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The Response of Gut Associated Lymphoid Tissues (GALT) Following Intranasal Administration of P. Multocida B2 in Rats

[1]S. Nor-Satinati, [1]A.B.Z. Zuki, [2]M. Zamri-Saad,
[1]A.J. Awang-Hazmi and [1]Saw Po Po
[1]Department of Veterinary Preclinical Sciences,
[2]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine,
Universiti Putra Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia

Abstract: The present study was conducted with the aim to investigate the response of GALT following oral administration of *P. multocida* type B2. A total of 12 adults, Spraque-dawley rats, supplied by the animal breeding unit, Faculty of Veterinary Medicine, UPM, were used in this study. The rats were divided into 2 groups, which consist of 6 rats per group. 0.5 mL of 10^4 CFU of *P. multocida* B2 was given to the rats in first group through intranasal route by using 1 mL syringe while the second group was given 0.5 mL normal saline using the same method. Both groups were kept in cages separately for 2 weeks before being euthanased. The number and size of Peyer's patches of the intestine were counted and measured macroscopically upon euthanasation. The intestine was then divided into 14 portions; proximal, middle and distal of each the duodenum, jejunum, ileum and colon and one each for caecum and rectum for histological evaluation. The Hematoxylin and Eosin staining was used to count the number of lymphocytes follicles and intraepithelial lymphocytes. This stain also used to measure the size of lymphoid follicles. The Methyl Green Pyronin was used to count the number of plasma cell. The results showed that intranasal administration of *Pasteurella multocida* B2 has slight influence on the development of GALT. However, the different in the parameters study between control and infected groups were not significant (p>0.05).

Key words: Rats, *P. multocida* B2, intranasal, GALT

INTRODUCTION

The mucosal lining of the respective tracts (digestive, respiratory, urogenital) is under constant threat of the penetration by all types of bacteria and viruses. No matter how the microbes and other antigens enter the body, they will eventually encounter the lymphoid system to initiate adaptive immune responses[1]. The mucosal immune system is functionally linked, so that exposure to an immunogen at one mucosal surface often results in an immune response to that immunogen at multiple mucosal sites[2]. Because of this, the draining lymph nodes usually show a high degree of immune reactivity.

In addition to these mucosa draining lymph nodes, the mucosa itself may contain organized lymphoid structures. It is because more than 50% of the total body lymphoid mass associated with mucosal surface[3]. These are collectively called Mucosal-Associated Lymphoid Tissues (MALT). The examples of MALT are, the Bronchus Associated Lymphoid Tissues (BALT) for respiratory tract[4] and Nasal Associated Lymphoid Tissues (NALT) and Gut Associated Lymphoid Tissues (GALT) for digestive tract[5].

The gastrointestinal tract is one of the largest immunological organs of the body containing more lymphocytes and plasma cells than the spleen, bone marrow and lymph nodes in combination. The gut associated lymphoid tissues comprise cells organized within the lymphoid follicles of the Peyer's patches as well as those distributed throughout the lamina propria and intestinal epithelium. The intraepithelial lymphocytes are any lymphocyte that found within an epithelium particularly the intestinal epithelium, whereas the lymphoid follicles are consists of a network of follicular dendritic cell, the spaces between which are packed with small recirculating B-lymphocytes. Bacteria belonging to the *Pasteurellaceae* family (i.e., organisms of the genera *Pasteurella, Mannheimia, Actinobacillus* and *Haemophilus*) have a high degree of host specificity. *Pasteurella* species are not commensals of human being although some strains may be present as a transient part of the normal flora.

Corresponding Author: A.J. Awang-Hazmi, Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia

Pasteurella multocida B2 is a gram-negative and bipolar staining rod. Transmission is by direct contact and to lesser extent, fomite, aerosol and sexual exposure. Disease susceptibility depends on host (host strains), environmental (shipping, experimentation, wide temperature fluctuations and high ammonia level) and bacterial factors (bacterial strains differ in many aspects including growth characteristics and colonization site) .The organisms have established a primary site of infection and they multiply before spread to other organs. Infection can spread directly trough tissues or via the lymphatic system to the blood stream. Bacteremia allows bacteria to spread widely in the body and permits them to reach tissues particularly suitable for their multiplication.

To our knowledge, the response of GALT following oral administration of *P multocida* type B2 has never been studied. Thus, this study was undertaken with the aim to evaluate the macroscopic and microscopic changes of GALT response to oral administration of *Pastuerella multocida* B2.

MATERIALS AND METHODS

Animals: Twelve adults, Spraque-Dawley rats weighing 200-250 g were used in this study. The rats were divided into two groups consisted of 6 rats in each group. The rats were kept in cages and given commercial pellet and water *ad-libitum*. Rats in the first group were subjected to intra-nasal administration of *P. multocida* type B2, while rats in the second group were used as control and were given oral administration of normal saline. The protocol was approved by the Faculty's Ethic Committee.

Preparation of *P. multocida* B2: *P. multocida* B2 used in this study has been first tested to examine the virulence of the bacteria. The bacteria have been diluted in normal saline and 3 mL was injected via intraperitoneum into three healthy adult mice. All the mice were died within 24 h of injection. After confirmation of the virulence of the bacterial, the bacterial culture was carried out on blood agar and the culture was incubated at 4°C for 24 h. Identification, biochemical test, cell morphology and colony morphology were observed and examine. A single colony of bacteria from the culture was subcultured on blood agar and incubated for another 24 h at the same temperature. The final step was to harvest all the bacteria from the colony in pure culture and wash three times with PBS solution by centrifugation. It was then wash with FPBS solution and standardized to 10^8 cells mL^{-1} bacteria using Mc Farland (0.5). Finally, dilution was done with PBS to get 10^6 CFU.

Innoculation protocol: The rats were anaesthetized with an IM injection of ketamine (50 mg kg^{-1}) and xylazine (5 mg kg^{-1}). *Pasteurella multocida* B2 was inoculated in rats of Group 2 by dropping the solution of *P. multocida* B2 using syringe 1 mL. Five drops of *P. multocida* B2 consist of 0.5 mL of 10^4 CFU of *P. multocida* B2 were dropped near the nostril of rats (1 drop = 0.1 mL of *P. multocida* B2). The same method was applied on rats in first group (control group) but using normal saline.

Gross examination: All the rats were euthanased with overdose of ketamine and xylazine at day 14 post-inoculation. Upon euthanasia, the small and large intestines of each rat were collected and examine macroscopically. The intestine was cut at the proximal end of duodenum and at the distal end of rectum. The intestinal contents were removed and washed with clean water. The intestinal lumen was then inserted with 10% buffered formalin and then the whole intestine was soaked into the solution for 30 min. The intestine was then cut open along the mesenteric border and the mucosal surface was examined. The number and size of follicles at each part of intestine were counted, measured and recorded.

Microscopic examination: The intestine was divided into 6 portions: the duodenum, jejunum, ileum, cecum, colon and rectum. Three samples were taken from each portion (the proximal, middle and distal parts) and fixed in 10% buffered formalin and process for histological examination. The tissues were sectioned at 0.4µ using Microtome (Leica 2045). The sections were then stained with Harris Hematoxylin and Eosin (H and E) and Methyl green pyronin. The number and size of follicle, Intraepithelial Lymphocyte (IEL) of villi and crypts were counted and recorded by using image analyzer (Synoptic Inc., UK). The Methyl green pyronin sections were used for plasma cell count.

Statistical analysis: All the data were analysed using Independent T-Test and One Way ANOVA. This was done using SPSS software, Version 11.0. The statistical analysis was based on the comparison between the groups (using Independent T-Test) and comparison within the groups (using One Way ANOVA). The data were considered significant at $p<0.05$.

RESULTS

Macroscopic evaluations

Number of peyer's patches: Results of the mean total number of Peyer's patches for each portion of intestine of control and infected group are showed in Fig. 1.

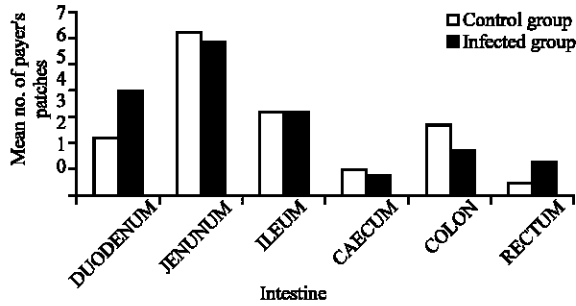

Fig. 1: The comparison mean number of Peyer's patches between control and infected groups in different part of intestine

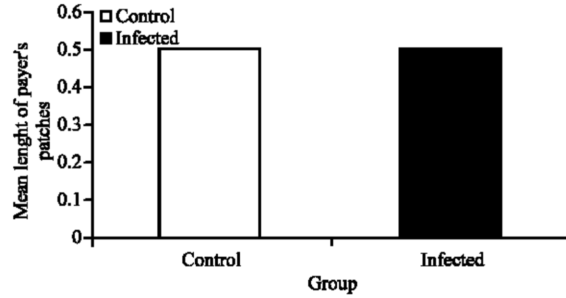

Fig. 4: The comparison mean length of Peyer's patches between control and infected groups for the whole part of intestine

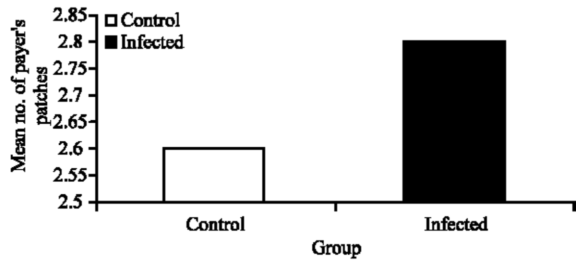

Fig. 2: The comparison mean number of Peyer's patches between control and infected groups for the whole part of intestine

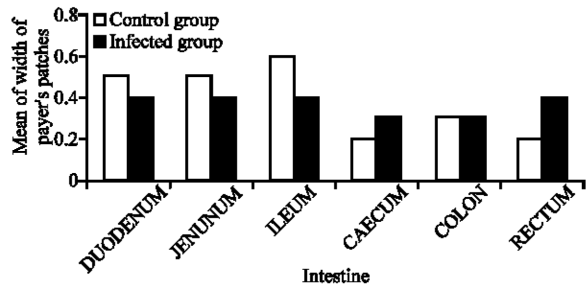

Fig. 5: The comparison mean width of Peyer's patches between control and infected groups for different part of intestine

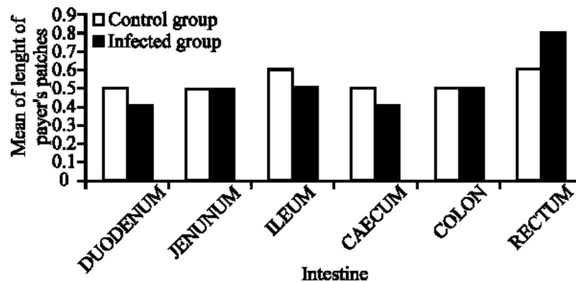

Fig. 3: The comparison mean length of Peyer's patches between control and infected groups for different part of intestine

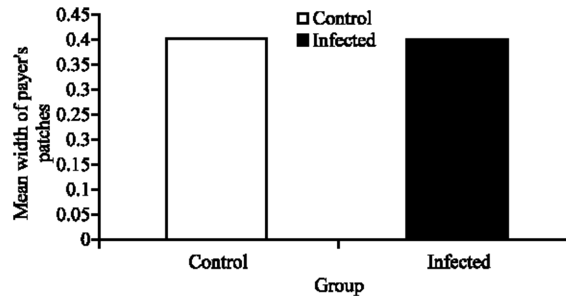

Fig. 6: The comparison mean width of Peyer's patches between control and infected groups for the whole part of intestine

For both group, the mean number of Peyer's patches was higher in the jejunum and was reduce toward caudal portions Fig. 1. While the mean number was higher at jejunum (5.8±3.5) and was lower in the caecum (0.8±0.8). Jejunum was significantly different ($p<0.05$) to other portions in control group and infected group. In general, the mean number of Peyer's patches of intestine in infected group was increased (2.8±1.9) as compared to control group (2.6±2.0) Fig. 2. However, the difference was not significant ($p>0.05$).

Length of peyer's patches: The length of Peyer's patches in both control and infected groups are showed in Fig. 4.3 and 4.4. The length of Peyer's patches was longer at rectum in both control (0.6±0.3) and infected groups (0.8±0.3) as compared to other portion Fig. 3. The Peyer's patches in rectum were significantly longer ($p<0.05$) than in other portions in infected group. Overall, the total mean length of Peyer's patches in control and in infected groups was similar Fig. 4.

Width of peyer's patches: For different part of intestine, the results showed that the small intestine had wider Peyer's patches as compared to large intestine both in control and infected groups. The width of Peyer's patches in ileum was bigger as compared to other portion in the control group Fig. 5. The mean width of Peyer's patches between control and infected groups for the whole part of intestine was the same (0.4±0.1) Fig. 6.

MICROSCOPIC EVALUATION

Number of lymphoid follicles: The results showed that colon had highest value of mean number of lymphoid follicles in both the control group (29.8±6.9) and in infected group (27.0±5.2) Fig. 7. In control group, the mean number of lymphoid follicles was lowest in rectum (0.8±1.3), whereas in infected group caecum had the lowest mean number of lymphoid follicles (1.3±1.0). In general, the mean number of lymphoid follicles in different part of intestine was slightly increased in infected group. However, the different was not significant ($p>0.05$) Fig. 8.

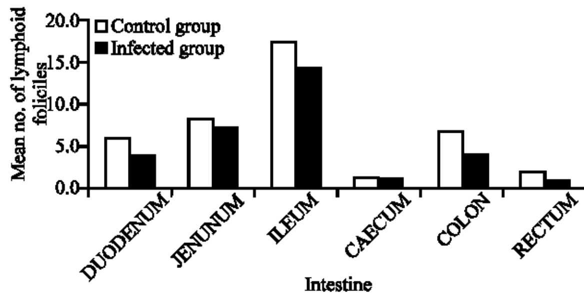

Fig. 7: The comparison mean number of lymphoid follicle between control and infected groups in different part of intestine

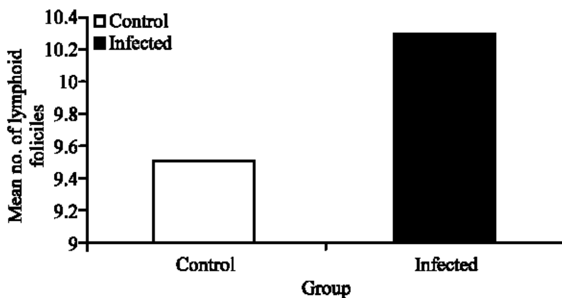

Fig. 8: The comparison mean number of lymphoid follicle between control and infected groups for the whole part of intestine

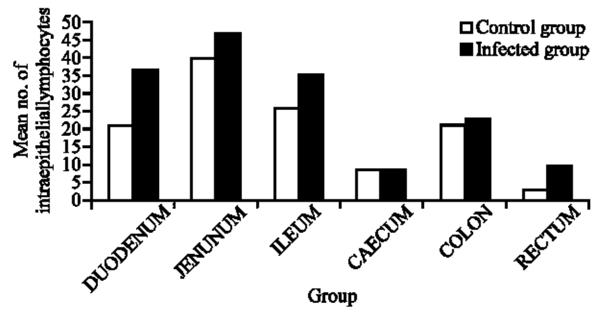

Fig. 9: The comparison mean number of intraepithelial lymphocyte between control group and infected group for different part of intestine

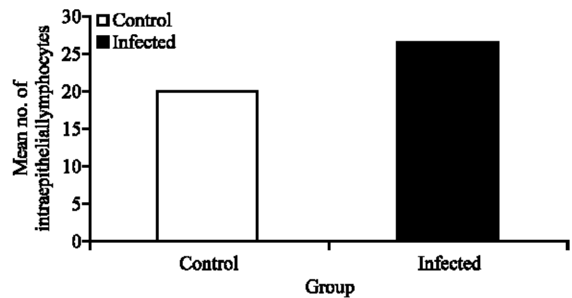

Fig. 10: The comparison mean number of intraepithelial lymphocyte between control and infected groups for the whole part of intestine

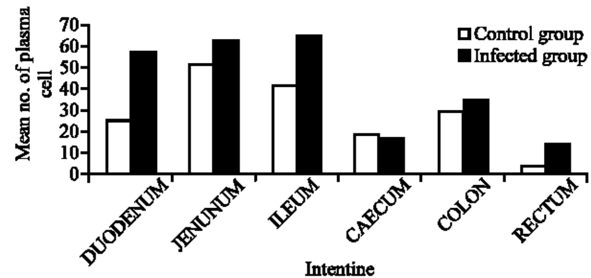

Fig. 11: The comparison mean number of plasma cell between control group and infected group for different part of intestine

Number of intraepithelial lymphocytes (IEL): Results showed that the mean numbers of IEL were increased in all portions of the intestine in infected group Fig. 9. The mean number of IEL was highest in jejunum as compared to other portions in both control group (39.2±11.0) and infected group (46.3±17.9). The number was declined toward caudally. The number of IEL in jejunum was significantly higher ($p<0.05$) than the rectum in both groups. The number of IEL in duodenum showed significant increased ($p<0.05$) after being infected with

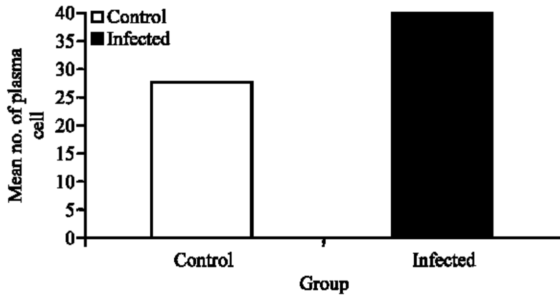

Fig. 12: The comparison mean number of plasma cell between control and infected groups for the whole part of intestine

Pasteurella multocida B2. Even though the total mean number of IEL in intestine on the infected group was higher, but different was not significant (p>0.05) Fig. 10.

Number of plasma cell: The mean number of plasma cell in infected groups was increased in duodenum (56.3±10.7), jejunum (62.3±17.0), ileum (64.0±27.2), colon (34.2±8.9) and rectum (13.0±8.5) except for caecum. The number of plasma cells in ileum was significantly different (p<0.05) to rectum in infected groups Fig. 11. In general, the total mean number of plasma cell in intestine of infected group was significantly higher (p>0.05) as control group Fig. 12.

DISCUSSION

The results of this study showed that all the immune cells (lymphoid follicles, Intraepithelial Lymphocytes (IEL) and plasma cell) were increased in infected group. However, only the number of plasma cell was statistically significant (p<0.05). This means that the gut associated lymphoid tissues had response to the *Pasteurella multocida* B2 through intranasal administration. When *Pasteurella multocida* B2 enters the body trough intranasal administration, the organisms have established a primary site of infection, the lungs. The organisms were multiplied before spreading to other organs. This was occurred via the lymphatic system to the blood stream. Bacteremia allows bacteria to spread widely in the body and permits them to reach tissues particularly suitable for their multiplication. A research by Nossal *et. al.*[6] revealed that, antigen is carried by lymphatic flow to medullary areas of the regional lymph nodes.

In addition, in the respiratory tract, NALT or BALT is exploited to enhance the local immunity and provides protection against inhaled antigens, but NALT or BALT may be less well developed than GALT. Liposomes have ability to enhance systemic and mucosal immunity following nasal administration. In this study, the distributions of the immune cells were more prominent in the small intestine than in the large intestine. This was due to the mucosa of the small intestine constitutes one of the larger compartments of the lymphoid system and is composed of both diffuse and organized lymphoid tissue[7]. Moreover, the intestinal IEL are a very large cell population in the epithelial layer of the small intestine[8].

The distribution of Peyer's patches, Intraepithelial Lymphocytes (IEL) and plasma cell were more prominent in jejunum portion as compared to other portions. This may be due to anatomical structure of the jejunum where the length of the jejunum is the longest as compared to other portion.

As compared to duodenum and jejunum in the infected group, ileum portion had lowest distribution of the IEL. This is because, the villi tend to be longest in the duodenum and become shorter and less numerous towards the ileum[9]. The results also showed that the lymphoid follicles were higher in ileum part. According to Young and Heath[9] the lymphoid follicles were least numerous in the duodenum and more prominent in the terminal ileum. Thus, the results are in accord with Young and Health[9].

Within the large intestine portion, this study showed that colon had higher number of immune cells. The colon is the major location of bacteria in the body. Roughly, 20% of the feces consist of bacteria, approximately 10^{11} organisms g^{-1} such as *Escherichia coli*, *Clostridium perfringens* and *Pseudomonas aeruginosa*[10]. This may explain why the immune cells were higher in the large intestine.

In general, the results of this study revealed the increased in the total mean number of the immune cells for the whole part of the intestine in infected rats. However, the increased was not significant (p>0.05) between the control and infected groups. This may be due to the infection was not severe enough to stimulate the GALT. The 10^4 CFU of *Pasteurella multocida* B2 broth was inoculated only once in the infected rats. The response is thought to be better if second inoculation is given to the infected group. The single inoculation of *Pasteurella multocida* B2 broth leads to primary response of immune system at mucosal layer of gastrointestinal tracts. A second response to second time of inoculation is more rapid and of greater intensity than primary response to first inoculation of *Pasteurella multocida* B2[11]. This is because the immune system having encountered the antigen before. Besides that, this study also need prolong time, which up to 5 weeks before euthanased the rat. This is because the lymphoid cells especially plasma cells will continue to increase and reach peaks in three to five

weeks post infection. In addition, administration of higher dose of *P. multocida* B2 should be considered. High dose of antigen may cause more severe infection on the mucosal surface and make the lymphoid tissues become actively produced in shorter time[1].

CONCLUSION

Pasteurella multocida B2 has slight influence on the development of GALT. However, the different in the parameters study between control and infected groups were not significant (p>0.05).

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