Electron microscopic study of ileum of mice infected experimentally with *Salmonella hadar*

A. A. Yousif[1] and M. M. N. AL-Naqeeb[2]

[1]College of Veterinary Medicine, University of Baghdad, [2]College Of Veterinary Medicine, University of Kufa, Iraq

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Abstract

Recently, *Salmonella hadar* has been isolated and identified from goat in Iraq. The purpose of the present study was to examine ultrastructural changes in the ileum epithelial cells of BALB/c mice experimentally infected with *S. hadar*. Mice were used as follows: Group A: 20 mice inoculated orally with phosphate buffer saline and considered as a control group. Group B: 20 mice inoculated orally with (100ID) by drenching the mice about 1 ml of the bacterial suspension which contain (1.5×10^9 cells) of *Salmonella hadar* and the ileum epithelial cells were examined by transmission electron microscopy at 24, 48, 72, 96 and 120 hours after infection. The ultra structural changes seen in the ileum of infected mouse at 24 hours were disorganization of the microvilli with severe cytoplasmic vacuolization, enlargement of the mitochondria and presence of intracellular *Salmonella*. Changes at 48 hours post infection, were detachments of many microvilli especially at the site of bacterial entry. Similar changes were observed after 72 hours but more severe; there was marked dilatation and proliferation of the endoplasmic reticulum with cytoplasmic vacuolization of the infected enterocytes. After 96 hours there were severe cytoplasmic vacuolization with accumulation of the bacteria within phagosomes and there was marked damage to the microvilli of the ileum. After 120 hours there was hypertrophy of goblet cell and thickening of the nuclear membrane and there was several *Salmonella* containing vacuoles.

Keywords: Salmonellosis; Electron microscopy; Ileum; Mice.
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دراسة بالمجهر الالكتروني عن اللفائفي في الفئران المصابة تجريبيا بسالمونيللا هدار

عفاف عبدالرحمن يوسف[١] و معن محسن نعمة صالح النقيب[٢]

[١] كلية الطب البيطري، جامعة بغداد، [٢] كلية الطب البيطري، جامعة الكوفة، العراق

الخلاصة

تم عزل وتوصيف *Salmonella hadar* من الماعز في العراق. كان هدف الدراسة معرفة التغيرات في البنيان الفوقي لخلايا ظهارة اللفائفي في الفئران البيض المصابة تجريبيا ببكتريا *S. hadar*. استخدمت الفئران كالاتي:٤٨ فأرة لحساب الجرعة المصيبة و٤٠ فارة استخدمت في الاصابة بالبكتريا قسمت المجموعة الاخيرة الى مجموعتين أ (٢٠ فارة) مجموعة السيطرة ومجموعة ب تم تجريعها فمويا بجرعة عالية من البكتريا 100× الجرعة المصيبة 1.5×10^7 خلية وفحص اللفائفي بالمكرسكوب الالكتروني النافذ بعد ٢٤و ٤٨و٧٢و٩٦و١٢٠ ساعة من التجريع. ظهرت تغيرات البنيان الفوقي لظهارة اللفائفي في الفئران المصابة بعد 24 ساعة بعدم ترتيب الزغيبات مع وجود فجوات سايتوبلازمية وتضخم في المايتوكوندريا ووجود عدد من السالمونيلا داخل الخلايا. كانت التغيرات بعد ٤٨ ساعة بانسلاخ الزغيبات وخاصة في مواقع دخول البكتريا وبعد ٧٢ ساعة لوحظت نفس التغيرات ولكنها كانت اشد. ولوحظ تمدد واضح والشبكة الهيولية الباطنة بفجوات السايتوبلازم في الخلايا المعوية وبعد ٩٦ ساعة بعد الاصابة لوحظ تفجي شديد في فجوات السايتوبلازمية مع تجمع عدد من البكتريا في الجسيمات المبلعمة مع تحطيم كامل لزغيبات اللفائفي. وبعد ١٢٠ ساعة لوحظت ضخامة لخلايا الكاسية مع تثخن في الغشاء النووي مع وجود العديد من السالمونيلا حاوية على الفجوات.

Introduction

Globally, salmonellosis has remained one of the three most common meat associated diseases in human (1) and the disease caused by *Salmonellae* organisms is the most common and important zoonotic diseases (2).

Salmonella hadar is now one of the five most frequently isolated serotypes in human and animals (3-5) it had been isolated firstly in the early 1950_s, from a stool sample of a subject with gastro-enteritis and fever (6).

Salmonella enterica serovars cause a variety of diseases ranging from self-limiting gastroenteritis to severe systemic infections. Virulence of these facultative intracellular pathogens is dependent on their ability to invade and replicate within non-phagocytic cells (7,8).

Considerable attention has been given to the role of bacterial adherence in colonization of mucous membranes and in the pathogenesis of many infections. Both scanning and transmission electron microscopies (SEM and TEM) have been used widely in the study of intestinal colonization by *Salmonellae* (9,10).

Mikula *et al*. (11) found that post oral infection of calves with *S. typhimurium* 4/5 strain at a dose (1×10^6 C.F.U./ml), appear in discontinuous and irregular of the brush border membrane of jejunal enterocyte.

Yass (12) study the pathogenesis of *S. typhimurium* infection in calves by using transmission electron microscopy, he found that the early ultra structural changes observed in microvilli of small intestine were characterized by local derangement with slight swelling of the proximal end in addition to the presence of many bacteria either associated or adherent to the microvilli, and this Intracellular *Salmonella* was usually intact and enclosed by a membrane. Complete disappearance of the microvilli was observed after 5 days.

Materials and methods

Bacterial strain

Salmonella hadar was isolated from Iraqi goats, diagnosed and confirmed according to (Standard Operating Procedure "SOP" (13) and serotyped in the national salmonella center in Iraq.

Estimating the infectious dose (ID)

Five colonies of *S. hadar* inoculated in 10 ml of brain heart infusion broth at 37 ˚C for 18 hours, then centrifuged in cold centrifuge at 8000 rpm (round per minute) for 15 minutes then the sediment washed three times with phosphate buffer solution (PBS) (pH=7.2) and re-suspended with 1 ml of PBS, then tenfold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}) were done. The viable count of the bacteria in each diluent was made according to method of Miles and Misra (14) and selected the diluents which had these concentrations for drenching the mice: (1.5×10^5 cells), (1.5×10^6 cells), (1.5×10^7 cells), (1.5×10^8 cells), (1.5×10^9 cells), (1.5×10^{10} cells) and (1.5×10^{11} cells). Forty eight mice were divided into eight groups, (each group contain 6 mice) seven groups of mice drenched orally with one of the calculated diluents (C.F.U./ml) above diluted with 1 ml PBS while the last group (6 mice) served as control and drenched with 1 ml PBS (pH=7.2). All groups were observed for 30 days to calculate the live and dead mice, the infective dose (ID) was estimated by choosing the group of mice which showed clinical signs resemble to those in *Salmonellosis* with no mortality after ensure that by culture. This dose was calculated according to (15,16).

Laboratory animals

Total numbers (40) mice (BALB/c) of both genders with age range (6 – 8) weeks old which adapted for two weeks before start of the experiment, all animals had negative fecal bacteriological culture for salmonella, then divided into two groups: Group (A): 20 mice inoculated orally with phosphate buffer saline and considered as a control group. Group (B): 20 mice inoculated orally with (100ID) by drenching the mice about 1 ml of the bacterial suspension which contain (1.5×10^9 cells) of *Salmonella hadar* (15).

Solutions

Phosphate buffer saline (PBS, pH=7.2): was prepared according to (17). 2.5 % Glutaraldehyde: was prepared by adding 2.5 ml of absolute formalin to 97.5 ml of PBS (pH=7.2) and used for electron microscope specimens.

Transmission electron microscope (TEM)

At the specified time of each experiment, the mouse was anesthetized by using the diethyl ether by inhalation. The abdomen was quickly opened, and then the ileum was removed as quickly as possible and drained then according to (18) the ileal tissue pieces (size approximately 1-2 mm^3) of the experimental and control mice were fixed in 3% glutaraldehyde in phosphate buffer saline (pH= 7.2) at 5 ˚C for 6 hours and subsequently post-fixed/block stained in aqueous 1% osmium tetroxide (OsO_4) for 6 hours at room temperature. The fixed tissues were embedded in epoxy resin and ultra-thin sections (approximately <0.1 μm in thickness) were cut using glass knife with an ultramicrotome. Ultra-thin sections obtained on 3 mm diameter copper grids, were stained with uranyl acetate and lead citrate stains for contrast and examined under Philips (CM-10) transmission electron microscope in (College of Medicine, Al-Nahern University).

Results

The ultrastructural examination of the brush border of the absorptive columnar epithelial cells that cover the villi of the control mice (group A) consisted of long, closely packed regular microvilli. The cytoplasmic region appeared normal with no ultrastructural changes (Fig. 1).

The ultrastructural changes which obeserved in the ileum of infected mice in group (B) at 24 hours post infection were disorganized with sever disruption of the M cell and disproportional of the microvilli with severe cytoplasmic vacuolization, dilation of endoplasmic reticulum, enlargement of the mitochondria and presence of intracellular *Salmonella* (Figs. 2 and 3).

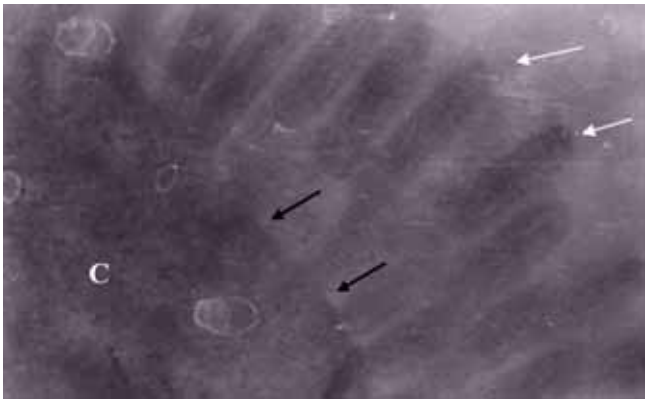

Fig. 1: Transmission electron micrograph. Ileum, mouse of control group (A), shows long, closely packed regular microvilli (white arrow), normal brush border (black arrow) with no changed cytoplasm of the enterocyte (C). (Uranyl acetate & Lead citrate) X 64000.

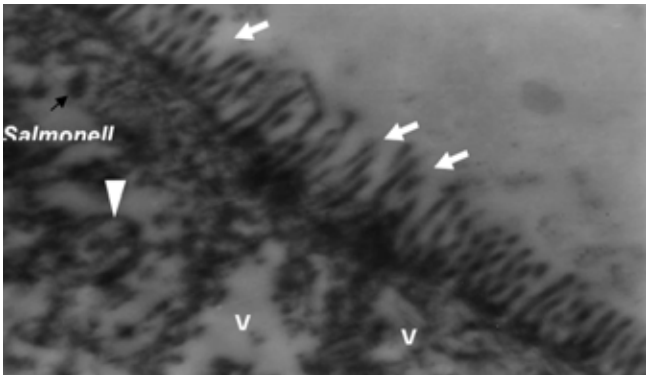

Fig. 2: TEM. Ileum, mouse (group B) at 24 hours post infection with *S. hadar*, shows detachment and disruption of microvilli (white arrow), severe cytoplasmic vacuolization (V) and enlargement of the mitochondria (white head arrow). (Uranyl acetate & Lead citrate) X 16000.

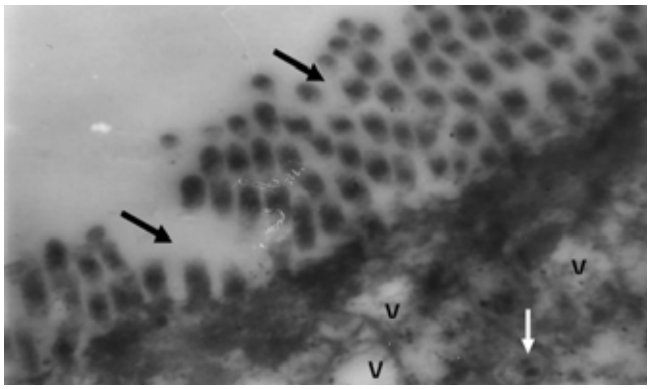

Fig. 3: TEM. Ileum, mouse (group B) at 24 hours post infection with *S. hadar*, shows disorganization and severe disruption of the M-cell (black arrow), also there is cytoplasmic vacuolization (V) and presence of *Salmonella* containing vacuole that contain two *Salmonella* (white arrow). (Uranyl acetate & Lead citrate) X 41000.

The ultrastructural changes at 48 hours post infection were detachments of many microvilli especially at the site of microorganism entry with vacuolar changes in the cytoplasm of the enterocytes (Fig. 4).

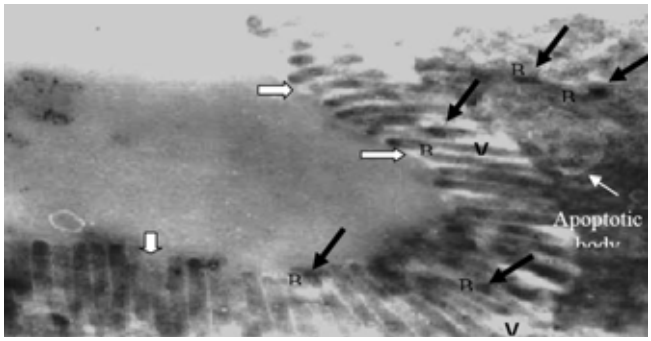

Fig. 4: TEM. Ileum, mouse (group B) at 48 hours post infection with *S. hadar*, shows bacterial attachment and invasion lead to loss of microvilli (white arrow) and vacuolation spaces (V), many intracellular invading bacteria (B) located in the lamina properia (black arrow), cell with apoptotic feature appear in field containing apoptotic bodies cell. (Uranyl acetate & Lead citrate) ((X 24500).

The ultrastructural changes in the ileum of group (B) mice that killed after 72 hours post infection were the same with that observed previously but was more severe, there was marked dilatation and proliferation of the endoplasmic reticulum with cytoplasmic vacuolization of the infected enterocytes; in other sections the endoplasmic reticulum

appeared to have dilated phagocytic vacuoles with lysosomes in addition to presence of many ribosomes detached and dispersed singly in the lamina properia and *S. hadar* is present in both an enterocytes and M cells (Figs. 5, 6 and 7).

At 96 hours post infection, there were severe cytoplasmic vacuolization with accumulation of the bacteria within phagosomes and there was marked damage to the microvilli of the ileum of group (B) mouse at the same time (Fig. 8).

Finally the ultrastructural changes in the ileum of group (B) mouse that killed after 120 hours post infection revealed hypertrophy of goblet cells, dilatation of endoplasmic reticulum, severe cytoplasmic vacuolization, thickening of the nuclear membrane and there were several *Salmonella* containing vacuoles (SCV) (Figs. 9, 10 and 11).

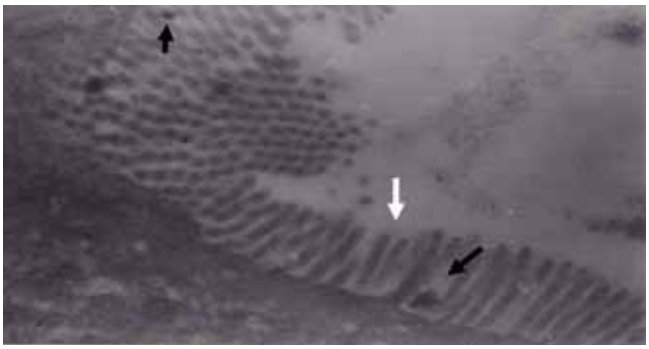

Fig. 5: TEM. Ileum, mouse (group B) at 72 hours post infection with *S. hadar*, shows an invading *Salmonella hadar* located in both an enterocytes and M-cells (black arrow) with detachment of the some microvilli (white arrow). (Uranyl acetate & Lead citrate) X 16000.

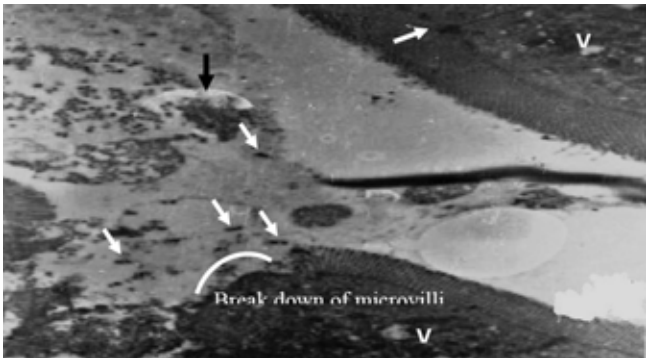

Fig. 6: TEM. Ileum, mouse (group B) at 72 hours post infection with *S. hadar*, shows breakdown of microvilli (white line), invading bacteria (white arrow), cytoplasmic vacuolization of enterocytes (V), and several phagosomes (black arrow) in addition to cellular debris. (Uranyl acetate & Lead citrate) X 3400.

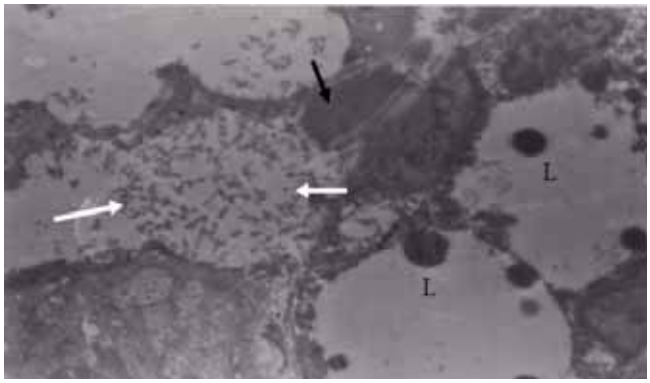

Fig. 7: TEM. Ileum, mouse (group B) at 72 hours post infection with *S. hadar*, shows dilation of the endoplasmic reticulum (black arrow), presence of phagocytic vacuole that co ain many ribosomes (white arrow) in addition to presence of many lysosomes (L) which detached and dispersed singly in the lamina properia. (Uranyl acetate & Lead citrate) X 3400.

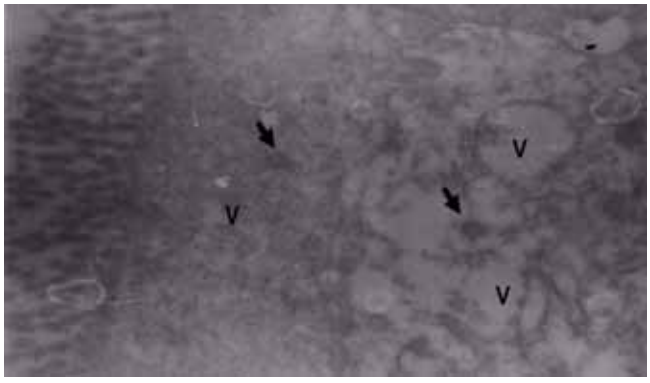

Fig. 8: TEM. Ileum, mouse (group B) at 96 hours post infection with *S. hadar*, shows severe cytoplasmic vacuolization (V) with accumulation of the bacteria within phagosome (black arrow). (Uranyl acetate & Lead citrate) X 16000.

Discussion

The early ultrastructural changes observed in the microvilli by transmission electron microscope in the mice of group A which infected with *S. hadar* were characterized by local derangement with little swelling on the proximal end. One of the interesting findings is the ballooning of the microvilli and the appearance of this ultrastructural change may be due to the attachment of *S. hadar* to their membrane. While, when the infection was became more advanced, the microvilli became short, more effacement from epithelial cells in some location; these observations in the microvilli were similar to that mentions by (19) who

studied the ultrastructural changes of ileum microvilli of rabbit infected with *S. typhimurium*, and also was similar to that recorded by (12).

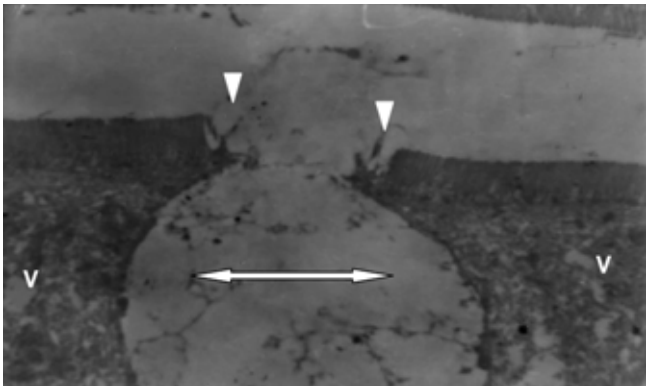

Fig. 9: TEM. Ileum, mouse (group B) at 120 hours post infection with *S. hadar*, shows severe hypertrophy of goblet cell (double head arrow), severe cytoplasmic vacuolization (V) and detachment of some microvilli (white head arrow). (Uranyl acetate & Lead citrate) X 4400.

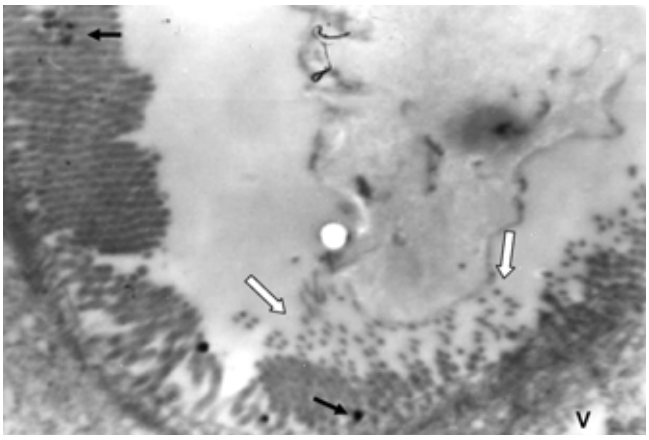

Fig. 10: TEM. Ileum, mouse (group B) at 120 hours post infection with *S. hadar*, shows brush border disruption at site of invasion; microvillus enlongation, invading bacteria (black arrow), bacterial attachment and invasion led to disruption of M-cells (white arrow) and vacuolation spaces (V). (Uranyl acetate & Lead citrate) X 12500.

The effacement of the brush border of the enterocytes of the microvilli by *S. hadar* resulted in the loss of brush border enzymes (amino oligo peptidase, aspartate amino peptidase, dipeptidyl amino peptidase, carboxy peptidase and γ glutamyl transferase which hydrolyze small peptides that are formed in the intestinal lumen by the action of proteases to free amino acids during the process of absorption), carrier protein and surface area as mentions by (20).

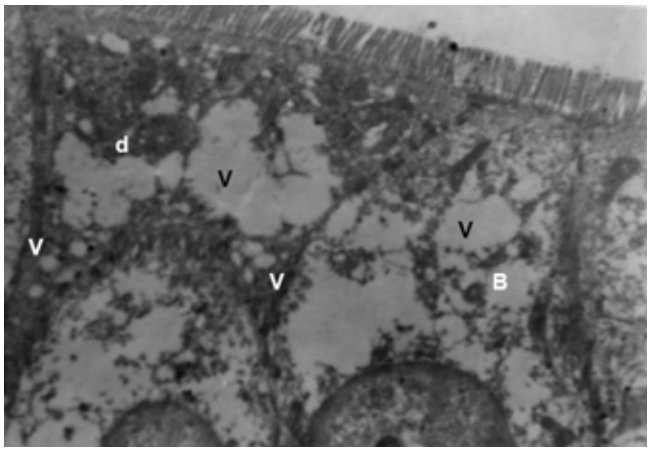

Fig. 11: TEM. Ileum, mouse (group B) at 120 hours post infection with *S. hadar*, shows highly dilatation of endoplasmic reticulum (d), vacuolization of the cytoplasm (**V**), and there is some invading bacteria (B). (Uranyl acetate & Lead citrate) X 4400.

The shortening and loss of microvilli is in accordance with decreased alkaline phosphatase activity, this enzyme is located in the plasma membrane of the microvilli and is considered as a measurement of the digestive-absorptive surface (21).

The fragmentation and vesiculation of microvilli may be due to increases in cytoplasmic Ca^{2+} concentration which lead to actin filaments of the microvillus core cytoskeleton to be broken into short filament as mentioned by (22). The ultrastructural changes was observed in the epithelial cells were probably due to the presence of many intracellular *Salmonella* that produced cytotoxin inside the cells; these observations is similar to that observed in ileum of the infected calves with *S. typhimurium* (12) and also with that found in the ileum of swine which infected with *S. typhimurium* DT104 (23). The loss of microvilli reduced the intestinal absorption surface, this together with the functional deficiency and destruction of many epithelial cells, may be resulted in decrease absorption and caused gastroenteritis and diarrhea. The ultrastructural changes observed in the cytoplasm of the enterocytes which manifested by vacuolization of cytoplasm, displacement of organelles and swollen mitochondria, these changes in the host cells were occurred as a result of the effect of *S. hadar* endotoxin that is part of the outer membrane or metabolic substances released from the bacteria as mentioned by (24).

Finally this study concluded that *S.hadar* has high ability to invade and established colonization in the intestine of mice, it is an invasive strain.

References

1. Cooper GL. Salmonellosis infection in man and the chicken: Pathogenesis and the development of live vaccines- a review. Vet Bull. 1994;64:123–143.
2. OIE. Manual of diagnostic tests and vaccines for Terrestrial Manual: Salmonellosis. OIE, France 2008.1267-1283.
3. Valdezate S, Echeita A, Diez R, Usera MA. Evaluation of phenotypic and genotypic markers for characterization of the merging gasteroenteritis pathogen *Salmonella hadar*. Eur. J Clinic Microbial Infect Dis.2000;9:275–281.
4. Cailhol J, Lailler R, Bouvet P, Lavieille S. Gauchard F, Sanders P, Brisabois A. Trend in antimicrobial resistance phenotypes in non-typhoid *Salmonella* from human and poultry origins in France. Epidemiol Infect.2006;134(1):171-178.
5. Yousif AA, Alshemmari IGM, Mahdi MS. Epidemiological study on *Salmonella spp* isolated from goat in some provinces in middle of IRAQ. (Unpublished manuscript submitted).
6. Hirsch W, Gerichter CB, Bregman E, Lubling P, Altman G. A new *Salmonella* type (*S. hadar*). Acta Med Orientalia.1954;13:41.
7. DeLeo F, Otto M. Bacterial Pathogenesis. Methods and Protocols, in Methods in Molecular Biology, Humana Press, a part of Springer Science+Business Media, LLC.2008.
8. Ohl ME, Miller SI.. *Salmonella*: A model for bacterial pathogenesis. Annu Rev Med.2001;52:259–274.
9. Santos RL, Tsolis RM, Baumler AJ, Adams LG. Pathogenesis of *Salmonella*-induced enteritis. Brazilian J Med Biological Res.2003; 36:3-12.
10. YashRoy RC. Mechanism of infection of a human isolate *Salmonella* (3,10:r:-) in chicken ileum: Ultrastructural study". Indian J Med Res. 2007;126:558-566
11. Mikula E, Pilipeinec I, Timkovicova M. Electro-microscopic studies of the intestinal tract in calves after experimental *Salmonella* infection. Folia Vet.1988;32:70-97.
12. Yass AA. Experimental study on the pathogenesis of *Salmonella typhimurium* infection in calves. [dissertation]. College Vet Med. Univ Baghdad. Iraq.1990.
13. Anonymous. Standard Operating Procedure "SOP" Bacteriological Inter-laboratory Comparison Study on the detection of *Salmonella* in food organised by crl-*salmonella*. food study ii, crl-*Salmonella*, Bilthoven, Netherlands, 2007.
14. Miles AA, Misra SS. The estimation of the bactericidal power of blood. J Hyg.1938;38:732-749.
15. AL-Naqeeb MMN. Study the pathogenesis of *Salmonella hadar* which isolated from goats in mice. [master's thesis]. College Vet Med. Univ Baghdad. Iraq.2009.
16. Blaser MJ, Newman LS. A review of human salmonellosis: I. Infective dose. Reviews of Infectious Diseases.1982;4:1096–1106.
17. Luna HT, Lee G. Manual of histological staining method of the Armed Forces Institute of Pathology. 3rd ed. The Blakiston Division. McGraw–Hill Book Co. New York. USA.1968.
18. Sabatini DD, Bensch K, Barrnett R. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol.1963;17:19-59.
19. Worton KJ, Candy DC, Wallis TS, Clarke GJ, Osborne MP, Haddon SJ Stephen J. Studies on early association of *Salmonella typhimurium* with intestinal mucosa *in vivo* and *in vitro*: relationship to virulence. J Med Microbiol.1989;29:283-294.
20. Tobey N, William H, Roger Y, Tai-In H, Cindy H. Human intestinal brush border peptidases. Gastroenterology.1985;88:913-926
21. Cousemeni W, Ducatelle R, Debouck P, Hoorens J. Pathology of experimental CV777 corona virus enteritis in piglet, I–Histopathological and Histochemical study. Vet Path.1982;19:46-56.
22. Embage H, Batt R, Saunders J, Getty S, Hart C. Interaction of enteropathogeni *E. coli* O111 with rabbit intestinal mucosa *in vitro*. Gastroenterology.1989;96:1079-1086.
23. Meyerholz DK, Stebel TJ, Ackermann, MR, Carlson SA, Jones BD Pohlenz J. Early Epithelial Invasion by *Salmonella enterica* Serovar Typhimurium DT104 in the Swine Ileum. Vet Pathol.2002;39:712-720.
24. Yamamoto I. Ultrastructural changes in mesenteric lymph nodes of mice infected with *Salmonella enteritidis*. J Infect Dis.1966;116:8-20.