Pertanika J. Trop. Agric. Sci. 37 (2): 215 - 221 (2014)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

The Responses by Gut-Associated and Bronchus-Associated Lymphoid Tissues of Buffalo Calves Following Oral Exposure to *Pasteurella multocida* B:2

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ABSTRACT

This report describes the mucosal immune response in the gastro-intestinal and respiratory tracts of buffalo calves following oral exposure to live wild-type *Pasteurella multocida* B:2. Nine buffalo calves of approximately 8 months old were treated with intramuscular injections of dexamethasone for 3 consecutive days before they were divided into 3 groups. Calves of group 1 were exposed orally to 50 ml inoculums containing 10^9 colony forming units (CFU)/ml of live wild-type *P. multocida* B:2. Calves of group 2 were exposed intra-trachea with 5ml of the same inocula while calves of group 3 were given 50ml of PBS orally. At the end of day 7 post-exposure, all surviving calves were killed and organs of gastro-intestinal and respiratory tracts were processed for histology examination. The presence of lymphoid nodules, the size of the nodules and the number of lymphocytes were noted. Both oral and intra-trachea exposures elicited mucosal responses in both gastro-intestinal and respiratory tracts. Oral exposure stimulated significantly ($p<0.05$) superior mucosal response in the gastrointestinal tract, while intratracheal exposure stimulated significantly ($p<0.05$) superior mucosal response in the respiratory tract. Overall, oral exposure was able to stimulate the distance mucosal sites such as the respiratory tract and provides potential use for oral administration of live vaccine against haemorrhagic septicaemia.

Keywords: Mucosal immunity, oral administration, gastro-intestinal tract, respiratory tract, *Pasteurella multocida* B:2, buffalo calves

ARTICLE INFO

Article history:
Received: 4 June 2012
Accepted: 30 July 2013

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ISSN: 1511-3701

INTRODUCTION

Mucosal immune system is a critical component of animals and human defense against pathogenic organisms, especially organisms that use the mucosal surfaces as portal of entry. The mucosal membranes mediate an interface between the body and environment, which presents a variety of innate and adaptive immune defense mechanisms against microorganisms (Holmgren, 1991; Bowersock *et al.,* 1999; Gerdts *et al.,* 2001). *Pasteurella multocida* B:2 enters the hosts through the respiratory and/or oral routes leading to septicaemia (Rhoades & Rimler, 1991; Lee *et al.*, 2000). In the process of entering, *P. multocida* B:2 was found to stimulate the mucosal associated lymphoid tissue (MALT) (Siti-Raudah *et al*., 2005). Similarly, oral administered antigens have shown to elicit mucosal immune response in distant sites such respiratory, reproductive and urinary tracts (Bowersock *et al.,* 1999). This report describes the mucosal lymphoid tissue response in the gastrointestinal and respiratory tracts following oral administration of buffalo calves with live wild-type *P. multocida* B:2.

MATERIALS AND METHODS

Nine clinically healthy local buffalo calves of approximately 8 months of age were used in this study. The calves were de-wormed subcutaneously with ivomectin® (0.2mg/kg body weight) for three consecutive days, while nasal swabs were collected from all the calves at the time of arrival and then at weekly interval to ensure that they were free of *P. multocida* (Townsend *et al.,* 1998).

Meanwhile, stock culture of *P. multocida* serotype B:2 isolated from a bovine case of haemorrhagic septicaemia (HS) was used to prepare the inocula (Zamri-Saad *et al.,* 2006) of 10^9 colony forming unit (cfu)/ml (Alcamo*,* 1997). At the start of the experiment, the buffalo calves were further subdivided into three groups. All the calves were kept in individual pens but calves of groups 1 and 2 were kept in the same vicinity, while group 3 was kept separated. Calves of group 1 were exposed orally to 50ml of the inoculums while calves of group 2 were exposed intra-trachea to 5ml of the same inoculums. Calves of group 3 were the sham group that was exposed orally to 50ml of sterile PBS.

The calves were observed for adverse response or exaggerated clinical syndrome. Calves that showed severe clinical disease were euthanized; otherwise, the experiment was terminated on day 7 post-infection in accordance with the Guidelines for Animal Care and Use Committee, Universiti Putra Malaysia [AUP12R148]. During post-mortem examination, tissue samples from the nasal mucosa, bronchus and lungs of the respiratory tract, and tissues of oesophagus, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, colon, caecum and rectum of the gastrointestinal tract were collected and placed in 10% neutral buffered formalin for at least 12 h, embedded in paraffin, sectioned at 4µm, stained with hematoxylin and eosin [HE].

Attempts were made to identify the gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT) in at least 5 microscopic fields before the sizes of BALT and GALT were determined by measuring the diameters. The numbers of lymphocytes were determined by counting the cells using the NIS element imaging software version 2.33. Data were exported to excel and subsequent predictive analysis software (PASW) for analysis.

The mean numbers of lymphocyte and the length of the diameter of the lymphatic nodule between the orally exposed, the intra-tracheal exposed and the sham-dosed calves were compared using One-ANOVA and statistical significance was considered when $p<0.05$. All the analyses were done using PASW 17.

RESULTS

Bronchus-associated Lymphoid Tissue

Both oral and intra-trachea exposed calves of groups 1 and 2 showed the presence of BALT in the respiratory tract but the calves of group 1 did not have the lymphoid aggregate in the nasal mucosa (Table 1). The calves of group 3 had only few lymphocytes found scattered in the lung parenchyma. In general, the size of lymphoid nodules and number of lymphocytes of calves of group 2 was significantly ($p<0.05$) larger than those of groups 1 and 3, while group 1 was significantly ($p<0.05$) larger than group 3 (Tables 1 and 2).

Gut-associated Lymphoid Tissue

The calves of group 1 showed significantly ($p<0.05$) larger size of lymphoid nodules in reticulum, abomasums, duodenum, jejunum, ileum and rectum (Fig.1) when compared to

TABLE 1
Mean size (µm per area) of lymphatic nodule in the respiratory tract of buffalo calves exposed to live wild-type *Pasteurella multocida* B: 2

Organ	Oral	Intra-trachea	Oral Sham-dose
Nasal mucosa	$0.00\pm0.00^{a,b}$	300.01 ± 0.59^{a}	$0.00\pm0.00^{a,b}$
Bronchus	219.00 ± 1.00^{a}	515.38 ± 0.66^{a}	0.00 ± 0.00^{a}
Lung	136.28 ± 1.00^{a}	608.44 ± 1.07^{a}	0.00 ± 0.00^{a}

Values with different superscript in the same row signifies significant difference $p<0.05$

TABLE 2
Mean lymphocyte counts (per unit area) in the bronchus-associated lymphoid tissue (BALT) of buffalo calves experimentally exposed to live wild-type *Pasteurella multocida* B:2

Organ	Oral	Intra-trachea	Oral Sham-dose
Nasal mucosa	45.0 ± 1.00^{a}	106.0 ± 1.00^{a}	8.3 ± 1.51^{a}
Bronchus	256.0 ± 3.05^{a}	425.3 ± 0.26^{a}	28.6 ± 0.84^{a}
Lung	163.7 ± 0.89^{a}	359.3 ± 1.13^{a}	7.4 ± 0.56^{a}

Values with different superscript in the same row signifies significant difference $p<0.05$

the calves of groups 2 and 3 (see Table 3). When present, the sizes of lymphoid nodules in the duodenum and jejunum of calves of group 2 were significantly ($p<0.05$) bigger than those of group 3. Similarly, the numbers of lymphocyte in the reticulum, abomasums, duodenum, jejunum, ileum and rectum were significantly ($p<0.05$) more in the calves of group 1 compared to those of groups 2 and 3 (Table 4). The numbers of lymphocytes in the reticulum, abomasums and colon of calves of group 2 were significantly ($p<0.05$) more than the numbers in calves of group 3.

DISCUSSION

This study on the response of mucosal immunity of the respiratory and gastro-intestinal tracts was based on the presence and size of lymphoid nodules and the number of lymphocytes presence in those tracts. The lymphoid nodules have been described in

TABLE 3
Mean size of the lymphoid nodule (μm per area) along the gastro-intestinal tract of buffalo calves exposed to live wild-type *Pasteurella multocida* B:2

Organ	Oral	Intra-trachea	Oral Sham-dose
Oesophagus	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Rumen	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Reticulum	71.97±1.09[a]	0.00±0.00[a,b]	0.00±0.00[a,b]
Omasum	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Abomasum	230.00±2.64[a]	0.00±0.00[a,b]	0.00±0.00[a,b]
Duodenum	301.58±1.38[a]	89.95±0.14[a]	48.65±0.91a
Jejunum	442.61±0.51[a]	310.80±0.72[a]	0.00±0.00a
Ileum	899.92±5.63[a]	165.72±0.43[a]	314.88±0.24[a]
Colon	249.83±0.32[a]	233.37±0.54[a]	239.85±1.20[a]
Rectum	659.29±0.25[a]	0.00±0.00[a,b]	0.00±0.00[a,b]

Values with different superscript in the same row signifies significant difference $p<0.05$

TABLE 4
The mean number of lymphocytes in the lymphoid nodules (per unit area) of the gastrointestinal tracts of buffalo calves exposed to live wild-type *Pasteurella multocida* B:2

Organ	Oral	Intra-trachea	Oral Sham-dose
Oesophagus	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Rumen	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Reticulum	262.3±1.04[a]	42.3±0.30[a]	0.00±0.00[a]
Omasum	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]
Abomasum	436.0±2.00[a]	24.3±0.88[a]	0.00±0.00[a]
Duodenum	233.3±0.30[a]	0.00±0.00[a]	59.5±0.00[a]
Jejunum	259.3±1.13[a]	229.3±0.70[a]	101.2±2.54[a]
Ileum	474.3±1.47[a]	126.3±1.04[a,b]	126.3±3.25[a,b]
Colon	353.3±1.70[a]	346.3±1.13[a]	0.00±0.00[a]
Rectum	652.7±3.35[a]	0.00±0.00[a]	93.0±1.41[a]

Values with different superscript in the same row signifies significant difference $p<0.05$

the respiratory tract and gastro-intestinal tracts of calves (Saw *et al*., 2004; 2005), while the diffuse lymphoid tissue, solitary lymphocytes, intraepithelial lymphocytes, lymphoid nodule and Peyer's patches have been used as tools for assessment of mucosal immune response (Shewen *et al.,* 2009). Following oral and intra-trachea exposure of calves to live *P. multocida* B:2, both GALT and BALT were stimulated in the size and number of cells compared to the non-exposed calves. Needless to say, those exposed orally showed significantly better response by GALT while those exposed intra-trachea showed significantly better response by the respiratory tract. This finding re-emphases and confirms the previous reports that concluded the most effective way of inducing mucosal immunity is the delivery of antigen at the portal of entry of the microorganism (Bowersock *et al.,* 1999). In contrast to the speculations of problematic nature of ruminants gastro-intestinal mucosa and possibility of microbial degradation by the rumen (Shewen *et al.,* 2009), oral administration of live *P. multocida* B:2 elicited more diffuse lymphatic tissue count and wider lymphatic nodular diameter at the point of delivery as well as at the distant sites, as observed earlier following the intra-tracheal administration of *P. multocida* B:2 (Saw *et al*., 2005). This is evident by comparable lymphatic nodules in the respiratory tract and wider organ coverage in gastrointestinal tract.

Nevertheless, the responses by mucosal immunity of both gastro-intestinal and

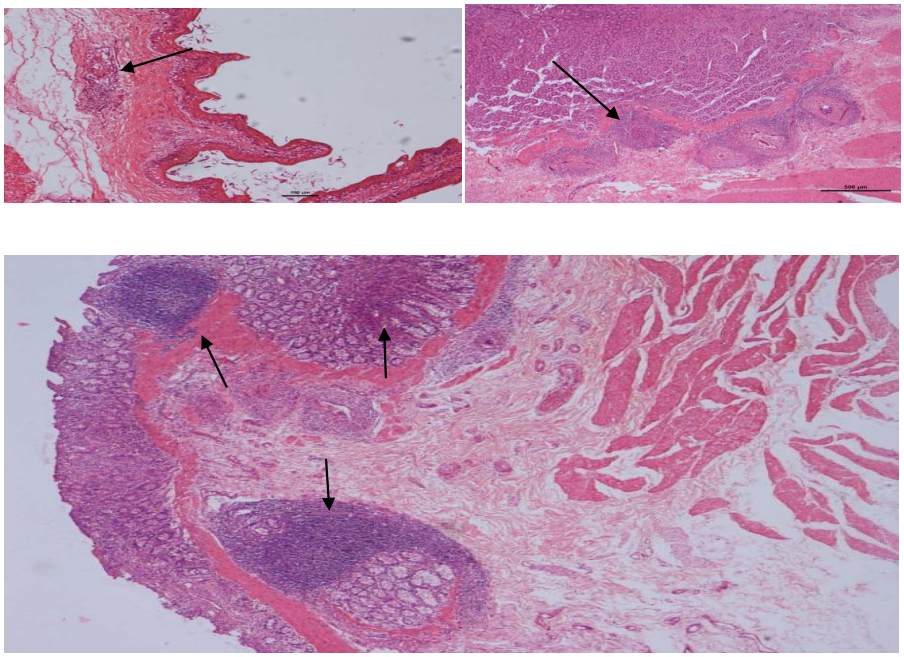

Fig.1: The gut-associated lymphopid tissue (arrows) observed in the reticulum (above, left), jejunum (above, right) and rectum of buffalo calves exposed orally to live wild-type *Pasteurella multocida* B:2

respiratory tracts were significant and in agreement to the earlier reports that there is a common mucosal pathway which enables administration of antigen at a mucosal site to stimulate mucosal immune response in the distant mucosal sites (Bowersock *et al.,* 1999). Therefore, further study should focus on stimulating mucosal associated lymphoid tissue using orally administered vaccine or antigen and assess the protective capacity provided by such vaccination programme in the control strategy of haemorrhagic septicaemia.

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