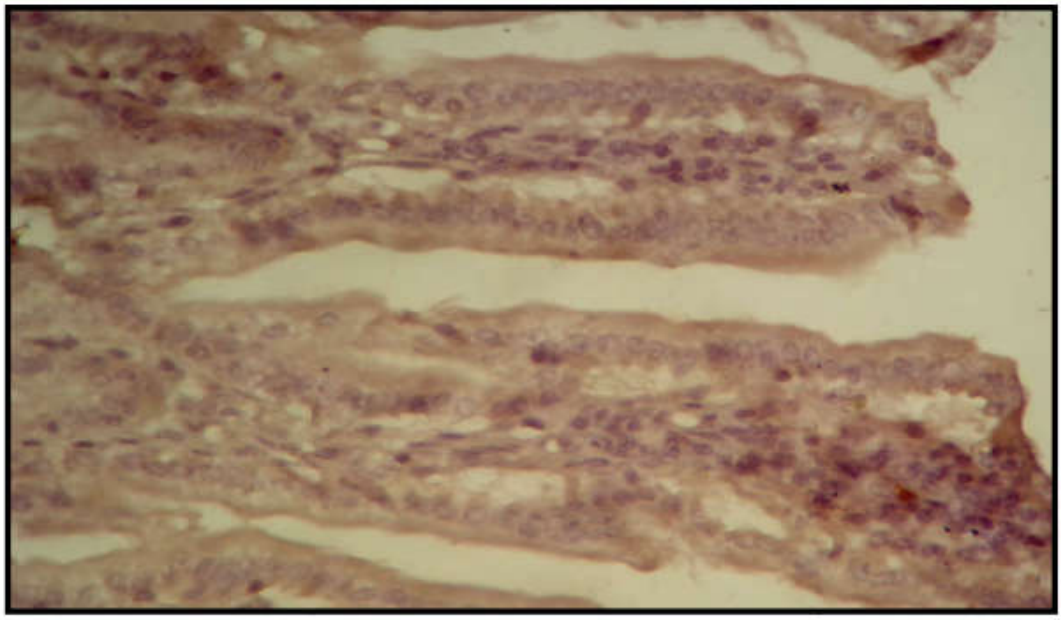

Figure (6) duodenum of the infected animals at 1st day post infection reveal that there is no destruction of inter-epithelial tight junction indicated by normal Claudine which appear as a dark brown intra-cytoplasmic and intercellular deposits of the stain 500X

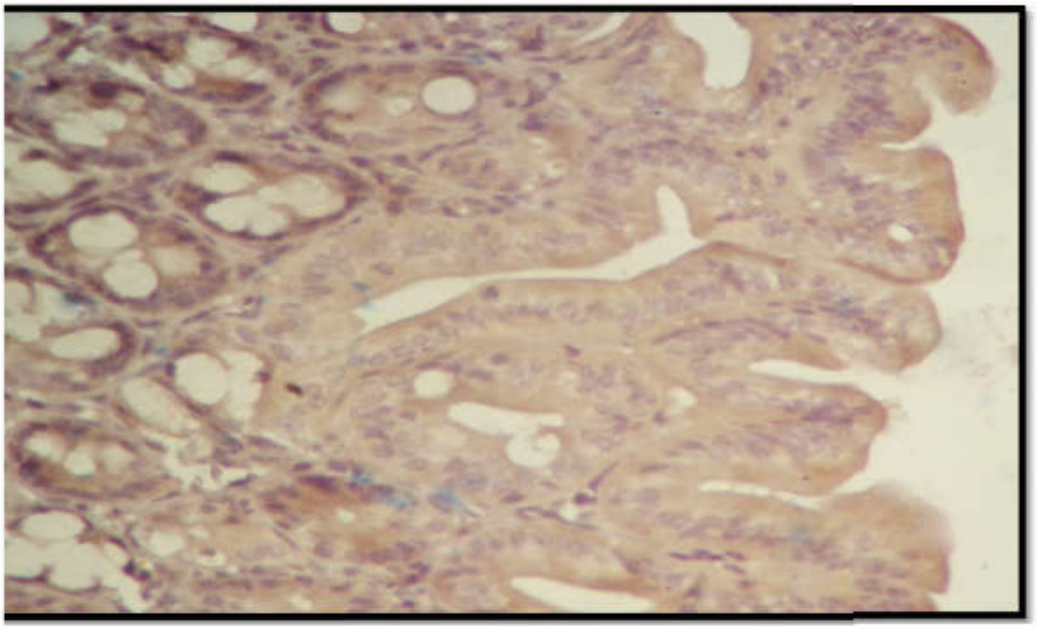

Figure (7) section of colon at 1st day post infection shows intact epithelial cells in both superficial mucosa and colonic glands revealed by intact epithelial tight junction (T) stained by DAB stain 500X

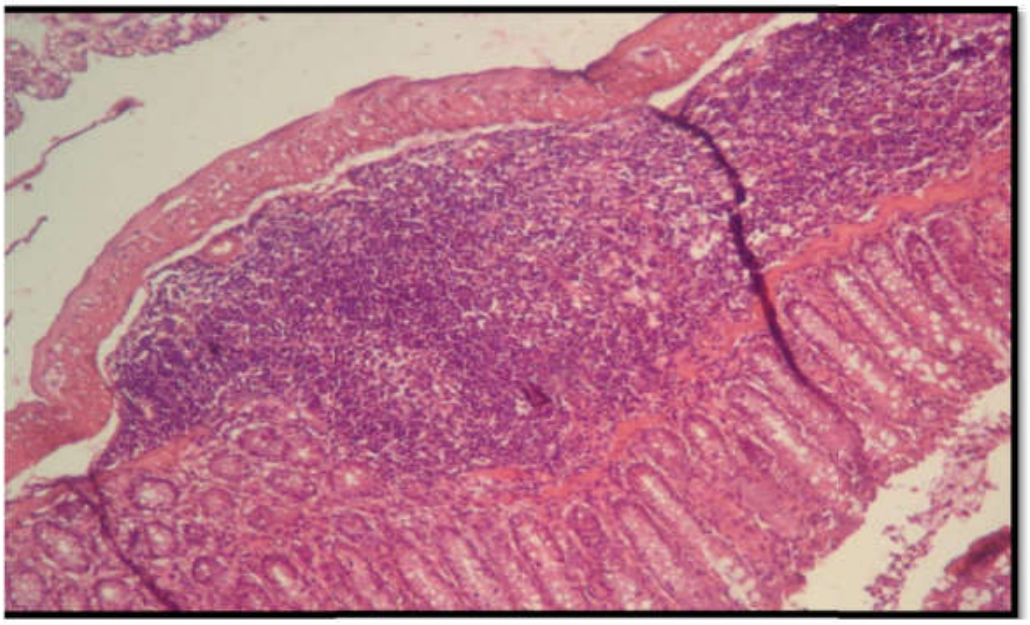

Figure (8) duodenum of infected animals after 3 days of infection shows massive infiltration of inflammatory cells in submucosa (IN) and superficial sloughing of mucosal epithelium (S). H&E 125X

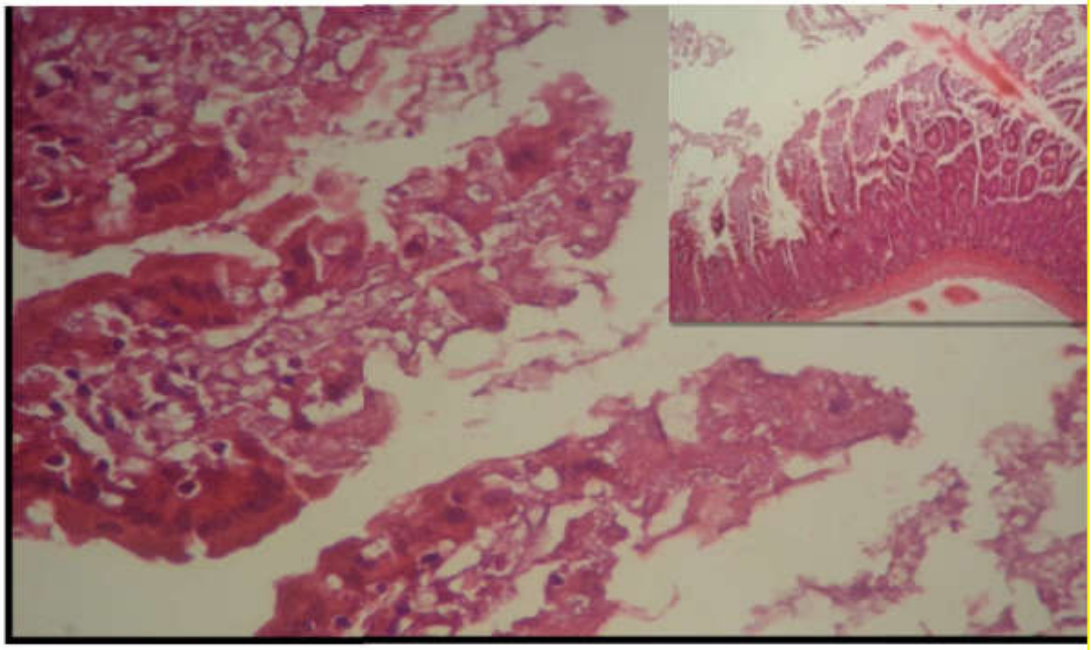

Figure (9) duodenum of infected animals after 3 days of infection shows superficial sloughing of mucosal epithelium. H&E A) 125X B) 500X

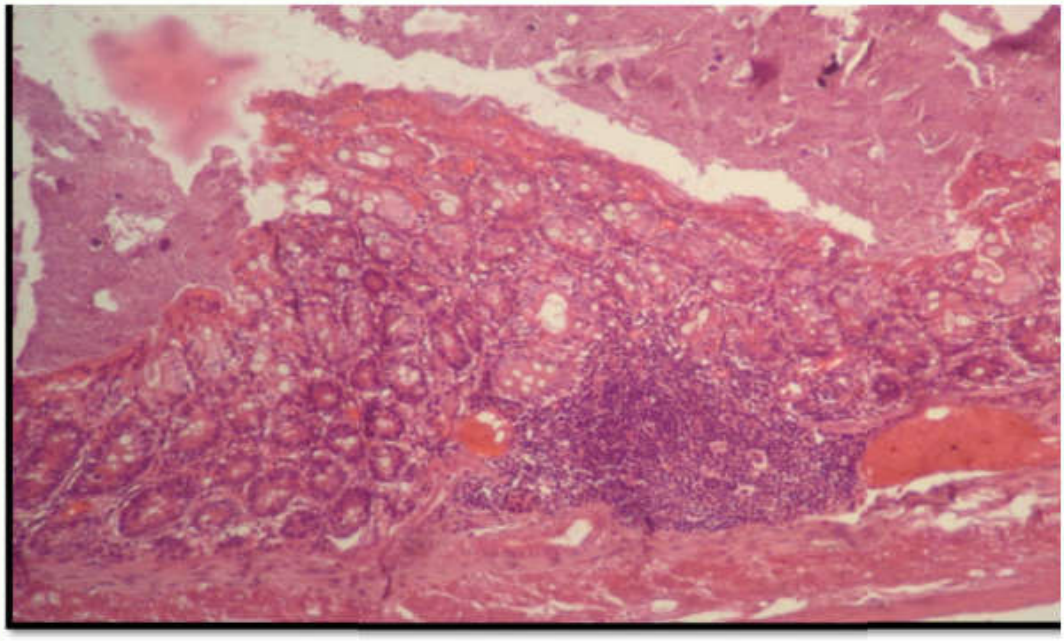

Figure (10) colon of infected animals after 3 days of infection in which there were mucosal hemorrhage (H) congestion of submucosal blood vessels (CO) and infiltration of inflammatory cells in the mucosa (IN) H&E 125X

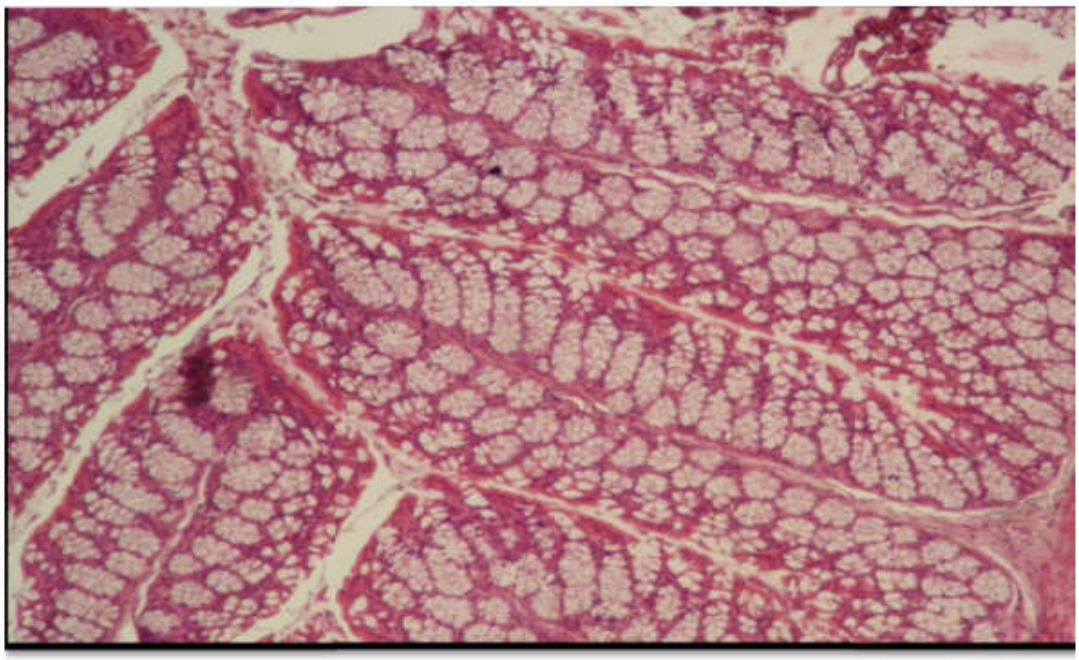

Figure (11) another area of colon of infected animal after 3 days of infection in which there were massive mucin production (M) with mucinous degeneration of the colonic glands (MD) H&E 125X

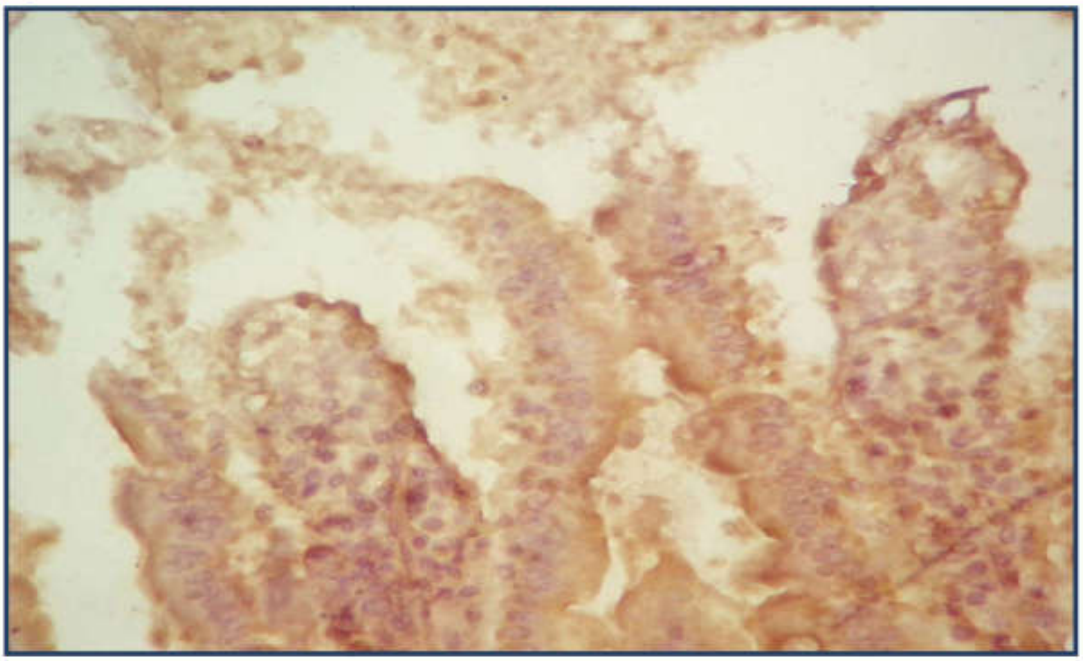

Figure (12) duodenum of the infected animals at 3rd day post infection reveal that there is a superficial sloughing (S) of the epithelial cells due to destruction of inter-epithelial tight junction from both lateral sides of the cells (L) and the basal site (B) revealed by the destructed tight junction in the aforesaid sites according to the DAB stain 500X

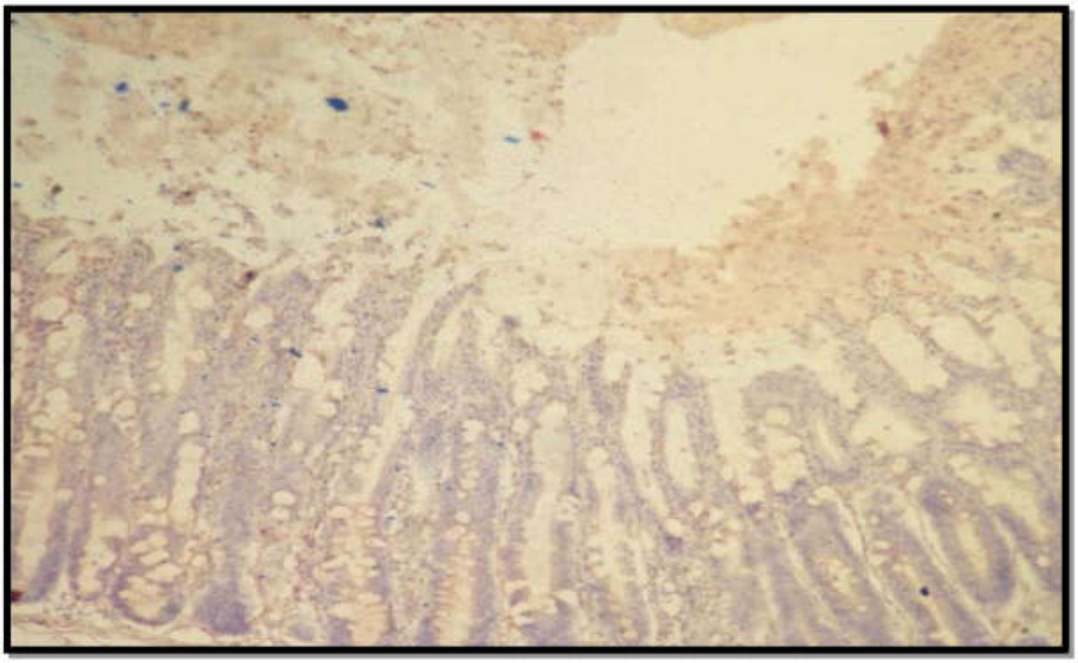

Figure (13) section of colon at 3rd day post infection shows sloughing of the superficial epithelial cells where the sloughed individual epithelial cells appear to express Claudine in the cytoplasmic portion (CL) only as it stained by DAB stain 500X

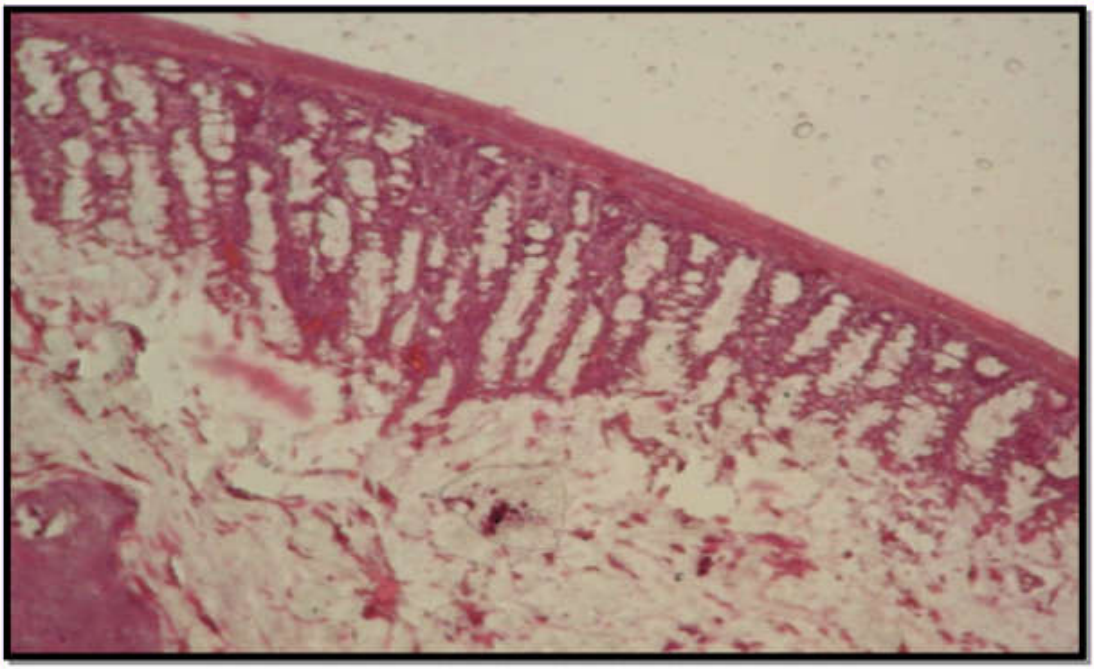

Figure (14) duodenum of infected animals after 7 and 14 days of infection shows massive mucinous degeneration of intestinal glands with massive mucin production **M** Excessive glandular destruction **ED**. H&E A) 125X B) 500X

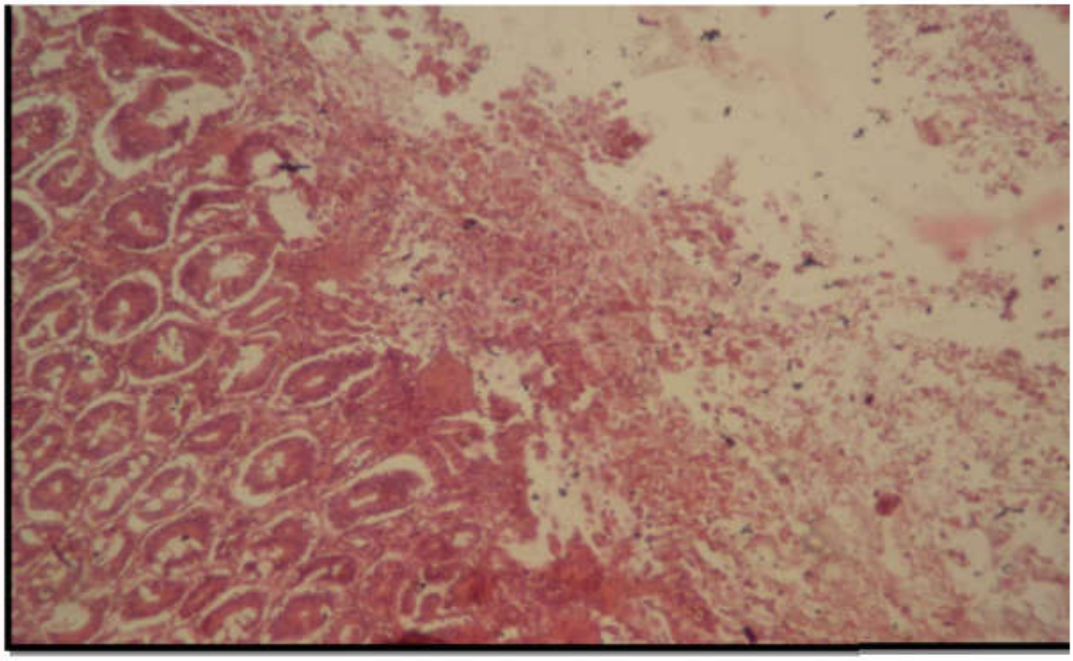

Figure (15) colon of 7 days infected animals in which there were marked sloughing of superficial epithelium (ES) admixed with acute inflammatory cells (N) H&E A)125X B) 500X

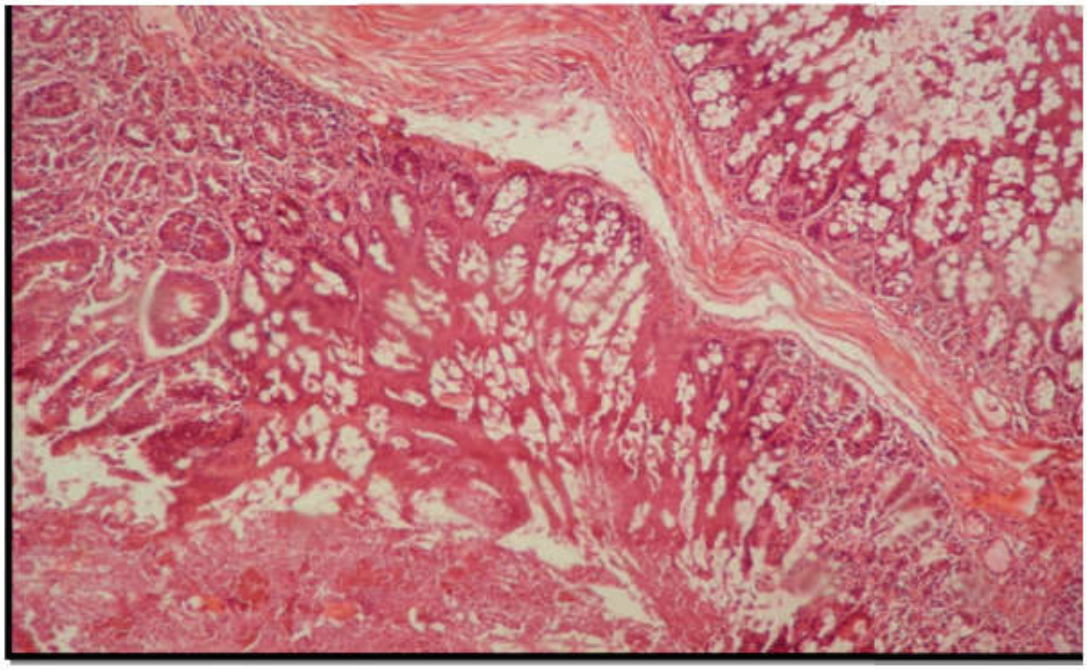

Figure (16) colon of 14 days infected animals in which there were multiple site of mucosal epithelial necrosis (EN), infliltration of inflammatory cells in the mucosa (IN) and massive mucin production (M) H&E 125X

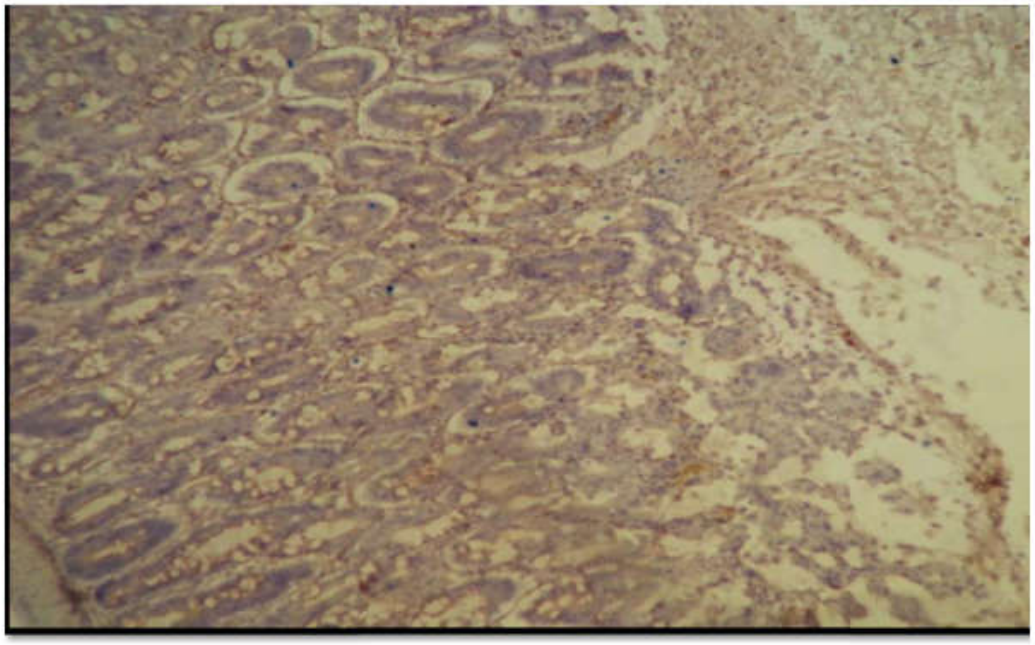

Figure (17) a section of colon at 7th day post infection shows marked sloughing of the superficial epithelial cells as it appears as clusters of sloughed cells due to marked destruction of interepithelial tight junction (Anti Claudine DAB stain) 500X

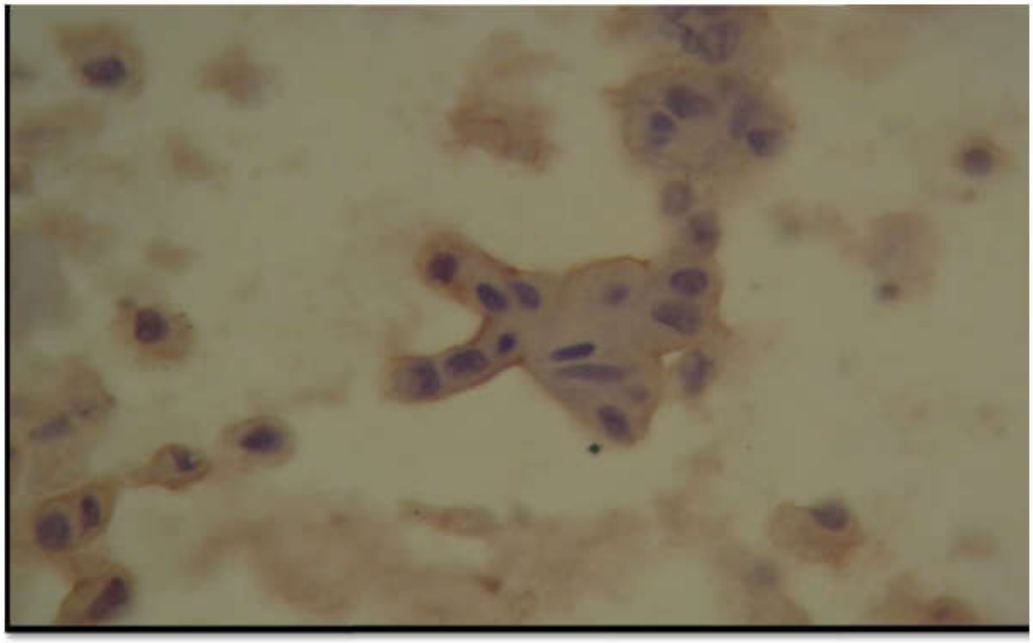

Figure (18) Anti Claudine immunohistochemistry (DAB) stain for a section of colon at 14th day post infection shows that the destruction of epithelial tight junction is not only involve the superficial epithelium but even involve the epithelium of the colonic glands 1250X

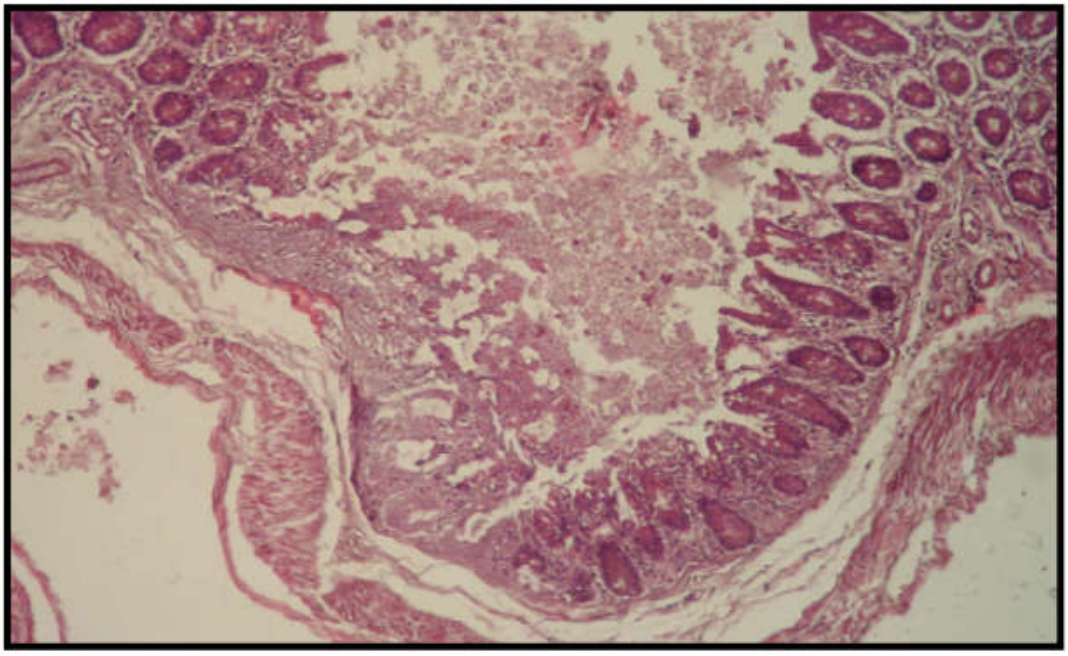

Figure (19) colon of 21 days infected animals in which there were an area of complete mucosal sloughing (MS), with fibrosis (F) and glandular atrophy (GA) indicating Ulcerative colitis H&E 125X

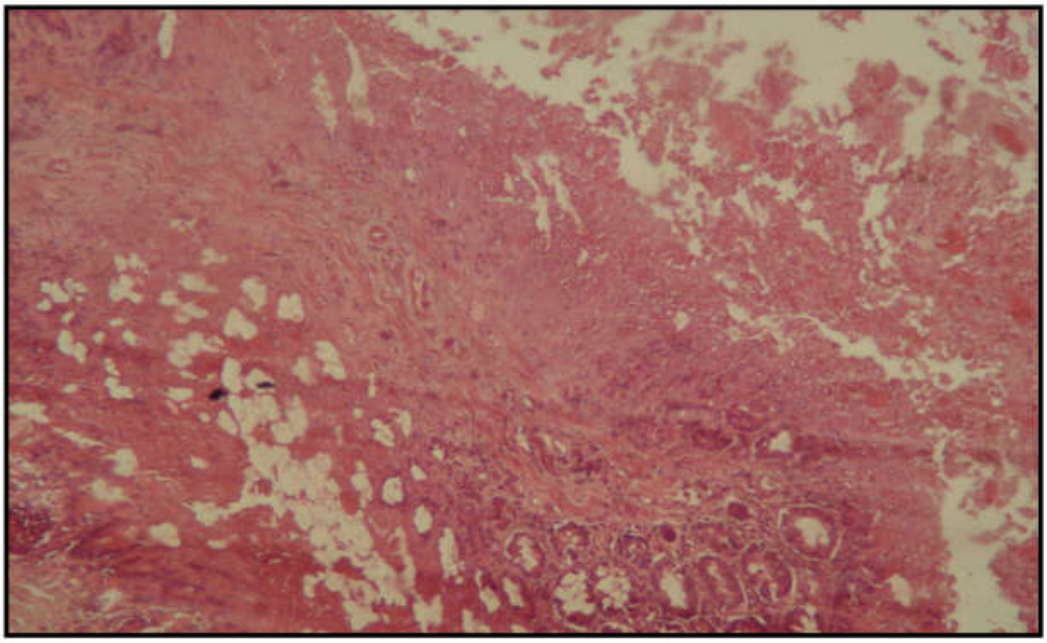

Figure (20) colon of 28 days infected animals in which there were an area of complete mucosal sloughing (MS), with complete fibrosis of ulcerated area (F) and glandular atrophy (GA) indicating Ulcerative colitis H&E 125X

DISCUSSION

Intestine represents the first site of the bacterial (O111 STEC) colonization since the oral administration of the pathogen was the rout that used in this study, because it represent the most common rout of infection with STEC, as well as in order to study the net effect of the O111 STEC on the mice and prevent the colonization inhibition by the other intestinal micro-flora, animals of the study were given streptomycine this agreed with [10][11] who reported that there are several mechanisms explaining enhanced colonization of non indigenous bacteria in streptomycin-treated animals include: first decreased competition between resident and nonindigenous bacteria for nutrients and attachment sites, second environmental changes in the gut favoring colonization and growth of pathogens such increase in pH and decrease in short chain volatile fatty acids.

One limitation in the use of streptomycin in *in vivo* models of infection is that the pathogen being studied should be resistant to the antibiotics. In order to allow excretion of residual streptomycin, mice were allowed to recover for another 24 hours after antibiotic treatment before infecting with EHEC this agreed with [10]

The gross pathological changes in the intestine were the yellowish watery content of the intestine which was appear in the third day post administration this

indicated that the diarrheal state occur after bacterial colonization in the intestine this result was agreed with [12] and it was attributed to the virulence factors which carried by O111 STEC particularly intimin which is produced by LEE locus of enterocyte effacement which is the pathogenicity island [13]. The LEE encodes type III secretion apparatus, secreted proteins, chaperones, and an outer membrane protein called intimin [13]. One of the secreted proteins forms hollow filaments through which EPEC delivers effector molecules directly into host cells [14][15]. One effector, Tir (translocated intimin receptor), is inserted into the host plasma membrane, where it serves as a receptor for the outer bacterial protein intimin [14][16].

[17] reported that EPEC infection triggers physiological changes in the intestinal epithelium, including altered ion transport, increased paracellular permeability, and initiation of inflammatory responses [18][19].

This study proved that the colonization of the STEC O111 had been involve multiple areas of small and large intestine including the cecum this result was agreed with [20] who reported that bacteria enter the gastrointestinal tract and then transit to the primary site of colonization (the cecum in mice) where they then elaborate Stx.

Stx production may occur during transition of the organism through the intestines. Stx was found in the cecum and at other sites where STEC were abundant early in infection. Furthermore Stx will be more abundant in the cecal and large intestinal luminal contents than in the corresponding tissue; these observations may indicate that toxin is absorbed into the circulation from both the cecum and the large intestine. At least a portion of the toxin produced within the gastrointestinal tract enters the bloodstream because (1) marked epithelial damage was evident in infected mice, and (2) parenteral inoculation of antitoxin antibody protected mice from the systemic manifestations of disease [20].

Multiple sites of mucosal epithelial erosions, sloughing was appear in the small intestine in addition to necrosis and ulcerations was appear in the colon including the cecum which appear to be related to elaboration of the bacterial toxins these results were agreed with [21][22]. [21][22][23] who reported that the Stx family of AB5 toxins (composed of a single A or active subunit noncovalently associated with a pentameric ring of B or binding subunits) contains two subgroups, Stx1 and Stx2, B subunit of Stxs forms a homopentameric ring structure as it binds the cellular toxin receptor, globotriaosylceramide (also known as Gb3 or CD77).

The A1 fragment of the A subunit is translocated into the host cell cytoplasm, where it acts as an N-glycosidase to remove a single adenosine residue from the 28S ribosomal RNA of the 60S ribosome. Alteration of the 28S rRNA prevents binding of elongation factor to the ribosome so as to inhibit protein synthesis. Stx-mediated inhibition of cellular protein synthesis generally results in death of the intoxicated cell.

Therefore the presence of the Gb3 is quite important for the bacterial Stx to exert its influence this interpret the isolation of the O111 STEC from fecal material of apparently healthy adult cows where they were act as a reservoir for the STEC because they lack Gb3 while STEC cause diarrhea in calves because their intestinal epithelial cells express Gb3 on their surfaces this is reported by [20].

In mice, [24] used Gb3 knockout mice to demonstrate that the effects of Stx injection are indeed dependent on the expression of Gb3 [24]. The concept that Gb3 may be differentially distributed in murine tissues in comparison to human tissue was supported by the work of [25]., who reported that the Gb3 synthase gene is expressed in the kidney, lungs, brain, gastrointestinal tract, and spleen [25]. They and others have shown that the actual glycolipid Gb3 is present in the kidney (tubules but not glomeruli, despite reports of toxin- associated glomerular damage, as well as the lung, brain, and spleen [25] [26]

Epithelial sloughing which was appear in multiple site of small and large intestine which is finally result in areas of ulceration particularly in the colon including cecum result from destruction of intestinal epithelial tight junction proteins particularly the Claudin this results was in line with [10][27].

[10] reported that the immune-staining of the intestinal and colonic Claudine revealed that there were no alteration in Claudine of intestinal or colonic tight junction in the mice experimentally infected with STEC in the 1st day post infection but the main alteration and redistribution of the Claudine start in the 3rd day post infection then continue diminished in the intercellular area but later on its expression was increased intracellularly but the extracellular portion remain diminished till the end of experiment.

Results of the present study was also in line with [28] who reported that the immune- fluorescent staining of the T84 cell culture for the tight junction proteins reveal reorganization of the Claudine was observed as a complete fragmentation of the cell boundaries and focus formation also they reported that claudin-1 was lost from cell-cell contact sites, this change could be observed as the absence of the brightly stained cell boundaries, indicating a loss and/or redistribution of claudin-1 [28].

[29] reported that TJ protein alterations caused by STb may be due to actin rearrangement, as a loss of organization in the perijunctional F-actin ring has been identified to be a critical event in the mechanism controlling transepithelial electrical transport (TER) and paracellular permeability [29][30][31] decreasing (TER) and increase permeability will be the cause of diarrhea and in the same time for systemic distribution of the bacterial toxin with blood circulation reaching other organs in the body.

Inflammation which was found in different areas of the small and large intestine was in response to O111 STEC infection and to the destruction of the mucosal epithelial cells which result from infection with elaboration of proinflammatory cytokines that trigger acute inflammation this is in agreement with [10][32][33] as well as the increase in the number of goblet cells and crypt abscess occur in response to inflammation and infection with STEC leading some to propose that this adaptive response allows goblet cell-secreted mucin to form a viscous gel that traps microorganisms and irritants and limits their access to the epithelium [34]. In chemically induced intestinal inflammation, the expression and secretion of mucin increased with disease progression and differed in the proximal colon and the distal colon [35] all these changes were consistent to what occur in the human infection with EPEC and agreed with [19][36].

التغيرات المرضية المناعية لأمعاء الفئران المصابة بجراثيم الاشريكيا القولونية المنتجة للذيفان

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كلية الطب البيطري ،جامعة بغداد ، بغداد ،العراق.

الخلاصة

لغرض دراسة امراضية حالات الاسهال المرضية المتسببة بواسطة جراثيم الاشريكيا القولونية المنتجة للذيفان، جرعت فئران مختبرية ذات فئات عمرية تتراوح بين 8- 10 اسابيع بالاشريكيا القولونية ذات النمط المصلي O111 بالجرعة المصيبة غير القاتلة والتي تم تحديدها مسبقا حيث كانت ($3,16X10^8$ CFU).
بعدها تم فحص الفئران من ناحية العلامات السريرية الناتجة من حقن الجرثومة وكذلك تمت دراسة التغيرات

المرضية النسجية بالاضافة الى الكيمياء النسجية المناعيةالدراسة المناعية النسيجية الكيمائية لغرض ملاحظة درجة الضرر الحاصل في الروابط بين الخلايا الطلائية. اظهرت النتائج ان هناك ضرراً وضحاً في الرباط الوثيق بين الخلايا الطلائية والذي بدوره ادى الى انسلاخ الخلايا الطلائية في كل من الامعاء الدقيقة و القولون مع التاب التهاب واضح وتنكس مخاطي ونزف. بدأت التغيرات المذكورة انفا منذ اليوم الثالث بعد تجريع البكتيريا واستمرت لغاية اليوم الرابع عشر، بعدها تطورت التغيرات الى ضهور قرح متعددة شملت مناطقمتفرقة من الامعاء الدقيقة والغليضة. فياليوم الثامن والعشرين والي يمثل نهاية التجربة كانت التغيرات متمثلةً بتليف في مناطق التقرحات. نستنتج مما ذكر اعلاه ان تجريع الفئران المختبرية بالاشريكيا القولونية النمط المصلي O111 يؤدي الى تغيرات تراجعية تشمل كلاً من الامعاء الدقيقة والغليظة.

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