RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences
(P-ISSN 1595-093X/ E-ISSN 2315-6201)
Abubakar *et al*/*Sokoto Journal of Veterinary Sciences* (2016) 14(1): 21-27
http://dx.doi.org/10.4314/sokjvs.v14i1.4

Immunohistochemical evaluation of lesions in the gastrointestinal tract of buffalo (*Bubalus bubalis*) calves orally exposed to *Pasteurella multocida* B:2

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Abstract

The gastrointestinal lesions and bacterial distribution of buffalo calves were evaluated histologically using immunoperoxidase, following oral exposure to wild-type *Pasteurella multocida* B:2 at 10^9cfu/mL in phosphate buffered saline. The lesions were basically of mild to severe mucohaemorrhagic abomasitis and enteritis.The lesions were confirmed to be associated with the inoculated *P. multocida* B:2, using the immunoperoxidase technique. *P. multocida* B:2 antigen was detected not only in the bacterial clusters in the gastric pits, intestinal epithelia and capillaries, Brünner's glands and Crypt of Lieberkühn but was also seen interacting with infiltrating neutrophils and macrophages intracellularly and on the surface of erythrocyte in congested vessels and haemorrhages. We observed higher localization and distribution of the immunoperoxidase reaction with increased severity of lesions along the gastrointestinal tract. This suggest intensity increases with increased amount of *P. multocida* B:2 or antigen in the tissue, which possibly leads to increase tissue damage.

Keywords: Buffalo calves, Gastrointestinal lesions, Immunoperoxidase, Oral infection, *Pasteurella multocida* B:2
Received: 23-08- 2015 Accepted: 02-03-2016

Introduction

Pasteurella multocida B:2 causes an acute, fatal and septicaemic disease of cattle and buffaloes mostly referred to as haemorrhagic septicaemia (HS). It is an important disease in tropical regions of the world, especially in African and Asian countries. Conventional methods of identification such as serotyping, biotyping, antibiogram determination and pathogenicity as well as molecular methods and characterization are applied in parallel for rapid epidemiological investigations of HS outbreaks (Shivachandra *et al.*, 2011)

Immunoperoxidase has been recognised as an important tool not only to show the presence of antigen and vividly localize bacteria, it is also best at demonstrating antigen in dead solubilized bacteria (Haritani *et al*., 1989). Immunoperoxidase also has superior advantage of revealing the relationship of the lesions to bacteria distribution in the tissues (Haritani *et al*., 1990). However, discrepancies might exist in correlating bacterial isolation and immunoperoxidase localization in tissues, possibly due to difficulty in collecting samples at the same site for both bacteriological and immunoperoxidase examinations. Further, immunoperoxidase may be used to determine relative bacterial concentration in the tissue as samples containing low bacterial concentration may show negative reaction (Haritani *et al*., 1990).

Immunoperoxidase has been used to show relationship between pneumonic lesions caused by *Pasteurella multocida* and bacterial distribution in the tissue in experimental situation (Haritani *et al*., 1989). It has also beeb used to differentiate pneumonia due to either *Pasteurella multocida* and/or *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) in natural situations (Haritani *et al*., 1990). Previous studies have

showed strong evidence of possible localization of *Pasteurella multocida* B:2 in gastrointestinal tract (Abubakar *et al*., 2013).

This present study describes the relationship between lesions induced and bacterial distribution and/or localization along the gastrointestinal tract in buffalo calves exposed to oral *Pasteurella multocida* B:2.

Materials and Methods

Animals

Six clinically healthy buffalo calves, approximately 8 months-old were used. The animals were not previously immunized against haemorrhagic septicaemia. Upon arrival at the experimental house, anthelmintic (Ivomectin) was administered subcutaneously at the rate of 0.2mg/kg body weight for three consecutive days. Concurrently, deep nasal swabs were collected weekly for six (6) weeks and examined for the presence of *P. multocida* by bacterial isolation and polymerase chain reaction (Townsend *et al.,* 1998; Zamri-Saad *et al.,* 2006) to ensure the calves were free from *P. multocida* prior to start of the experiment. All calves were observed daily while temperature and respiratory rate were monitored. The experiment started after 4 weeks when all calves were negative for *P. multocida,* has low specific antibody titre as examined using the enzyme-linked immunosorbent assay (El-Eragi *et al*., 2001) and clinically appeared healthy.

The calves were kept in individual pens, fed on cut-grass and supplemented with palm kernel based pellet at the rate of 400g/calf twice daily. Drinking water was provided *ad libitum*. Access to veterinary care was available at all times and the animal`s well-being was assessed regularly.

Inocula preparation

A stock culture of *P. multocida* serotype B:2 isolated from a bovine case of HS (local laboratory source) was used (Zamri-Saad *et al.,* 2006). The organism was cultured on blood agar at 37^0C 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 by Alcamo (1997) to produce an estimated infection dose of 10^9 colony forming units (cfu)/mL of *P. multocida* B:2. The actual bacterial dose estimated by retrospectively plating out serial dilutions of bacteria onto blood agar was 2.56 x 10^9cfu/mL.

Experimental procedure

The buffalo calves were divided into two groups and were kept separately in individual pens in different vicinity with standard safety protocol to control possible bacterial escape at biosafety level 2. The calves of group 1 were exposed orally to 50 ml of the inoculum containing 10^9cfu/ml of live wild-type *P. multocida* B:2. Calves of group 2 were exposed orally to 50ml of sterile PBS.

All calves were observed at 6 h, 12h and then at 12 hourly interval for clinical signs of haemorrhagic septicaemia for up to 7 day post-infection in the first infection. The clinical signs observed include; demeanour, nature of respiration, nasal discharge, appetite, lacrimation and salivation. The rectal temperature and respiratory rates were also noted. Any calf that developed increased clinical signs with consistent high rectal temperature and laboured or dyspnoeic respiration was euthanized to minimise the degree and duration of suffering. Concurrently, nasal and rectal swabs were collected from all animals for possible isolation of *P. multocida* B:2. The same procedure was repeated two week post initial infection (day 14 post initial infection) and clinical signs, concurrently, rectal temperature, respiratory rate were noted, nasal and rectal swabs taken for next 7 days (day-21 post initial infection). Animals were observed for six weeks post initial exposure and experiment terminated at day-42 after first exposure.

Bacterial isolation

Nasal, rectal and tissue swab samples were taken aseptically on day zero (0) to seven (7) post initial infection and then on days 14 (second infection day) to 21. These were cultured on blood agar and incubated at 37^oC for 24 h. Isolates were identified as *P. multocida* based on biochemical tests as previously described (Carter & Chengappa, 1980; 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, as earlier described (Zamri-Saad *et al.*, 2006).

Post-mortem and Immunoperoxidase examination

At the end of the experiment all calves were euthanized in accordance with the guidelines of the Animal Care Ethics Committee, Universiti Putra Malaysia. During necropsy, attention was focused on the gastrointestinal tracts. Representative tissue samples were collected from the oesophagus, abomasum, duodenum, jejunum, ileum, colon, caecum, rectum and associated lymph nodes of the gastrointestinal tract, and placed in 10 % neutral buffered formalin for at least 12 hours. The samples were processed routinely for histopathology using the paraffin embedded technique sectioned at 4 µm and stained with haematoxylin and eosin [H & E]). For immunoperoxidase staining, briefly, the sections were dewaxed by placing in oven at 60^0C for 15

mins and then in xylene for 5 mins. Rehydration was done through graded alcohol from 100% to 70%, prior to quenching activity of endogenous peroxidase with 3% H_2O_2 in distilled water (v/v) for 15 mins. Subsequently, slides were rinsed in PBS containing 0.05% v/v $Tween_{20}$ (PBS_T), and then digested with proteinase k (Sigma-Aldrich, USA) for 10 mins at 37^0C for antigen retrieval. Non-specific binding was blocked by incubation in 5% bovine serum albumin in PBS (v/v) at room temperature for 30 mins. Rabbit anti-*P.multocida* B:2 antiserum diluted 1:1600 of PBS_T was applied and incubated for 1h at 37^0C and then washed with PBS_T (3 X 5 mins). Anti-rabbit IgG conjugated with HRP was added for 30 mins at room temperature, slides were washed in PBS_T (3 X 5 mins) and incubated for at least 8 mins or till colour change with 3, 3'-diaminobenzidine (DAB) chromogen prior to being washed in water and counterstained with haematoxylin. Negative control slides were prepared using non-immunized rabbit serum. The intensity of bacterial and/or antigen localization were graded as follows; Very strong (Golden brown, +++); Strong (Brown, ++); Weak (Light brown, +) and no detectable reaction (-). While the distribution was scored three (3) when diffuse in distribution, two (2) when multifocal, one (1) when focal or minimal and zero (0) for no detectable reaction. Lesions were scored based on the method described by Abubakar & Zamri-Saad (2011). Briefly, severe lesion scored three (3), moderate scored two (2), mild scored one (1) and no detectable lesion scored zero (0). All experimental protocols and designs were approved by Animal Care and Use Committee of our institution.

Results

Clinical observations

Calves of group 1 showed signs typical of mild to severe haemorrhagic septicaemia including; pyrexia, anorexia, salivation, dyspnoea, congested ocular mucous membrane, submandibular oedema. One calf had diarrhea with severe clinical signs which incude; pyrexia, anorexia, salivation, dyspnoea, congested ocular mucous membrane, submandibular oedema and diarrhea but died 48 hours post inoculation. Two weeks after second infection, all calves survive infection and showed only mild clinical signs of reduced appetite and transient dullness.

Bacterial isolation

Pasteurella multocida B:2 was isolation from rectum on days 3-5 and in the nasal cavity at day-4 post infection and on days 17-19 (corresponding to days 4-6 post second inoculation), and in the lungs, liver, kidney, spleen, abomasum, duodenum, jejunum, ileum, caecum, colon and rectum of calf that died 48 hours post infection.

Pathology and immunohistochemical detection

Grossly, the abomasums were moderate to severely congested with areas of focal, petechial and ecchymotic haemorrhages; the intestines were mildly to moderately congested and haemorrhagic and contained large amounts of mucous. The rectum was congested with multifocal areas of petechial haemorrhages. The mesenteric lymph nodes were moderately enlarged, congested and oedematous (Table 1). Histologically, abomasum showed severe congestion and haemorrhages, disruption of epithelial lining, necrosis and fluid accumulation in submucosal glands and focal diffuse lymphatic tissue formation; *P. multocida* B:2 were immuno-detected in gastric pits, seen in the interglandular

Table 1: Severity of lesions and extent of specific immunohistochemical distribution in orally infected buffalo calves

Calf ID	Abomasum (distribution)	Small intestine (distribution)	Large intestine (distribution)	Mesenteric lymph node (distribution)
41(G1)*	3(3)	3(2)	2(2)	2(1)
42(G1)	1(0)	2(1)	2(1)	1(1)
43(G1)	1(0)	1(1)	1(1)	1(0)
44(G2)	1	1	1	1
45(G2)	0	1	0	0
46(G2)	0	1	0	0

Histological lesions and immunoperoxidase detection distribution in Buffalo calves, both scored 0-3, where, 0 =no significant lesions/detection and 3 severe lesions/diffuse distribution. Scores were assigned to individual organ for each calf. G1, calves infected with *P. multocida*B:2 Orally; G2, calves administered sterile PBS (negative control). Numbers in parentheses are distribution of specific immuno-reaction of *P. multocida*B:2. All calves of G2 showed negative immuno-reaction. *Died at 48 h after infection.

Table 2: Showing Spearman's rho (r) correlation between severity of lesions in organs and distribution of immunoperoxidase reaction with *P. multocida* B:2 or antigen

Organs	Spearman's rho (r)	p-value
Abomasum	1.000	0.001
Small Intestine	0.866	0.333
Large Intestine	0.500	0.667
Mesenteric lymph nodes	0.500	0.667

Guildford's rule of strength of correlation [Negligible (r=0.0-0.29), Low (r=0.3-0.49), Moderate (r=0.5-0.69), Strong (r=0.7-0.89), Very strong (r=0.9-1.0)]

Table 3: Showing specific immunohistochemical reaction and bacterial distribution in lesions induced by experimental oral exposure to *Pasteurella multocida* B:2

Organ/Lesion	Bacterial distribution in the tissue and/or lesion	Immunoperoxidase reaction intensity
Abomasum (acute mucohaemorrhagic abomasitis)	Gastric pits	+++
	Epithelial capillaries and	+++
	Interglandular space	+++
	Submucosal capillaries	++
	Submucosal glands	+
Duodenum (acute mucohaemorrhagic duodenitis)	Epithelial surface and capillaries	+++
	Brünner's glands (submucosal glands)	++
	Intersubmucosal glandular space	++
	Interacting with erythrocytes	+
Jejunum (acute mucohaemorrhagic jejunitis)	Epithelial surface and capillaries	+++
	Submucosal glands	+++
Ileum and Ileocaecocolic Junction (acute mucohaemorrhagic ileitis)	Epithelial surface and capillaries	++
	Submucosal glands	++
	Diffuse lymphatic tissues and lymphatic nodules	++
Caecum (acute mucohaemorrhagic typhlitis)	Epithelial surface	++
	Crypt of Lieberkühn	++
	Cytoplasm of Infiltrating neutrophils and macrophages	++
	Surfaces of erythrocytes	+
Colon (acute mucohaemorrhagic colitis)	Crypt of Lieberkühn	+
	Interglandular space	++
	Blood capillaries in the muscularis mucosae	+
	Cytoplasm of infiltrating macrophages, neutrophils and plasma cells	++
Rectum (acute haemorrhagic proctitis)	Epithelial surface and blood vessels	++
	Intestinal glands	+
Mesenteric lymph nodes	Capsular blood vessel	++
	Medullary cord, trabeculae and diffuse lymphatic tissue	+

+++ = Very strong reaction (Golden brown); ++= Strong reaction (Brown); += Weak reaction (Light brown)

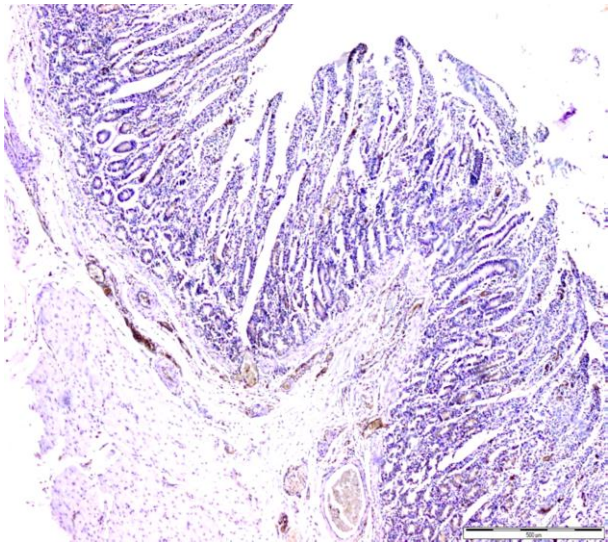

Plate 1: Segment of Small Intestine showing immunolocalization of *Pasteurella multocida B:2* and/or antigen in the epithelia surface, lamina propria, capillaries, blood vessel and intestinal glands. Bar= 500µm IHC counter stain with haematoxylin

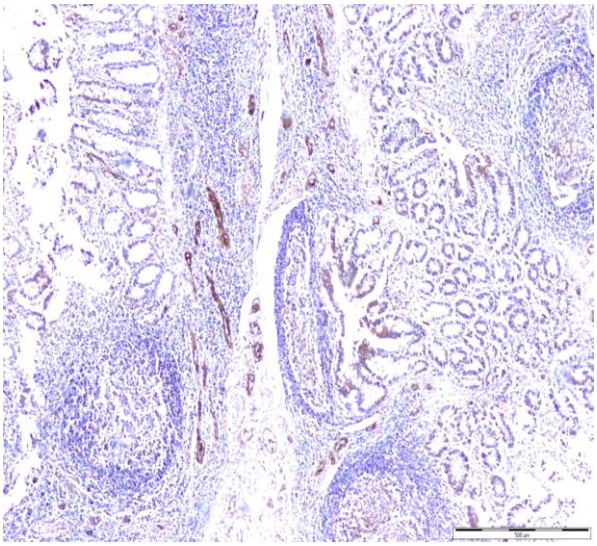

Plate 2: Segment of Small Intestine showing immunolocalization of *Pasteurella multocida* B:2 and/or antigen in the intestinal glands, gut associated lymphoid tissue and blood vessels. Bar= 500µm IHC counter stain with haematoxylin

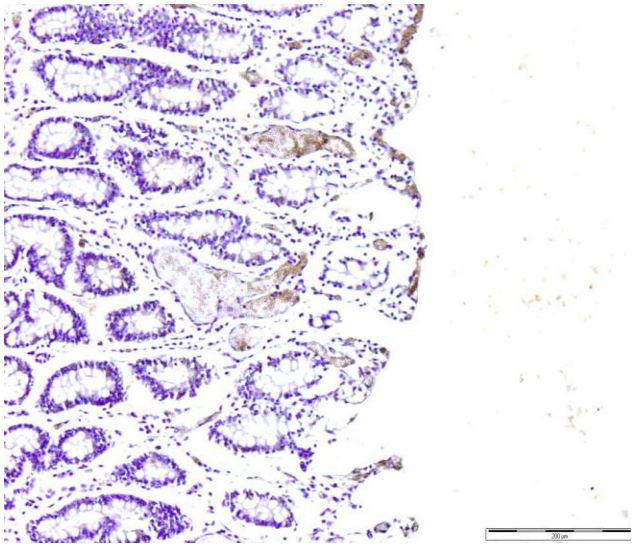

Plate 3: Segment of large intestine showing immunolocalization of *Pasteurella multocida B:2* and/or antigen in the epithelial surface, blood capillaries and intestinal glands. Bar= 50µm IHC counterstained with haematoxylin

space and in blood capillaries (Table 3) with very strong bacterial or antigen distribution correlation with lesion severity (r=1.0, p=0.001) (Table 2). Small intestines also showed moderate congestion and haemorrhages in disrupted epithelia and severe infiltration with inflammatory cells, predominately neutrophils and macrophages with few plasma cells. Bacterial organisms were seen in the epithelial surface (Plate 1), submucosal glands (Plate 2) and detected on the surface of erythrocytes in the duodenum. Ileum showed severe infiltration of aggregate lymphoid nodules with moderate congestion and haemorrhages in the lamina propria and severe inflammatory infiltrates in the epithelial surface. Bacterial clusters were seen on the epithelial surface and capillaries, and detected in lymphoid nodules and submucosal glands (Plate 2). Correlation of bacterial distribution with lesion severity in the small intestine was strong (r=0.866, p=0.333). The large intestine showed extensive necrosis of the intestinal gland, proliferation of goblet cells, disrupted epithelia with infiltration of neutrophils and macrophages. Bacterial were on the epithelial surface in the intestinal glands, the cytoplasm of neutrophils and macrophages and on the surface of erythrocytes of the vascular wall in the caecum and colon (Plate 3), with moderate correlation (r=0.5, p=0.667) when lesion scores were compared with distribution of *Pasteurella multocida* B:2 (Table 2). All calves in G2 showed negative reaction to *P. multocida* B:2.

Discussion

The present study described the relationship between lesions and bacterial distribution and localization in the gastrointestinal tract of buffalo calves exposed orally to *Pasteurella multocida* B:2. Lesions in the gastrointestinal tract due to *Pasteurella multocida* B:2 have been reported earlier (Abubakar & Zamri-Saad, 2011). The clinical features, gross and histologic changes observed in this study were characteristics of haemorrhagic septicaemia. Immunoperoxidase evaluation of pneumonic lesions induced by *Pasteurella multocida* have been described (Haritani *et al*., 1989) and have been used to

differentiate between pneumonic lesions caused by *Pasteurella multocida* and/or *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) (Haritani *et al*., 1990). *Pasteurella multocida* B:2 has been detected using immunoperoxidase technique in the respiratory tract of goats experimentally exposed intratracheally (Shafarin *et al*., 2009). Immunohistochemical evaluation of lesions in gastrointestinal tract induced by either natural or experimental *Pasteurella multocida* B:2 is probably reported for the first time.

We observed higher localization and distribution of the immunoperoxidase reaction with increased severity of lesions along the gastrointestinal tract, this suggests intensity increases with increased amount of *P. multocida* B:2 or antigen in the tissue, which possibly lead to increased tissue damage. The level of severity in the different segments of the gastrointestinal tract may suggest levels of bacterial concentration and potential sites of bacterial attachment, colonisation and proliferation since strong immunoperoxidase reaction implie higher bacterial concentration (Haritani *et al*., 1990). Furthermore, all surviving calves appeared clinically healthy at the end of the experiment and showed mild bacteria distribution or negative immunoperoxidase reaction. In addition, they showed mild to moderate gross and histologic changes. This may possibly be due to increase in neutralising antibodies, developed after first exposure. However, these may not suggest complete absence of *P. multocida* B:2 as low bacterial concentration may not be detected by immunoperoxidase (Abubakar *et al*., 2013). Bacterial concentration has been reported to increase in stressful situations led to increased bacterial proliferation and colonisation in immuno-suppressed or naive animals (Shafarin *et al*., 2008). These calves showed mild and transient clinical features post second exposure, which could be due to development of strong immunity following initial exposure. However, *P. multocida* B:2 was isolated in the rectum which may possibly be excreted in the faeces.

These findings re-emphasise previous reports of possible transmission of *P. multocida* B:2 through the gastrointestinal tract and confirmed bacterial isolation and immuno-detection of *P. multocida* B:2 or antigen in the gastrointestinal tract. Further understanding of the response of the gastrointestinal system to *P. multocida* B:2 will elucidate the pattern of occurrence and possibly transmission and maintenance of the organism in animal population. The findings will open a new paradigm in control and prevention strategy of haemorrhagic septicaemia.

Acknowledgement

This study was supported by Science Fund Grant, Ministry of Agriculture, Malaysia.

References

Abubakar MS & Zamri-Saad M (2011). Clinico-pathological changes in buffalo calves following oral exposure to *Pasteurella multocida* B:2. *Basic and Applied Pathology,* **4**(4): 130-135.

Abubakar MS Zamri-Saad M & Jasni S (2013). Ultrastructural changes and bacterial localization in buffalo calves following oral exposure to *Pasteurella multocida* B:2. *Pakistan Veterinary Journal*, **33**(1): 101-106.

Alcamo IE (1997). *Fundamentals of Microbiology* . Fifth edition, Menlo Park, California: Addison Wesley Longman. Pp 35-45.

Carter GR & Chengappa MM (1980). Hyaluronidase production by type B *Pasteurella multocida* from cases of haemorrhagic septicaemia. *Journal of Clinical Microbiology,* **11**(1): 94-96.

El-Eragi AMS, Mukhtar MM & Babiker SH (2001). Specific antibodies of *Pasteurella multocida* in newborn calves of vaccinated dams. *Tropical Animal Health and Production,* **33**(4): 275-283.

Haritani M, Narita M, Murata H, Hashimoto K & Takizawa T (1989). Immunoperoxidase evaluation of pneumonic lesions induced by *Pasteurella multocida* in calves. *American Journal of Veterinary Research,* **50**(12): 2162-2167.

Haritani M, Nakazawa M, Hashimoto K, Narita M, Tagawa Y & Nakagawa M (1990). Immunoperoxidase evaluation of relationship of necrotic lesions and causative bacteria in lungs of calves with naturally acquired pneumonia. *American Journal of Veterinary Research,* **50**(12): 1975-1979.

Peter EJ, Martha J & Carolyn JH (1996). A minimal medium for growth of *Pasteurella multocida. FEMS Microbiology Letters,* **140**(2-3): 165-169.

Shafarin MS, Zamri-Saad M, SitiKhairani B & Saharee AA (2008). Proliferation and

transmission patterns of *Pasteurella multocida* B:2 in goats. *Tropical Animal Health and Production* **40**(5): 335-340.

Shafarin MS, Zamri-Saad M, SitiKhairani B & Saharee AA (2009). Pathological changes in the respiratory tract of goats infected by *Pasteurella multocida* B:2. *Journal of Comparative Pathology,* **140**(2-3): 194-197.

Shivachandra SB, Viswas KN & Kumar AA (2011). A review of hemorrhagic septicemia in cattle and buffalo. *Animal Health Research Reviews,* **12**(1): 67–82.

Townsend KM, Frost AJ & Lee CW Papadimitrious JM & Dawkins HJS (1998). Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *Journal of Clinical Microbiology,* **36**(4): 1096-1100.

Zamri-Saad M Ernie A & Sabri MY (2006). Protective effect following intranasal exposure of goats to live *Pasteurella multocida B:2*. *Tropical Animal Health and Production*, **38**(7-8): 541-548.