Pathogenesis of *Salmonella enterica* serovar Albany in experimental infected SPF BALB/c Mice

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(Received November 11, 2020; December 12, 2020; Available online July 12, 2020)

Abstract

Salmonellosis remains an important zoonotic disease and public health concern, *Salmonella enterica* serovar Albany is one of the motile serovars which has been identified from poultry and humans. However, its pathogenic potentials and shedding probability and duration from infected/colonized chickens have never been reported. To assess its pathogenic potentials and shedding probability 6 SPF BALB/c mice was inoculated with 0.1ml volume for each mice bacterial solution of 10⁸ CFU/ml of *Salmonella enterica* serovar Albany after 24 hours the segments of the duodenum, jejunum, ileum, caecum and colon were fixed to study the histopathology and the polymerase chain reaction (PCR) was used to confirm the *Salmonella enterica* serovar Albany in the intestinal mucus swabs. The control group consist of 6 SPF BALB/c mice were inoculated with 0.1ml of 0.9% normal saline, The pathogenesis incidence rate of the disease caused by *S. enterica* serovar Albany revealed that prominent blood vessels on caecum 100%, red intestinal serosal 100%, infiltration of inflammatory cells in the crypt of liberkhun and submuscular layer of small intestine 100%, enterocyte necrosis 100%, haemorrhagic enteritis 83.3%, cecitis 33.3%, colonitis 66.6%, villus atrophy 100%, crypt atrophy 100%, and detachment of epithelial tissue 50%, can occur as soon as 24 hours post infection. Infected *S. enterica* serovar Albany was also successfully re-isolated from the intestinal swabs which revealed that the mice is potentially shed the bacteria through feces.

Keywords: Salmonellosis, *Salmonella enterica* serovar Albany, Pathogenesis

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The recovered from intestines of the animals that admitted in this study for isolation of the bacteria. A group of 60 chickens were used in this experiment. The mice that were purchased before used were used in this experiment. The mice that were purchased before used were kept in nutrient agar slant at room temperature for 26 months in artificially contaminated poultry food. Salmonellosis remains an important zoonotic disease and public health concern. The disease is known to be most prevalent in areas of intensive animal husbandry, especially in swine, poultry, and cattle as well as in animal products such as poultry eggs, milk and fresh seafood. Infection with Salmonella often causes gastroenteritis with increase susceptibility in young children, older people and immunosuppressed person. Raw chicken products are an important part of international food trade. In poultry, the infection of Salmonella often affects a flock and is carried asymptomatically in the gastrointestinal tract of a proportion of the birds and subsequently may be transferred to carcasses during processing via fecal contamination. Furthermore, contamination of the carcasses, assist the transmission of the Salmonella to human which cause foodborne disease. Vast majority of foodborne cases are undiagnosed, however in the United States it has been estimated that foodborne diseases cause approximately 76 million illness with 325000 hospitalization and 5000 deaths each year. Consumption of contaminated poultry meat with Salmonella will contribute to the elevation of foodborne cases since poultry meat consumption is the highest record per capita meat consumption. According to the data documented by Laboratory Based Surveillance, Disease Control Division, Ministry of Health Malaysia, S. enterica serovar Albany was listed in top 10 non-typhoidal Salmonella serotypes identified in year 2003, 2004 and 2005 (12).

Recent study, there has been isolation of S. enterica serovar Albany from poultry meat samples which is collected from wet markets Pengkalan Chepa with antimicrobial sensitivity test show resistance towards Streptomycin and Tetracycline. Pathology and pathogenesis of S. Typhimurium in mouse model discovered in vivo intestinal disease or enterocolitis (5,13,14). While study of enteritis and typhoid fever using mouse models of Salmonella infection specifically on serotype Typhi was also done but for serotype Albany experimental infection study using animal models is still lack (15,16). Therefore study of pathology and pathogenesis on the disease caused by this pathogen is quite significant due to the fact that this bacterium can be potentially zoonotic.

Materials and methods

Preparation of bacteria inoculum

Salmonella enterica serovar Albany was used throughout this study. This bacteria culture was isolated from chicken carcasses obtained from a wet market. The culture is kept in nutrient agar slant at room temperature and was propagated on nutrient agar plate before used. Bacterial culture was diluted in sterile saline solution by using McFarland standard 0.5 to make 10^8 CFU/ml.

Experimental animals

Twelve SPF BALB/c mice between 12 weeks of age were used in this experiment. The mice that were purchased from University Sains Malaysia Laboratory Kubang Kerian, Kota Bharu, Kelantan were healthy and free from pathogen infection was housed under specific pathogen free (SPF) condition that are free of specified micro-organisms and parasites, but not necessarily free of others not specified and also their SPF BALB/c mice lacked facultatively anaerobic gram-negative bacteria, such as Escherichia coli, in their gut microbiota. Three mice were kept per cage at the laboratory animal room at the Faculty of Veterinary Medicine, University Malaysia Kelantan.
Animal infection  
The animals were divided into two groups which is the control group and experimental groups. Control group consists of 6 mice and experimental group consist of 6 mice. Both groups were given ad-libitum of commercial mice pallet and water. Animal infection was carried out by inoculating mice per orally using modified gavage needle by using indwelling catheter size 18 gauge.  
The control group (Group A) was inoculated with 0.1ml of 0.9% normal saline, while experiment group (Group B) was inoculated with 0.1ml volume for each mice bacterial solution of 10³ CFU/ml of bacteria. Water and food were removed 4 hours prior to the experimental infection. Then, drinking water was provided immediately after infection and food was provided 2 hours post-infection. The infected mice was examined twice daily for any clinical signs and mortality. Two mice were euthanized every day from the experiment group and two mouse euthanized from Control group on post inoculation. Then, post-mortem was performed immediately after euthanization and the organ samples such as the small intestine (duodenum, jejunum and ileum), caecum and colon were collected and the gross lesions were observed and recorded.  

Bacteriology  
Intestinal mucosal swabs were collected using sterile cotton swab from small intestine, caecum and colon. The swabs were immediately placed in 20ml of sterile buffered peptone water (BPW) at pH 7.0 and incubated at 37°C for 48 hours. 1ml of the solution was transferred into 10ml of tetraphionate broth (TTB) and incubated at 42°C for 24 hours before isolation. The sample spread onto selective media. Suspected *Salmonella* spp. colonies were taken for biochemical test. Polymerase chain reaction (PCR) was used to confirm the *Salmonella* spp.  

DNA extraction  
For extracting DNA from an isolate, the preserved isolate was inoculated onto 5% citrated bovine blood agar at first which was then incubated for 24 hours at 37°C. Three to four colonies from the agar plate were transferred to a 1.5 ml Eppendorf tube containing 200 µl de-ionized water and mixed well. The tube was vortexed and heated at 99 °C for 15 minutes and immediately transferred onto ice. The suspension was then centrifuged at 15000 rpm for 2 minutes. After centrifuge, 100 µl of the supernatant was transferred into a new Eppendorf tube which was used as DNA template.  

Polymerase Chain Reaction  
The sequences of specific gene (invA) of the primer set used to detect *S. enterica serovar* Albany were (F) 5’-AGCACAACTGCTTAAATGTG-3’ and (R) 5’-GGTAGGATTCCACAGCGTGATCT-3’ published elsewhere for the identification of *Salmonella*. PCR was performed by using a PCR mastermix (Vivantis Technologies Sdn. Bhd.) and was prepares in a final volume of 25 µL containing 0.5 pmol of each primer, 9.5 µL nucleus free water, 12.5 µL of mastermix solution and 2 µl of DNA extract. the mixture then subjected to 35 cycles of amplification in a thermal cycler (Table 1). The PCR product were analysed on 1.5% (w/v) agarose gel electrophoresis at 100 volt for 30 minutes for 8 wells agarose gel and at 85 volt for 45 minutes for 20 wells agarose gel (Figure 1).  

Table 1: PCR cycling protocol  
| Steps                  | Target gene       |
|------------------------|-------------------|
| Initial denaturation   | 94 °C at 3 min    |
| Denaturation           | 94 °C at 30 sec   |
| Annealing              | 50 °C at 30 sec   |
| Extension              | 72 °C at 30 sec   |
| Final extension        | 72 °C at 3 min    |
| Hold                   | 4 °C              |

**Figure 1:** PCR result from re-isolation of *S. enterica serovar* Albany from experimental mouse. Lane 1 is a ladder, lane 2 is positive control, lane 3 to lane 7 is samples from small intestines swab from 6 of the experimental mouse and lane 8 is negative control.  

Histopathology  
Segments of the duodenum, jejunum, ileum, caecum and colon were fixed in 10% buffered formalin for 48 hours. After 48 hours, the samples were sectioned, stained with hematoxylin and eosin (H&E) stains. The sections were observed for microscopic changes using a light microscope (Table 2).
Table 2: Standard tissue processing protocol of Leica®

| Station | Reagent   | Time |
|---------|-----------|------|
| 1       | Formalin  | 1h00 |
| 2       | Formalin  | 1h00 |
| 3       | Alcohol 70% | 1h30 |
| 4       | Alcohol 80% | 1h30 |
| 5       | Alcohol 96% | 1h30 |
| 6       | Alcohol 100% | 1h00 |
| 7       | Alcohol 100% | 1h00 |
| 8       | Alcohol 100% | 1h00 |
| 9       | Xylene    | 1h30 |
| 10      | Xylene    | 1h30 |
| 11      | Paraffin  | 2h00 |
| 12      | Paraffin  | 2h00 |

Results

Post Infection Clinical Signs

Mice inoculated with $10^8$ CFU/ml of bacteria per orally with *S. enterica* serovar Albany did not cause diarrhea. However, it causes depression between two to four hours post-infection. There was no death occur in this experiment, all mice were euthanized to proceed pathological examination.

Bacterial isolation

All suspected colonies from all samples of experimental mouse show urease reaction negative, H$_2$S positive (TSI), motility test (SIM) positive, oxidase test negative, indole negative, Methyl Red positive, and Simmons citrate positive. All isolates were further identified by PCR confirmation. All samples from the control mouse show no growth of suspected *Salmonella* spp. colonies on XLD agar.

PCR Confirmation

Eighteen samples from small intestine, caecum and colon swabs from the experimental mouse show positive for *S. enterica* serovar Albany. The results for gross lesion, histopathology and morphological diagnosis as in table 3.

Gross lesion of the small intestine of the control mice and gross lesion of small intestine of the experimental mice after 3 days post infection. Mesenteric blood vessels of the experimental mice are congested and serosal layer are reddened. Control mice absence of mesenteric blood vessels congestion and absence of serosal layer reddening; Figure 2.

Grossly, there is no evidence of ulceration on the intestinal mucosal layer. *S. enterica* serovar Albany colonizes along the small, caecum and colon, *S. enterica* serovar Albany was re-isolated from all part of the intestines and confirmed by PCR. Therefore, pathological changes are able to be seen in all part of the intestines. Experimental infection of *S. enterica* serovar Albany revealed that this bacteria cause enteritis, cecitis and colonitis. The inflammations are predominantly by macrophages. Haemorrhages and epithelial layer erosion are seen in small intestine; Figure 3. Inflammations at the colon are predominantly also by macrophages; Figure 4 and histopathological analysis of the colon post-infection; Figure 5.

Table 3: Gross and histopathology lesion summary after 1 day post-infection

| Histopathology lesion                                      | %     |
|-----------------------------------------------------------|-------|
| Prominent blood vessels on caecum                          | 6/6 (100%) |
| Red intestinal serosal                                     | 6/6 (100%) |
| Infiltration of inflammatory cells in the crypt of ilerkhun| 6/6 (100%) |
| Infiltration of inflammatory cells in the submuscular layer of small intestine | 6/6 (100%) |
| Enterocyte necrosis                                        | 6/6 (100%) |
| Haemorrhagic enteritis                                     | 5/6 (83.3%) |
| Cecitis                                                    | 4/6 (66.6%) |
| Haemorrhagic cecitis                                       | 2/6 (33.3%) |
| Colonitis                                                  | 4/6 (66.6%) |
| Haemorrhagic colonitis                                     | 2/6 (33.3%) |
| Villus atrophy                                             | 6/6 (100%) |
| Crypt atrophy                                              | 6/6 (100%) |
| Detachment of epithelial tissue                            | 3/6 (50%) |

Discussion

Many studies suggested that poultry, especially chicken could be the most common reservoir of *Salmonella* spp. (17) and it’s a hallmark of *Salmonella* enteritis in human diarrhoea (17,18). Result from this study reveals that experimental infection of *S. enterica* serovar Albany in mice do cause lesion such as enteritis, cecitis and colonitis right after 24 hours post infection. *S. enterica* serovar...
Albany is a serovar rarely isolated from infectious processes (13).

However in Malaysia it is listed top in top 10 non-typhoidal Salmonella serotypes identified in year 2003, 2004 and 2005 stated by Laboratory Based Surveillance, Disease Control Division, Ministry of Health Malaysia.

Figure 3: Histopathological analysis of the small intestine after 1 day post-infection. A and B are section of the small intestine from control mice stained with H&E. C and D are section of the small intestine from experimental mice at after 1 day post-infection stained with H&E. Arrows in C and D show the haemorrhages on the villus. In C and D there are also infiltrations of the inflammatory cells in the lamina propria predominately by macrophages and also infiltrated with PMN. Abbreviation; L: lumen, Cr: crypt of Liberkhun, v: villus, S.M: submucosa, M: muscular layer, L.P: lamina propria.

Figure 4: Histopathological analysis of the caecum after 1 day post-infection. A is a section of caecum from the control mice treated with 0.1ml of 0.9%normal saline, stained with H&E. B is a section of caecum from the experimental mice at p.i day 1 of 0.1ml, 10⁸ CFU/ml of S.enterica serovar Albany stained with H&E. Arrows in B shows the infiltrations of the inflammatory cells in the lamina propria and submucular layer, predominantly by macrophages. B, necrosis of the crypt and vacoulattion of the goblet cells. Abbreviation; L: lumen, S.M: submucosa, M: muscular layer, L.P: lamina propria, G: goblet cell.

Figure 5: Histopathological analysis of the colon after 1 day post-infection. A is a section of colon from the Control mice treated with 0.1ml of 0.9%normal saline, stained with H&E. B is a section of colon from the experimental mice at p.i day 1 of 0.1ml, 10⁸ CFU/ml of S.enterica serovar Albany stained with H&E. Arrows in B shows the infiltrations of the inflammatory cells in the lamina propria and submucular layer, predominantly by macrophages. B, necrosis of the crypt and enterocyte necrosis. Abbreviation; L: lumen, S.M: submucosa, M: muscular layer, L.P: lamina propria.

Oral ingestion of S.enterica serovar Albany form undercooked poultry meat cause salmonellosis. Findings such as enteritis, cecitis and colonitis in this study resemble the findings in human salmonellosis.

Results from similar study revealed that oral infection of S.enterica serovar Albany do not cause diarrhea in mice unlike in human (19). However, a previous study-found that feeding of chicken contaminated with S.enterica serovar Albany to ocelot in captivity cause salmonellosis and necropsy of the ocelot revealed ileum, villi and crypt necrosis as well as severe mononuclear lymphocytic infiltration (20, 21).

Mice villus necrosis resemble the finding in the study of ocelot, however in this inflammatory cells infiltration is different from previous finding. Macrophages were found to be predominant inflammatory reaction in this study. Bacteria re-isolation is 100% re-isolated from all experimental mouse’s small intestine, caecum and colon swabs. This finding is resemble the finding form the study in ocelot that feed with contaminated chicken meat with S.enterica serovar Albany, where Salmonella serotype isolated from the feed sample is same as Salmonella serotype isolated from the ocelot feces (17,20).
As *Salmonella enterica* serovar Albany was isolated from all part of the intestines, it reveals that mice do shed *Salmonella* spp. through their feces asymptotically.

**Conclusion**

The pathogenesis of the disease caused by *S. enterica serovar* Albany revealed that haemorrhagic enteritis, cecitis and colititis can occur as soon as 24 hours post infection. Infected bacteria sp. also was successfully re-isolated from the intestinal swabs which revealed that the mice is potentially shed the bacteria through feces.

**Acknowledgement**

The authors are grateful to the Middle East University, Amman, Jordan for the financial support granted to cover the publication of this article.

**Conflict of interest**

None

**Ethical clearance**

All animal experiments were performed in accordance with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and the Ethical Committee approval was obtained from ethical committee of University Malaysia Kelantan-Malaysia.

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