Egyptian Journal of Veterinary Sciences

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Pathogenicity and Reservoir Potential of *Salmonella enterica serovar Rissen* in Murine Models: Implications for Environmental Dissemination

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Abstract

THIS RESEARCH was conducted to study the pathogenicity of *Salmonella enterica serovar Rissen* in mice and its potential role in rodents as reservoirs for its environmental dissemination. A total of 18 mice divided into infected group which were group A and B which consisted of six mice each. Necropsy was conducted on day 2 and day 5 post-Infection (PI). The control group consisted of three mice day 2, and three mice day 5 for necropsy. The control group was given 0.1ml of normal saline. While the infected group was given 0.1ml of 4.9 x 109 CFU *Salmonella enterica serovar Rissen* orally. The study showed that infection of S. enterica serovar Rissen in mice caused enteric lesions and systemic infection. *Salmonella enterica serovar Rissen* did not cause diarrhea and mortality but depression during the period of the experiment. All infected mice showed reddened serosal layer of the small intestine especially in the jejunum and ileum. Histopathology analysis of these organs revealed hemorrhagic enteritis, hemorrhagic cecities, cecities, colitis, interstitial nephritis, hemorrhagic hepatitis and splenitis. *Salmonella enterica serovar Rissen* was isolated from the kidney and spleen 2 days PI, and from the urine and kidney of the mice 5 days PI through PCR confirmation. The clinical signs and histopathological findings highlight the organism's impact on multiple organs, particularly the intestines, liver, and kidneys. The successful isolation of the pathogen from various tissues supports the hypothesis that rodents, including mice, may act as reservoirs for this serovar, posing risks for environmental dissemination.

Keywords: *Salmonella enterica serovar Rissen*, pathogenicity, dissemination, experimental infection.

Introduction

Salmonellosis is a major foodborne disease causing significant economic losses and public health concerns globally [1]. Most human infections are caused by Salmonella enterica serovar Typhimurium and Enteritidis, with other serovars more prevalent in Southeast Asia, Latin America, and Europe [2,3]. Changes in serovar prevalence are due to international travel, migration, and global food and livestock trade [4]. Knowledge of prevalence and molecular epidemiology of specific serovars in a specific region had facilitated recognition and emerging pathogens [5,6]. Recently, *Salmonella enterica serovar Rissen* has been recognized as common serovars among human and pig production in different parts of the world, especially in Asian countries [5,7]. Recently, *Salmonella enterica serovar Rissen* had caused outbreaks in humans associated with white pepper consumption [8]. Among patients diagnosed with *Salmonella enterica serovar Rissen* infection, they develop diarrhoea [9], enteritis, gastroenteritis, some urinary tract infections, and other infections, including peritonitis and pneumonia [8,10]. Asymptomatic carrier pigs and rodents play an important role in excreting *Salmonella* to the environment and increasing contamination in the production chain [11,12]. The potential reservoir for spreading *Salmonella enterica serovar Rissen* is unknown, although frequently isolated from pig farms, food products and water sources [13]. *Salmonella enterica serovar Rissen* had been isolated from fish, shrimp, mussels, cuttlefish, squid and lobster, thus contributing as the second

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(Received 29 June 2024, accepted 17 November 2024)
DOI: 10.21608/EJVS.2024.300026.2210

most predominant serovar in seafood after *Salmonella enterica serovar* Weltevreden in Cochin, India [14]. Previous studies highlighted the diverse route of Salmonella contamination in seafood. Human infection with *Salmonella enterica serovar Rissen* is at risk due to consumption of pig or pork products and traveling to Thailand as this serovar is predominant in this area [4,13,15].

Pathology and pathogenicity aspects of *Salmonella enterica serovar Rissen* is not fully understood. This research was conducted to provide information on pathogenicity of *Salmonella enterica serovar Rissen* in mice as a model for rodents.

Material and Methods

Animals and Experimental designs

Total 18 BALB/c mice (Bagg Albino' mice of genotype/c) (14-week-old males) were purchased from the Animal Research and Service Centre, University Sains Malaysia and they were divided into three groups by six animals for each group A and B and C. A and B used for inducing post mortem infection while group C used as control group without any infection. The mice used were free from pathogen infection and susceptible to Salmonella infections [12,16] and housed in cages in the laboratory animal room at the Faculty of Veterinary Medicine. Commercial pellet and water are provided ad-libitum. The mice were euthanized using chloroform then cervical dislocation. They grouped into two groups (A and B). After induced post infection in these animals' post mortem changes were examined for intestinal and systemic changes after 2 days in group A and after 5 days in group B.

Bacterial Inoculum

Salmonella enterica serovar Rissen was used throughout this study. The bacteria were isolated from the intestine of a weaner pig with diarrhoea. Bacterial culture is kept in nutrient agar slant and was propagated on nutrient agar plate before use. Bacterial culture was diluted in sterile saline solution by using McFarland standard 0.5 to make 1.5 X 10^8 CFU/ml.

Animal Infections

Two hours prior to bacterial inoculation, the mice were deprived of food and water. Infection was carried out by inoculating mice using a gavage needle with 4.9 x 10^9 *Salmonella enterica serovar Rissen* in a 0.1ml volume. Water and food were provided ad libitum two hours following infection. Mice in both groups were examined twice daily to observe clinical signs.

Pathogenicity study

A postmortem was conducted immediately after euthanasia and the small intestine, caecum, colon, liver, spleen and kidney were collected. Gross lesions were observed and described during the necropsy procedure. The tissues were fixed in 10% Buffered formalin for 24 hours and placed into the tissue processing machine and processed using the standard method according to the manufacturer's instructions, embedded in a paraffin block, sectioned with a microtome, mounted on microscopic slides and stained with haematoxylin and eosin stains. Histopathologic lesions for every organ were described [17].

Isolation and Identification of Salmonella enterica serovar Rissen

Samples collected from were intestinal content, liver, spleen, kidney, and urine samples from each mouse under study [18,19]. They samples were homogenized in Rappaport-Vassiliadis Soy Peptone Broth and incubated at 42°C for 24 h. A loop of tissue suspension was cultured on nutrient agar and sub-cultured on xylose lysine deoxy chocolate agar and incubated at 37°C for 24 hours. The suspected Salmonella colonies were selected and further confirmed with biochemical tests. PCR was conducted to confirm the presence of Salmonella serovar Rissen from suspected bacterial colonies or direct detection of the bacteria from RVS broth. PCR was conducted using SRF (5' CAC CGG TAT AAG AAT TGG AT 3') with products size (282 bp) and SRF-1 primers (5' GGA AAA ATC CGG CGA CGA GA 3') with products size (282 bp) that were synthesis by First Base Integrated DNA Technologies. The PCR setting program was started at Initial denaturation at 92ºC for 5m, final denaturation at 92ºC for 0.5 m, annealing at 50ºC for 0.5 m, initial extension at 72ºC for 0.5m, final extension at 72ºC for 5m and hold at 12ºC.

Results

Clinical signs

In group A, four out of 6 mice developed signs of depression, while in group B, all the mice showed signs of depression. Clinical signs were not observed in control group mice of day 2 and day 5 PI. The control group did not show clinical signs. None of the mice showed clinical signs of diarrhoea and mortality during days 2 and 5 PI. However, both infected groups A and B developed signs of depression, ruffled fur, hunched posture, reduced motility and feeding activity.

Bacterial Isolates

Twenty samples were collected from infected groups A and B which consisted of intestinal content, liver, spleen, kidney, and urine samples. On day 2 PI, bacterial growth was not observed from the liver, spleen, and kidney samples on XLD agar and nutrient agar. On day 5 PI, there was bacterial growth from all samples. Intestinal content and urine samples showed mixed bacterial colonies of yellow and pinkish colour on XLD agar. However, the

colonies are not suggestive of *Salmonella enterica serovar Rissen* by biochemical tests. The reactions on TSI medium were acid slant, acid butt and gas production; citrate positive; MR positive; VP negative; and indole negative. The bacteria colonies from intestinal content and urine samples were confirmed as *Citrobacter spp*.

Identification of Salmonella enterica serovar Rissen through PCR confirmation:

Twenty samples consisting of intestinal content, liver, spleen, kidney and urine samples homogenized in RVS broth were used for direct detection of *Salmonella enterica serovar Rissen* through PCR confirmation. On day 2PI the bacteria were detected in the kidney and spleen (Fig. 1), while on day 5PI, the bacteria were detected in the urine and kidney (Fig. 2).

Gross Pathology:

Gross Lesion from the infected group showed intestinal lesion on days 2 and 5PI (Fig. 3). No significant lesion was observed in the control group (Fig. 3A). On day 2 PI, four out of six mice showed severe diffused reddening of the serosa layer of the small intestine especially at the jejunum and ileum, as in (Fig. 3B). Two out of six mice showed mild localized reddening of the serosa layer of the small intestine. Five out of six mice showed prominent blood vessels and mild reddening of the serosa layer of the caecum. One of the mice showed moderate reddening of the serosa layer of the colon. On day 5 PI, all six mice showed severe reddening of the serosa layer of the small intestine (Fig. 3C). All six mice showed prominent blood vessels of the caecum and some of the mice showed reddening of the serosa layer of the caecum and colon (Fig. 3D).

Histopathology

Histopathological test of the control group revealed normal villi, no infiltration of inflammatory cells (Fig. 4A). The infected group A, 2-day PI, revealed congested blood vessels and blunted villi, detachment of enterocytes from swollen villi and infiltration of monocular cells (Fig. 4B). On day2 PI, the infected B exhibited detachment of enterocytes from villi into the intestinal lumen, villi atrophy and presence of red blood cells in the lamina propria (Fig. 4C).

Histological changes of the caecum in the infected group at day 2 PI exhibited congested blood vessels and hemorrhages of the lamina propria with extravasations of red blood cells (Fig.5A), whereas group B of infected mice 5day IP exhibited hemorrhages of the lamina propria, submucosal and serosal layers with extravasations of red blood cells and infiltration of mononuclear cells at the mucosa and submucosal layers (Fig. 5B).

Microscopy of the liver in infected group revealed no significant lesions (Fig. 6A). Note the normal structure of the portal triad, sinusoids and hepatocytes. Histopathology of the liver in the infected group A at day 2 PI revealed thrombosis in the central vein and focal infiltration of mononuclear cells near the central vein primarily consisting of plasma cells and lymphocytes, all mic developed necrotizing hepatitis and one of them developed haemorrhagic hepatitis (Fig. 6B). Group B 5day revealed congestion and thrombosis of the blood vessels infiltration of mononuclear cells near the blood vessels and multifocal infiltration of inflammatory cells near the hepatocytes, sinusoids filled with red blood cells, and the presence of several Kupffer cells (Fig. 6C).

Histopathogical aspects of the spleen in the control group revealed no sigenificsnt lesions, and normal structure of white and red pulp, no neutrophils were observed (Fig. 7A). Group A 2day PI revealed lymphoid depletion and splenitis, wherase group B exhibited depletuion of white pulp (Fig. 7B). Histopathology of the spleen from a mouse in the infected group B at day 5 PI showing depletion of white pulp area (Fig. 7C). All six mice developed splenitis and five of 6 developed lymphoid atrophy. Kidney of the control group revealed no infiltration of the inflammatory cells in the cortex. Group A revealed multifocal infiltration of mononuclear cells in the renal cortex, presence of lymphocytes and plasma cells in the renal cortex (Fig. 8).

Discussion

The aim and the objective of this research work based on the study of pathogenicity and pathogenic effect of *Salmonella enterica serovar Rissen* in pathogen free mice to approve the hypothesis of a mouse model infection of human disease and act as reservoirs for this serovar that can infect and disseminate to cause various pathological changes in the parenchymal organs mainly the gastrointestinal, hepatic and kidney tissues leading to severe abnormalities and dysfunctions.

The study showed that *Salmonella enterica serovar Rissen* isolated from the intestine of a weaner pig with diarrhoea is pathogenic in BALB/c mice when inoculated orally with $4.9x\ 10^9$ CFU/ml of bacteria inoculum. The infected mice from both groups which were day 2 (group A) and day 5 (group B) post-infection showed depression, ruffled fur, hunched posture, and reduced motility and feeding activity. However, diarrhoea and mortality were not observed. Mice inoculated with10^6 to 10^8 wild types of *S. Typhimurium* into different types of mouse strain developed sign of spinning and rotary motion as this bacteria gain assessed to the central nervous system and caused meningitis which composed of acute and chronic inflammatory cells; neutrophils and macrophages [20].

In this experimental study, mice infected with *Salmonella enterica serovar Rissen* developed systemic disease without diarrhoea and inflammatory response predominantly consisted of mononuclear lymphocytes. Infiltrations of inflammatory response with predominance of mononuclear lymphocytes without diarrhoea was observed when mice were infected with *S. Typhimurium* [21].

All mice infected with *Salmonella enterica serovar Rissen* showed enteric and systemic salmonellosis with pathological lesions mainly in the intestine on day 2 PI. followed by day 5PI. respectively. However, there was no growth of bacterial colonies from the liver, spleen and kidney on day 2PI. in comparison to day 5PI. on XLD agar. Molecular analysis by PCR, however, revealed the presence of *Salmonella enterica serovar Rissen* infection in the spleen and kidney on day 2PI. The nonbacterial isolation may be due to either low bacterial load for attachment or the resistant evoked by local and systemic defence mechanisms. In spite of the low bacterial number, the virulence factors of this organism had caused splenitis and interstitial nephritis since day 2PI. It is believed this serovar had tropism towards urinary tract infection as in the experimental infection in mice, this organism is detected in urine and caused interstitial nephritis meanwhile in human natural infection this organism caused urinary tract infection and had been isolated from urine specimens too [8]. *Salmonella enterica serovar Rissen* had caused haemorrhagic enteritis, enteritis, haemorrhagic caecitis, caecitis, and colitis. However, this organism was not isolated from the intestinal content on day 2 and 5 PI. either due to the low bacteria count in the intestinal lumen or this intracellular bacterium had already invaded the intestine (enterocytes) and caused septicaemia. The small intestine played a role as site of colonization and attachment in the pathogenesis of enteric bacterial pathogens [22].

The pathological changes in the liver caused by *Salmonella enterica serovar Rissen* infection in this study were similar to that caused by *S. Typhimurium* in an experimental infection of mice when acute congestion and swelling of hepatocytes with areas of parenchyma necrosis scattered in the liver was detected [23]. The study conducted by the aforementioned researchers differ in which they used 10^2 to 10^3 of *S. Typhimurium* in 0.2ml saline solution for primary infection of male Swiss-Webster strain intraperitoneally. However, it is noted that in this current experimental study there was proliferation of Kupffer cells on day 5PI. which play a role in ingestion of the bacteria and invading the hepatocytes [19]. *Salmonella enterica serovar Rissen* had caused splenic congestion and infiltration of band neutrophils in red pulp area and foci necrotic area at red pulp area on day 5PI. In contrast, by day 5PI. of mice infected with *S. Typhimurium* caused multiple micro abscesses composed entirely of PMNs in the liver and spleen and later transformed into granuloma on day 6 or 7 PI [14]. The multifocal infiltration of mononuclear cells around the hepatocytes is observed in the early stage of this study and it is believed to develop into micro abscesses composed of Polymorphonuclear leukocytes and in later phase the abscesses are replaced by granuloma of a chronic infection.

Researchers indicate that rodents in farms played an important role in disseminating *Salmonella enterica serovar Rissen* to the environment [20]. These rodents are responsible for spreading Salmonella spp. in domestic animals. *Salmonella enterica serovar Rissen* (12.8%) was the second most isolated serovar from rodents in the farms after *S. Typhimurium* [25,26]. Salmonellas usually excreted to the environment through faecal materials. In this study, results indicated that on day 5PI. the organism was excreted through the urine where dissemination of this organism to the environment occurred. Besides, as early of day 2PI. this organism was detected in kidneys and caused interstitial nephritis. Since rodents play a role in disseminating *Salmonella enterica serovar Rissen* to the environment, it is important to have rodent control program in the food producing farm [27]and prevent further spreading of the organism to the environment [22], consequently causing salmonellosis. Ingestion of this organism originating from food-producing farms will cause salmonellosis in both animals and human; which is of public health concern [28]. Bacterial shedding and dissemination to environment followed gastrointestinal and urinary infection. However, this organism has the same behaviour in human cases with enteritis [29,30].

Conclusion

The experimental studies in this research work approves that the clinical signs and pathological changes after infection with *Salmonella enterica serovar Rissen* in rodents can highlight the organism's impact on multiple organs, particularly the intestines, liver, and kidneys. The bacterium serovar pathway in vivo study causing enteric lesions and systemic infection was a clear finding and the successful isolation of the pathogen from various tissues supports the hypothesis that rodents, including mice, may act as reservoirs for this serovar, posing risks for environmental dissemination.

Acknowledgments

This research is supported by Faculty of Veterinary Medicine, University Malaysia Kelantan, Kota Baharu, Kelantan, Malaysia. The research team gratefully acknowledge use of facilities at the Faculty of Veterinary Medicine and Veterinary Clinic for helping to support the completion of the work.

Declaration of Conflict of Interest

The authors declare no conflict of interest.

Funding statement

This research work was funded by Ministry of Education Malaysia through its Fundamental Research Grant Scheme.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Faculty of Veterinary Medicine, Benha University, Egypt (ethics approval number; 49/11/2023).

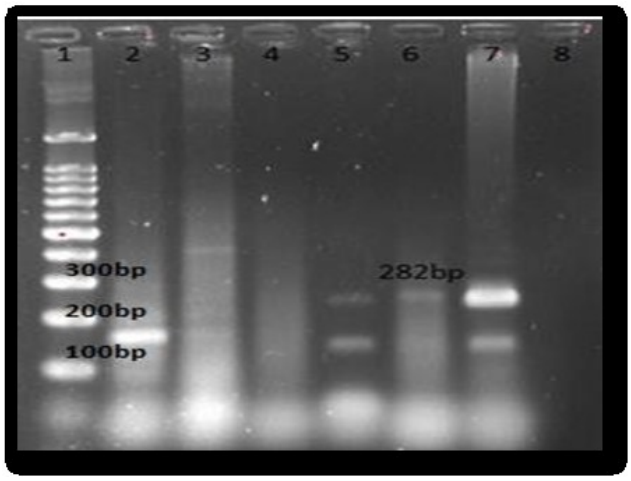

Fig. 1. Gel electrophoresis of nucleic acid from *Salmonella enterica serovar Rissen* identified in infected group A at day 2 PI. *Salmonella enterica serovar Rissen* nucleic acid Lane 1: DNA ladder; 2: intestinal content; 3: urine; Lane 4: liver; 5: spleen; 6: kidney; 7: control.

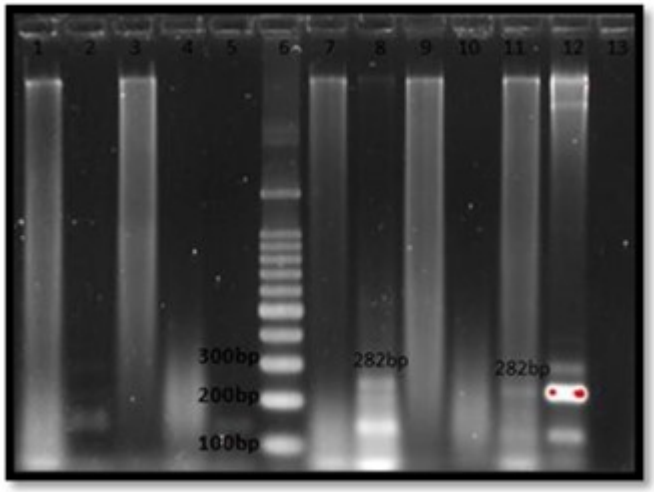

Fig. 2. Gel electrophoresis of nucleic acid from *Salmonella enterica serovar Rissen* identified in infected group B at day 5 PI.: *Salmonella enterica serovar Rissen* nucleic acid DNA. Lane 1: intestinal content; 2: urine; 3: liver; 4: spleen; 5: kidney; 6:100bp DNA ladder; Lane 7: intestinal content; Lane 8: urine; Lane 9: liver; Lane 10: spleen; Lane 11: kidney; Lane 12: control.

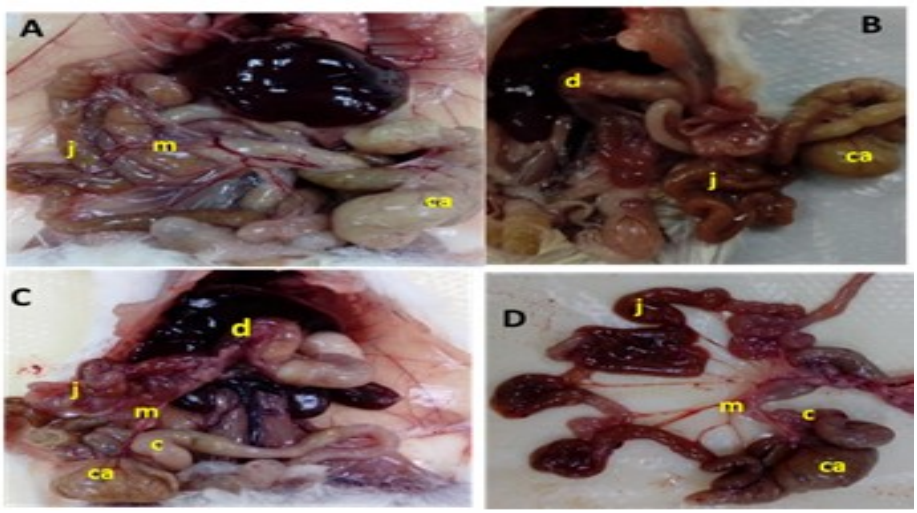

Fig.3. Gross lesion of the small and large intestine from a mouse. [j: jejunum; m: mesenteric blood vessels; ca: caecum; d: duodenum; c: colon]. (A) Control group. Note the normal appearance of the serosal layer of the small intestine, caecum and colon. (B) group A. Note the severe diffused reddening of the serosal layer of the duodenum, jejunum and ileum. There is mild reddening of the serosal layer of the caecum. (C): Group B. Note the severe diffused reddening of the serosal layer of the duodenum, jejunum and ileum. There are prominent blood vessels of the caecum and mild reddening of the serosal layer of the colon. (D): Group B Note diffused reddening of the serosal layer of the small intestine and mild reddening of the serosal layer of the colon.

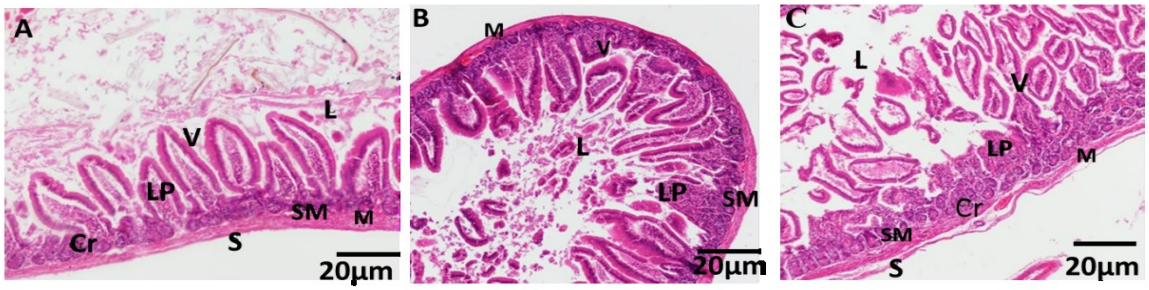

Fig.4. Microscopy of the small intestine from a mouse. (A) control group. Note the normal villi appearance and no infiltration of inflammatory cells. (B): Group A at day 2 PI. showing congested blood vessels and the villi which are blunted. Note there is detachment of enterocytes from villi which appeared swollen and infiltrated with mononuclear cells. (C): Group B at day 5 PI. X40.

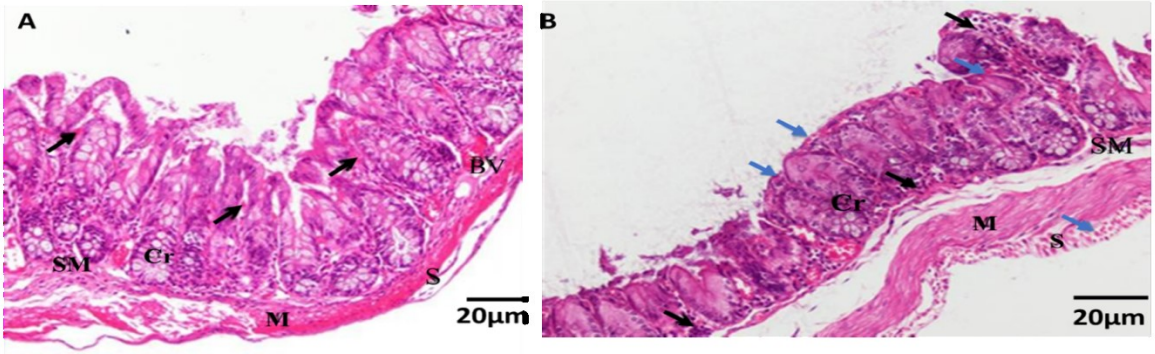

Fig.5. Histopathology of the caecum from a mouse in the infected. Fig.A; Group A at day 2 PI. showing congested blood vessels and hemorrhages of the lamina propria with extravasations of red blood cells [black arrow]. Fig.B : Group B at day 5 p.i. showing hemorrhages of the lamina propria, submucosal and serosal layers with extravasations of red blood cells [blue arrow] and infiltration of mononuclear cells [black arrow] at the mucosa and submucosal layers. [Cr: crypt; SM: submucosa; M: muscularis; S: serosal; BV: blood vessel; SM: submucosa]. Hematoxylin and eosin: 40×, Scale bars: 20 µm.

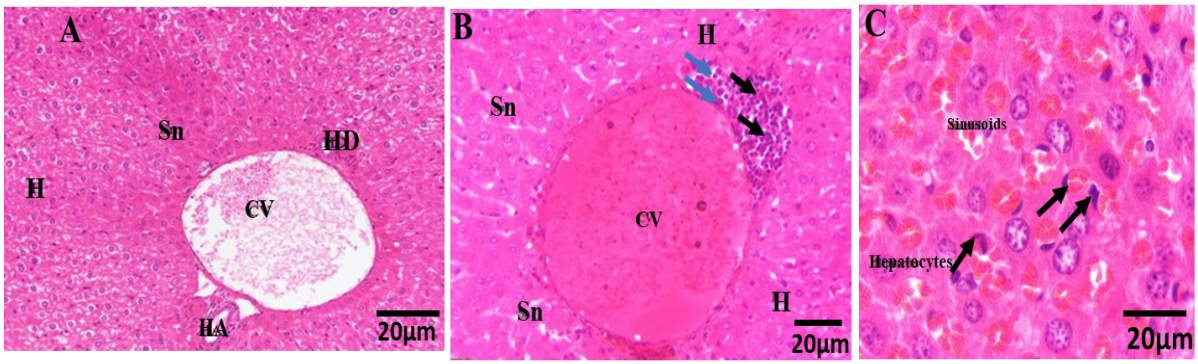

Fig.6. Microscopy of the liver from a mouse. (Fig.A) Control group reveals normal structure of the portal triad, sinusoids and hepatocytes, the normal structure of the portal triad, sinusoids and hepatocytes. (Fig.B); Infected group A at day 2p.i. showing thrombus formation in the central vein and focal infiltration of mononuclear cells near the central vein primarily consisting of plasma cells [blue arrow] and lymphocytes [black arrow]. Fig. C: Infected group B at day 5 PI. showing sinusoids filled with red blood cells and presence of several Kupffer cells. [CV: central vein; H: hepatocytes; Sn: sinusoid; HA: hepatic artery; HD: hepatic duct; Sn: sinusoid]. Hematoxylin and eosin: 40×, Scale bars: 20 µm.

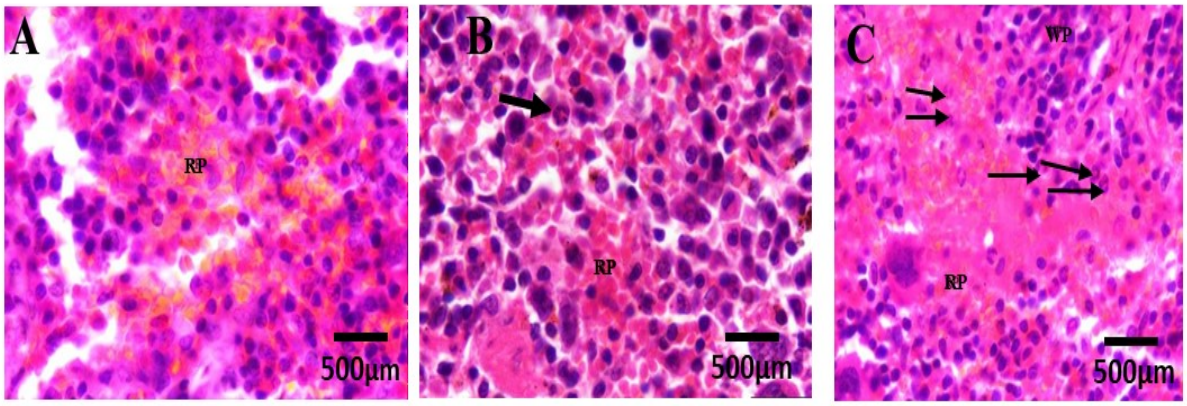

Fig.7. Microscopy of the spleen from a mouse. Fig.A: Control group showing structure of the red pulp. Note there is no presence of neutrophil. Fig.B: Infected group A at day 2 p.i. showing presence of a neutrophil [black arrow] in the red pulp. Fig.C: Infected group B at day 5 p.i. showing necrotic area at the red pulp area and increase in the number of neutrophils presence. [Cap: capsule; RP: red pulp; WP: white pulp]. Hematoxylin and eosin: 40×, Scale bars: 500 µm.

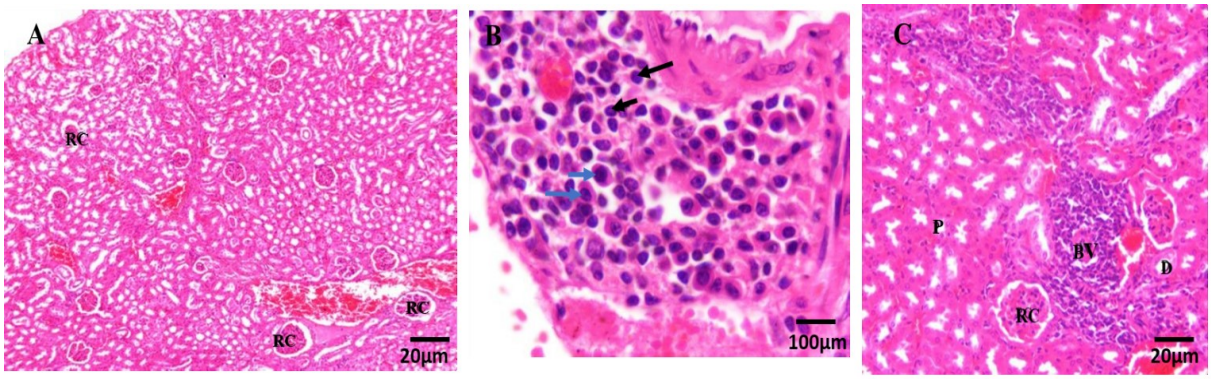

Fig.8. Microscopy of the kidney from a mouse. Fig.A; control group. Note no infiltration of inflammatory cells in the renal cortex. Fig.B: Infected group A at day 2 p.i showing presence of lymphocytes [black arrow] and plasma cells [blue arrow]in the renal cortex. Fig.C: Infected group B at day 5 p.i. showing perivascular cuffing with mononuclear cells and infiltration of mononuclear cells in the renal cortex. [RC: renal corpuscle; P: proximal convoluted tubule; BV: blood vessels; D: distal convoluted tubule]. Hematoxylin and eosin: 40×, Scale bars: 20 µm.

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القدرة المرضية وإمكانية وجود مستودع لسلالة *Salmonella enterica Serovar Rissen* في نماذج الفئران: الآثار المترتبة على الانتشار البيئي

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الملخص

أجريت هذه الدراسة لدراسة مرضية ونشوء بكتريا *Salmonella enterica serovar Rissen* في الفئران والدور المحتمل للقوارض مثل الفئران كمستودعات محتملة في نشر هذا الكائن الحي إلى البيئة. تم استخدام 18 فأرًا في هذه الدراسة، مقسمة إلى مجموعة مصابة وهي المجموعة أ والمجموعة ب والتي تتكون من ستة فئران لكل منهما. تم التشريح في اليوم الثاني واليوم الخامس بعد الإصابة (PI). تتكون المجموعة السيطرة من ثلاثة فئران في اليوم الثاني وثلاثة فئران في اليوم الخامس للتشريح. أعطيت المجموعة السيطرة 0.1 مل من محلول ملحي عادي. بينما أعطيت المجموعة المصابة 0.1 مل من 4.9×10^9 CFU/ml *Salmonella enterica serovar Rissen* عن طريق الفم. أظهرت الدراسة أن إصابة الفئران بجرثومة *Salmonella enterica serovar Rissen* تسببت في آفات معوية وعدوى جهازية. لم تسبب جرثومة *Salmonella enterica serovar Rissen* الإسهال والوفيات ولكنها تسببت في الاكتئاب خلال فترة التجربة. أظهرت جميع الفئران المصابة طبقة مصلية محمرة في الأمعاء الدقيقة وخاصة في الصائم واللفائفي. كشف التحليل النسيجي لهذه الأعضاء عن التهاب الأمعاء النزفي، والتهاب الأمعاء، والخراجات النزفية، والخراجات، والتهاب القولون، والتهاب الكلى الخلالي، والتهاب الكبد النزفي والتهاب الطحال. تم عزل سلالة *Salmonella enterica serovar Rissen* من الكلى والطحال بعد يومين من الإصابة، ومن بول وكلى الفئران بعد خمسة أيام من الإصابة من خلال تأكيد تفاعل البوليميراز المتسلسل. تسلط العلامات السريرية والنتائج النسيجية المرضية الضوء على تأثير الكائن الحي على أعضاء متعددة، وخاصة الأمعاء والكبد والكلى. يدعم العزل الناجح للممرض من أنسجة مختلفة الفرضية القائلة بأن القوارض، بما في ذلك الفئران، قد تعمل كخزانات لهذا المصل، مما يشكل مخاطر للانتشار البيئي.

الكلمات الدالة: سلالة *Salmonella enterica serovar Rissen*، المرضية، الانتشار، العدوى التجريبية.