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RESEARCH ARTICLE

Ultrastructural Changes and Bacterial Localization in Buffalo Calves Following Oral Exposure to *Pasteurella multocida* B:2

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ABSTRACT

This study describes the ultrastructural changes and distribution of *P. multocida* B:2 in the respiratory and gastrointestinal tracts of buffalo calves following oral exposure and compared with intratracheal exposure. Nine 8-month old buffalo calves were divided into 3 groups. At the start of the experiment, dexamethasone (1mg/kg) was injected intramuscularly into buffaloes of all groups for three consecutive days. Then, calves of Group 1 were exposed orally to 50ml of the inoculum containing 10^9 colony-forming unit (CFU)/mL of live *P. multocida* B:2. Calves of Group 2 were exposed intra-tracheal to the same inoculum while calves of Group 3 were exposed orally to PBS. Transient and mild clinical signs were observed in calves of Groups 1 and 2. *Pasteurella multocida* B:2 was isolated from the nasal mucosa for up to 5 days post-infection. Only calf that was killed at 48 h post-infection had *P. multocida* B:2 in both respiratory and gastro-intestinal tracts showing ultrastructural changes typical of acute cellular injury, with degeneration of endothelium and vascular walls. There were deciliation in the respiratory and microvilli degeneration in the gastrointestinal tracts. The study concludes that oral exposure may not play major role in the development of hemorrhagic septicemia. Nevertheless, the buffalo calf that succumbed to hemorrhagic septicemia carried *P. multocida* B:2 in the gastro-intestinal organs.

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INTRODUCTION

Hemorrhagic septicemia (HS) is an acute, fatal, septicemic disease of cattle and buffaloes caused by specific serotypes of *Pasteurella multocida* and serotype B:2 is the main cause of the disease in Asia (Hopkins *et al*., 1998; Benkirane and DeAlwis, 2002). The disease occurs as devastating epizootics in many Asian and African countries, with resultant high morbidity and mortality leading to huge economic loss (OIE, 2008).

This is a typical septicemic disease that developed following the production of endotoxin (Horadagoda *et al.,* 2002; Farooq *et al.,* 2011). It has been reported that *P. multocida* has an affinity for respiratory tract mucus membrane (Lettellier *et al.,* 1991) and a better affinity to non-ciliated respiratory epithelial cells (Pijoan and Trigo, 1990). Therefore, the role of respiratory route in HS has been well established (Zamri-Saad and Shafarin, 2007). However, oral route may play a role in the epidemiology and pathology of HS (Abubakar and Zamri-Saad, 2012). Inflammatory reactions were observed along the gastrointestinal tract while *P. multocida* B:2 has been isolated from the small and large intestines of buffalo calves that died of HS. Although ultrastructural changes such as deciliation, vacuolation, capillary congestion and fibrin deposit in the upper respiratory of rabbits due to *P. multocida* D:1 and A:3 were previously reported (Al-Haddawi *et al.,* 1999; Al-Haddawi *et al.,* 2001), the detailed pathological changes in the organs of gastrointestinal tract, particularly the ultrastructural lesions in buffalo calves died of HS have, so far, not been described. This report describes the ultrastructural changes and bacterial localization in the respiratory and gastro-intestinal tracts of buffalo calves following oral exposure to *P. multocida* B:2.

MATERIALS AND METHODS

Animals: Nine clinically healthy buffalo calves of approximately 8 months of age were selected from a herd

with no history of HS. Upon arrival to the experimental house, the calves were kept in individual pen, fed on cut-grass and supplemented with palm kernel-based pellet at the rate of 400g/calf twice daily. Drinking water was given *ad libitum*. Access to veterinary care was available at all times and the animal well-being was assessed regularly, in accordance with the Guidelines of the Animal Care Ethics Committee, Universiti Putra Malaysia [AUP No. 12R148].

Immediately upon arrival, anthelmintic (Ivomectin, UK) was administered subcutaneously at the rate of 0.2mg/kg body weight for three consecutive days. Concurrently, deep nasal swabs were collected weekly and examined for the presence of *P. multocida* by bacterial isolation and polymerase chain reaction (Townsend *et al.*, 1996; Zamri-Saad *et al.*, 2006) to ensure that calves were free from *P. multocida* prior to start of the experiment. All calves were observed daily for signs of disease while temperature and respiratory rate were monitored to ensure that they were healthy. The experiment started only when all calves were found negative from carrying *P. multocida*, has low specific antibody titer as examined using the enzyme-linked immunosorbent assay (El-Eragi *et al.*, 2001) and appeared clinically healthy for a period of 4 weeks.

Inocula preparation: A stock culture of *P. multocida* serotype B:2 isolated from a bovine case of HS was used (Zamri-Saad *et al.*, 2006). The organism was cultured on blood agar at 37°C for 24 h before the brain heart infusion broth was seeded with four uniformly sized colonies and incubated, with shaking for 18 h. The bacterial concentration was determined using the method described previously (Alcamo, 1997) to produce an estimated infection dose of 10^9 colony forming unit (cfu)/ml of *P. multocida* B:2. The actual bacterial dose was estimated retrospectively by plating out serial dilutions of bacteria onto blood agar and was to be 1.7×10^9 cfu/ml.

Experimental design: At the start of the experiment, the buffalo calves were further divided into three groups. Calves of Groups 1 and 2 were kept in individual pens within the same vicinity while those of Group 3 were kept separated. Prior to the start of the experiment, all calves were subjected to intramuscular dexamethasone injections at the dose rate of 1mg/kg for three consecutive days. At the end of dexamethasone treatment, the calves of Group 1 were exposed orally to 50ml of the inoculum prepared earlier containing 10^9 cfu/ml of live wild-type *P. multocida* B:2. Calves of Group 2 were exposed intra-tracheal with 5ml of the inoculum containing 10^9 cfu/ml of live wild-type *P. multocida* B:2 (Khin *et al.*, 2010) while calves of Group 3 were exposed orally to 50ml of sterile PBS. All calves were observed at 6h, 12h and then at 12-hourly interval for clinical signs of HS for up to 7 days post-infection. Survivors were subsequently euthanized and necropsy was focused on brain, liver, kidney, spleen, heart, lymph nodes, and organs of the respiratory and gastro-intestinal tracts.

Bacteriological examination: Nasal and rectal swabs were taken prior to the start of the experiment and subsequently on daily basis for a period of seven days. Tissues of trachea, lungs and associated lymph nodes of the respiratory tract, each section of the entire gastrointestinal tract, the associated lymph nodes, the liver, kidney and spleen and brain samples were collected aseptically, cultured on blood agar and incubated at 37°C for 24 h. Isolates were identified as *P. multocida* on the basis of morphological criteria and biochemical test used previously (Peter *et al.*, 1996). Total DNA from representative sections of respiratory and gastrointestinal tract tissues were purified and used as templates in the PCR to determine the serotype of *P. multocida* (Zamri-Saad *et al.*, 2006).

Immunohistochemistry (IHC) for *P. multocida* B:2: Similar respiratory and gastro-intestinal tract organ samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4µm and subjected to IHC (Haritani *et al.*, 1989). The sections for IHC were dewaxed in oven and then in xylene before rehydration through graded alcohol from 100 to 70%. Rabbit anti-*P. multocida* B:2 antiserum and anti-rabbit IgG conjugated with HRP were diluted 1:1600 in PBS_T. Negative control slides were prepared using non-immunized rabbit serum.

Electron microscopic examination: The same samples of respiratory and gastrointestinal tracts were trimmed into $1cm^3$ for scanning electron microscopy (SEM) and $1mm^3$ for transmission electron microscopy (TEM) and placed in small glass vials. The samples were fixed in 4% glutaraldehyde buffered solution while soft tissues were placed in 2.5% glutaraldehyde buffered solution for 12-24hrs at 4°C. The samples were further processed for electron microscopic examination according to Cheville (1994). The samples for SEM were then viewed with ESEM scanning electron microscope (Philip XL30 ESEM) at accelerating voltage of 20Kv and photographed.

For TEM, ultrathin sections (80-90 nm) were cut from selected area with diamond knife on an ultra microtome (Leica, EM KMR 2UTC, Germany). Subsequently the ultrathin section were then mounted onto mesh-copper grids for ultrastructural studies, stained with uranyl acetate and lead citrate and then examined under transmission electron microscope (Leo 912 AB EFTEM, Omega Filtering system, Germany).

RESULTS

Clinical observations: Clinical signs observed in Groups 1 and 2 were characteristic of HS, which included anorexia, pyrexia, dyspnea, depression and salivation. However, signs were more pronounced in Group 2, which were seen within 24 h post-intra-trachea inoculation and led to euthanasia of one calf for humane reason. The signs were mild and transient in Group 1 and peaked on day 3 post-inoculation. At day 5 post-inoculation all surviving calves showed regressed clinical features, and on day 7 all survivors recovered. Calves of Group 3 show no clinical signs throughout the study period.

Bacterial isolation: *Pasteurella multocida* B:2 was cultured and identified from the nasal mucosa of all calves of Groups 1 and 2 on day 4 and from four calves (3 of Group 1 and 1 of Group 2) on day 5 post-infection. The

organism was also isolated from several organs of the calf that was euthanized at 48 h pi, which included the submandibular and/or brisket edema fluid, lung, heart, liver, kidney, spleen, brain, esophagus, abomasum, jejunum, ileum, cecum and rectum (Table 1). However, *P. multocida* B:2 was not isolated from any calf at days 0 and 7 post-infection.

Table 1: Bacterial distribution in buffalo calves following experimental exposure to *Pasteurella multocida* B:2

Organ/Tissue	Culture & Isolation		PCR detection		IHC localization	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Edema fluid	-	++++	NA	NA	NA	NA
Brain	-	++	-	-	-	-
Heart	-	+++	+	+	-	-
Liver	-	+++	+	-	-	-
Kidney	-	+++	-	-	-	-
Lung & Bronchus	-	++++	+	+	+	+
Spleen	-	+++	+	+	-	
Trachea	-	-	-	-	-	+
Bronchial lymph node	-	-	-	-	-	-
Esophagus	-	+	+	+	-	-
Rumen	-	-	-	-	-	-
Reticulum	-	-	-	-	-	-
Omasum	-	-	-	-	+	-
Messenteric lymph node	-	-	+	-	+	+
Duodenum	-	-	+	-	-	-
Jejunum	-	+	+	+	-	-
Ileum	-	+	+	+	-	-
Colon	-	-	-	-	-	-
Caecum	-	+	-	-	-	-
Rectum	-	+	-	-	-	-

Note that no bacteria was isolated, detected or localized from control animals killed after 7-day experiment. NA = Not Applicable, PCR= Polymerase chain reaction, IHC= Immunohistochemical

Immunohistochemical detection of *Pasteurella multocida* B: 2: *Pasteurella multocida* B:2 was detected in the medullary sinus and around the medullary cord and was found attached to lymphocytes of the mesenteric lymph nodes (Fig. 1A) of the calf that was euthanized at 48 h pi. The stratified squamous epithelium of conical papillae and in the lamina propria of the omasum (Fig. 1B) was also immunohistochemical positive. Similarly, *P. multocida* B:2 was detected in the bronchiolar epithelium of the lungs (Fig. 1C) and lamina propria and pseudo-stratified columnar epithelium of the trachea (Fig. 1D). Other survived calves had no *P. multocida* in any organ.

Ultrastructural observations: Generally, the ultrastructural changes observed in both Groups 1 and 2 suggested mild to moderate alternations characteristic of acute cellular injury. Surface examinations showed moderate to severe deciliation in the upper respiratory tract of Group 2 and extensive degeneration and disruption of microvilli in the gastro-intestinal tract of Group 1 (Table 2).

In the esophagus, large numbers of *P. multocida* B:2 were seen attached to the epithelium (Fig. 2). In the brain, there was loss of organelles and degeneration of myelinated fibers, dilatation of mitochondria and cytoplasmic vacuolation. Nasal mucosa of Group 2 showed swollen epithelium and bacterial attachment to the epithelial and goblet cells surfaces (Fig. 3) with deciliation and excessive mucus secretion. There was

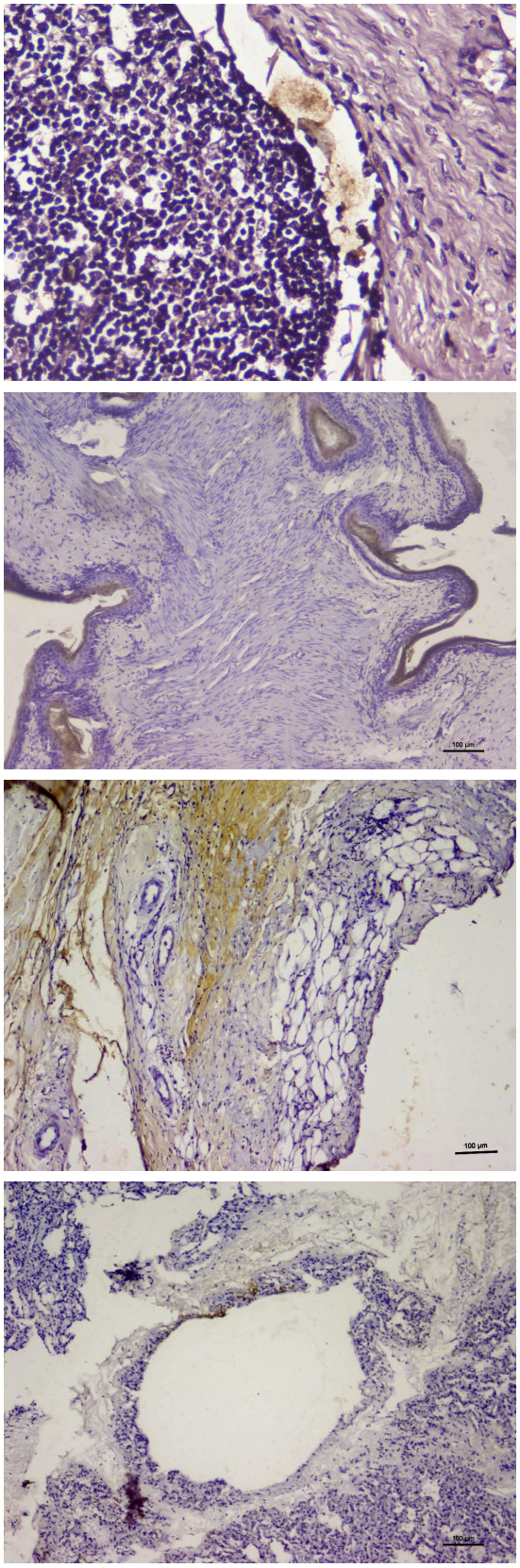

Fig. 1: Immunolocalization of *Pasteurella multocida* B:2 (arrows) in the medullary sinus and cord of the mesenteric lymph node (A), in the conical papillae and lamina propria of omasum (B), in the lamina propria and epithelium of the trachea (C) and in the bronchiolar epithelium in the lungs (D).

Table 2: Descriptive ultrastructural changes in the various organs/tissues examined

Organ/Tissue	Orally infected		Intratracheally infected	
	TEM	SEM	TEM	SEM
Nasal mucosa & Trachea	Central chromatin condensation	Mild loss of cilia *Pasteurella multocida* B:2 on the mucosa Goblet cell proliferation	Shortened cilia Irregular and invaginated nuclear membrane Peripheral and central chromatin condensation Nucleoplasmic rarefaction and loss of nucleolus	Complete loss of cilia with ulcerations Bacterial attachment with erythrocytes
Esophagus	Changes characteristics of cellular swelling, peripheral nuclear condensation.	Pure colonies of similar sizes of rods bacteria suggestive of *Pasteurella multocida* B:2	Mild cellular changes	Smooth surface with ridges
Intestinal segments	Showed capillaries with thin wall; Vascular interstitial space with intercellular vacuolation loss of cellular organelles and membrane; Degeneration and degranulation of mast cell	Severe disruption and loss of microvilli	Showed cellular changes characteristic of acute cellular swelling	Mild to moderate disruption and loss of microvilli
Brain (TEM Examine only)	Mild degeneration of myelinated nerve fibres; Vacuolations in large multipolar neuron; Cristolysis and dilatation of mitochondria		Moderate to severe degeneration of the myelinated nerve fibres; Vacuolations in large multipolar neuron; Cristolysis and dilatation of mitochondria	
Other organs (Lungs, Heart, Spleen, Liver and Kidney) (TEM examine only)	Degeneration; Loss of organelles; Cytoplasmic pallor with presence of erythrocyte in the intercellular space in the organs and/or tissue, Dilation of vessels and accumulation of plasma protein precipitation in the lumen and interstitium in the heart; Degeneration of endothelial cell and wall; In the case of the lungs the changes in the cytoplasm, mitochondria and presence of erythrocytes in the intercellular spaces were more severe in the intratracheally infected group compared to orally			

Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM).

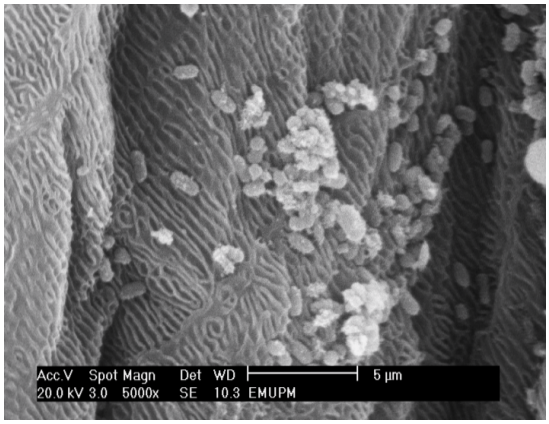

Fig. 2: Scanning electron micrograph of esophagus showing colonies of rod-shaped bacteria (0.7 x 2.0 µm) suggestive of *Pasteurella multocida* B:2

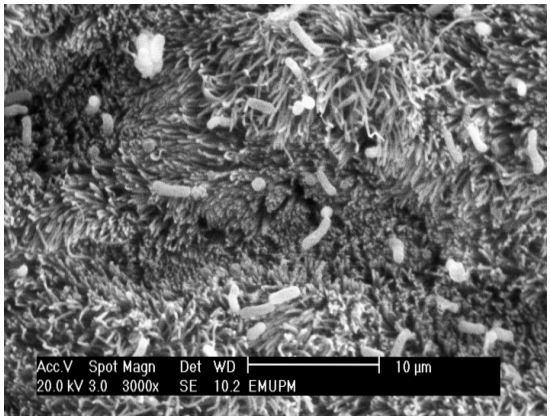

Fig. 4: Scanning electron micrograph of trachea showing *Pasteurella multocida* B:2 attached to the cilia.

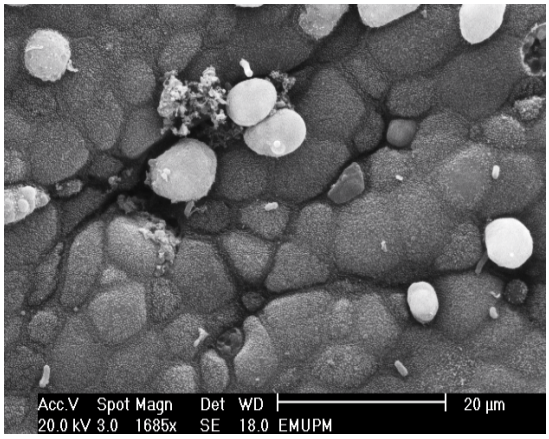

Fig. 3: Scanning electron micrograph of the nasal mucosae showing loss of cilia, distended intercellular space and attachment of *Pasteurella multocida* B:2 onto the mucosa and the goblet cell.

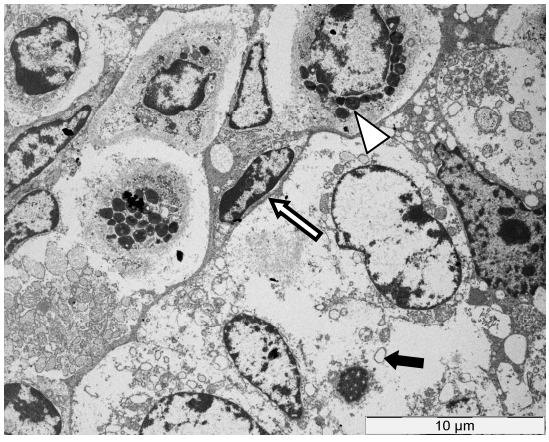

Fig. 5: Blood vessel with thin wall and distended surrounding vascular interstitial space, with endothelial cells (blank arrow) undergoing degeneration and degranulated mast cell (arrow head) in the jejunum. Note the intercellular vacuolation (arrow) and loss of cellular organelles and membrane.

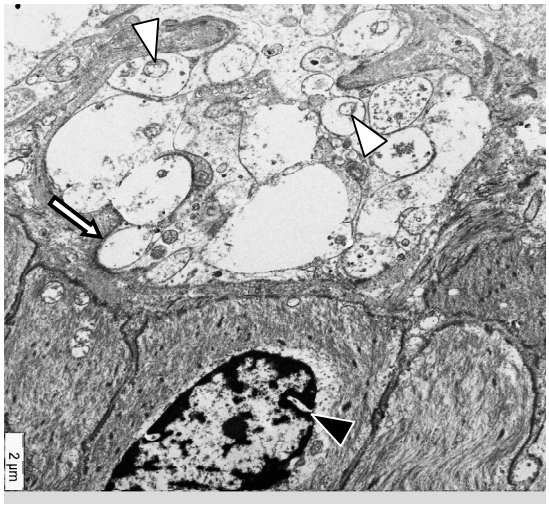

Fig. 6: Rectal epithelium showing cytoplasmic vacuolation (blank arrow), vessiculation (white arrow head) and rarefaction with peripheral nuclear condensation and invagination (black arrow head).

cytoplasmic vacuolation, vesiculation, expanded intercellular spaces with peripheral nuclear condensation and organelles leakage into extracellular spaces. Loss or shortened cilia, dilatation of endoplasmic reticulum and nuclear invagination were also observed. Scanning examination of the trachea showed large numbers of *P. multocida* B:2 attached to the cilia (Fig. 4). In the lung, transmission electron microscopic examination revealed severe cytoplasmic pallor, mitochondrial cristolysis, peripheral nuclear condensation, lysis of nuclear matrix and cytoplasmic vacuolation. In addition, there was loss of cell membrane of pneumocytes and presence of erythrocyte in the intercellular spaces. The changes observed in the mitochondria and vacuolation in the cytoplasm were typical of acute cellular swelling leading to subsequent events that were observed in the tissue. In the heart, endothelial wall were dilated and plasma protein precipitates were seen in the vascular lumen and the interstitium surrounding the vascular wall and the endothelial cells that are undergoing degenerative changes, which probably led to leakage of fluid into the interstitium and cardiac cells were disrupted. In small intestine, blood vessel wall was thin with distended surrounding perivascular area. The endothelial cells were also undergoing degeneration with nuclear chromatin condensation, and there were intercellular vacuolation, loss of cellular organelles, and mast cell degeneration and degranulation (Fig. 5). In the rectum there was cytoplasmic vacuolation, vesiculation, possibly due to persistent cellular injury leading to replacement of endoplasmic reticulum with vesicles and rarefaction with peripheral nuclear condensation and invagination (Fig. 6).

DISCUSSION

The clinical signs observed were typical of HS previously reported in both natural (DeAlwis, 1992; Khan *et al.*, 2011) and experimental (Horadagoda *et al.*, 2001; Khin *et al.*, 2010) situations. However, the signs observed following oral exposure were much milder. Nevertheless, the study described for the first time ultrastructural changes in the respiratory and gastro-intestinal tracts following oral exposure and compared with intratracheal route and/or respiratory route, which is believed to be the most common route of infection by *P. multocida* B:2. Generally, the changes observed suggest mild to moderate alterations, characteristic of acute cellular injury and in most cases endothelial cells were undergoing degeneration. Recent study implicated severe endotoxemia in the pathogenesis of HS in buffaloes, confirming the earlier observations (Horadagoda *et al.*, 2002). Endotoxemia and septicemia are associated with major hemodynamic alterations and organ injury (Watthaut *et al.*, 1994). Many inflammatory mediators such as cytokine, leukotriene's, platelet-activation factor, thomboxane and anaphytoxin were shown to be overwhelmingly generated during severe endotoxemia (Watthaut *et al.*, 1994), contributing in recruitment of neutrophils and tissue macrophages in lung, liver (Hewett *et al.*, 1992; Jaeschje *et al.*, 1991) and intestine (Musemeche *et al.*, 1991).

Following exposure to *P. multocida* B:2, the organisms were found in the respiratory and gastro-intestinal tracts for the first 5 days before they disappeared (Abubakar and Zamri-Saad, 2012). It is either the organisms were kill or suppressed by the body immune mechanisms or have developed evasion strategy by hiding in the tissue, this evasion strategy is currently being investigated in our laboratory. The calves showed regressed clinical signs and seem to have recovered. However, the one calf that had to be killed following severe clinical signs, showed presence of *P. multocida* B:2 in the lungs and along the gastro-intestinal tract. This finding suggests the possibility of clinically and/or sub-clinically ill animals may be shading the organisms through feces to susceptible animals but this needs to be further investigated. Ultrastructural changes indicated similar alterations in either orally and/or intratracheally infected calves, but milder in orally infected group.

Since all the calves exposed orally to *P. multocida* B:2 survived the infection and *P. multocida* B:2 were seen in the esophagus, localized along the gastro-intestinal tract and isolated from several intestinal segments, it is evident that transmission from gastrointestinal tract is strongly possible. Furthermore, it can be concluded that oral route of infection can manipulate the organisms better than intratracheal route, since the animals exposed via this route showed delayed, mild and transient clinical disease. This route of infection may play significant role in the epidemiology, especially in transmission of the organisms and consequently occurrence of hemorrhagic septicemia in ruminants.

Conclusion: Buffalo calves exposed oral or intratracheal show varying degree of lesions in the gastrointestinal tract. Although most calves survived the exposure, *P. multocida* B:2 was found in the organs of gastrointestinal tract. The shedding of *P. multocida* via the gastrointestinal tract of exposed buffalo calves is a possibility.

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