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Effect of Dexamethasone on Protective Efficacy of Live gdhA Derivative *Pasteurella multocida* B:2 Vaccine

[1,2]O. Rafidah and [1]M. Zamri-Saad

[1]Research Centre for Ruminant Diseases, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[2]Department of Veterinary Services and Animal Industry, Kota Kinabalu, Sabah, Malaysia

Corresponding Author: M. Zamri-Saad, Research Centre for Ruminant Diseases, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia Tel: (603) 89463453

ABSTRACT

The effect of dexamethasone (Dex) on protection of vaccinated buffaloes against challenge by wild-type *Pasteurella multocida* B:2 was studied. Sixty field buffaloes were selected and divided into 3 groups. Buffaloes of group 1 was exposed intranasally to 10^6 CFU mL^{-1} of the gdhA derivative *P. multocida* B:2. Buffaloes of group 2 were not vaccinated but were allowed to commingle with the buffaloes of group 1. Buffaloes of group 3 remained un-vaccinated and were kept separated. Serum samples were collected prior to and at 2-monthly intervals post-exposure for 12 months to determine the levels of IgG. At the end of the 12-month period, three buffaloes from each group were selected and injected intramuscularly with dexamethasone at the dose rate of 1 mg kg^{-1} b.wt. for 3 consecutive days. Following dexamethasone treatment, all selected buffaloes were challenged subcutaneously with 10^9 CFU mL^{-1} of wild-type *P. multocida* B:2. There was significant ($p<0.05$) increase in the IgG levels in groups 1 and 2 following the intranasal exposure. The dexamethasone treatment resulted in significant ($p<0.05$) and rapid reduction in the IgG levels in the control group 3. Groups 1 and 2 showed insignificant ($p>0.05$) reduction. Following challenge, all control group 3 succumbed the infection while buffaloes of groups 1 and 2 survived the challenge. In conclusion, dexamethasone injections did not significantly reduce the protective efficacy of the live attenuated gdhA derivative *P. multocida* B:2 but significantly predisposed unvaccinated buffaloes to the infection.

Key words: Live gdhA derivative *Pasteurella multocida* B:2, protective efficacy, dexamethasone

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute and highly fatal infection of ruminants in tropical countries (Kamarudin, 2005) mainly affects cattle and buffaloes. It is caused by specific serotypes of *P. multocida*, which is B:2 in Asia and E:2 in Africa (De Alwis, 1999). Carter and De Alwis (1989) concluded that the carriers and naturally acquired immunity are closely related and have an important bearing on the epidemiological cycle of the disease in endemic areas. Therefore, stress plays a role in HS outbreaks through activation of latent carriers (De Alwis *et al*., 1990).

Various stressful conditions such as transportation, climate change and housing management contribute significantly to the reduction of the defense mechanism, leading to outbreaks of HS (Zamri-Saad, 2005). The immunosuppression following stress increases the susceptibility to

infectious disease (Anderson *et al.*, 1999) by inhibiting a variety of macrophage functions (Goppelt-Struebe *et al.*, 1989). Glucocorticoid has been designated as the stress hormone because its level in circulation rises sharply in response to stress (Hardy *et al.*, 2005).

Although vaccination using plain bacterin, alum precipitated and oil adjuvant vaccine has been used routinely to control HS, outbreaks among vaccinated animals are not uncommon. Nevertheless, the naturally acquired immunity has been shown to be superior to vaccinial immunity (De Alwis, 1999). Therefore, a live-attenuated vaccine that has all the properties of the field strains but lost its virulence is expected to confer more solid, effective and long-term protective immunity (Myint *et al.*, 1987; Khin *et al.*, 2009; Rafidah *et al.*, 2010).

Dexamethasone, a potent synthetic glucocorticoid has been used extensively in experimental models evaluating potential immunomodulators in cattle, and is thought to mimic the immunosuppression induced by transport stress (Roth and Flaming, 1990). Therefore, in this report, dexamethasone was used to imitate glucocorticoid to induce the immunosuppressive action among buffaloes for study on protective efficacy of the live gdhA derivative *P. multocida* B:2 against challenge with the wild type *P. multocida* B:2.

MATERIALS AND METHODS

Preparation of gdhA derivative *Pasteurella multocida* B:2: The gdhA derivative *P. multocida* B:2 prepared earlier by Sarah *et al.* (2006) was grown on blood agar containing kanamycin-streptomycin for 46 h at 37°C. Then, 30 colonies were selected and inoculated into 500 mL brain heart infusion broth containing kanamycin and streptomycin and incubated at 37°C for 46 h with gentle shaking (150 rpm). The viable bacterial count was determined using the plate count technique of Alcamo (1997). The concentration was re-adjusted to give the final concentration of 10^6 CFU mL^{-1}.

Safety of the newly prepared live vaccine was tested in mice. Six white mice, weighing 10-20 g were inoculated intraperitoneal with 0.5 mL of the freshly prepared live vaccine. The mice were then observed for 10 days where survival of all mice indicated a safe vaccine.

Virulent wild-type *Pasteurella multocida* B:2: Wild-type *P. multocida* B:2 was isolated earlier from a buffalo that died in an outbreak of haemorrhagic septicaemia. The stock was subcultured onto blood agar without antibiotics before being propagated in BHI broth for 18 h at 37°C. The viable bacterial count was determined using the standard plate count technique and re-adjusted to 10^9 CFU mL^{-1} with sterile PBS.

Experimental procedure: Sixty healthy field buffaloes from a non-endemic area in Sabah that were kept grazing extensively with minimal human intervention were selected and divided into 3 groups of 20 buffaloes per group. Buffaloes of group 1 were exposed intranasal to 5 mL inoculum containing 10^6 CFU mL^{-1} of live gdhA derivative *P. multocida* B:2 by spraying directly into both nostrils. A booster dose was similarly administered 2 weeks after the first exposure. Buffaloes of group 2 were not vaccinated but were allowed to graze and commingle with buffaloes of group 1 in the same grazing area throughout the experimental period. Buffaloes of group 3 were similarly exposed to PBS and were allowed to graze separately from buffaloes of groups 1 and 2 throughout the experimental period.

Serum samples were collected randomly from a minimum of 30% of the buffaloes of each group prior to and at 2-month intervals post-vaccination for a period of 12 months. At the end of the

12-month post-vaccination, 3 buffaloes were randomly selected from each group and kept at a designated confinement facility. All selected buffaloes were injected intramuscularly with dexamethasone at the rate of 1 mg kg^{-1} for three consecutive days to induce stress. Serum samples were collected prior to and daily following the dexamethasone injections. At the end of the 3-day dexamethasone injections, all buffaloes were challenged subcutaneously with 1 mL inoculum containing 1.21×10^9 CFU mL^{-1} of live wild-type *P. multocida* B:2.

Following challenge, all buffaloes were monitored at 6 hourly intervals for assessments on the severity of clinical signs. Buffalo with continued high body temperature and/or depressed demeanor were humanely killed. On day 4 post-challenged, all surviving buffaloes were killed and post-mortem examinations were immediately conducted. The Animal Care and Use Committee of Universiti Putra Malaysia approved the experimental procedure.

Samplings: Serum samples were collected from all groups at 2-monthly intervals for 12 months and on daily basis following dexamethasone injection. The sera were subjected to indirect ELISA (Rafidah *et al*., 2011) to determine the group serum IgG level and pattern against *P. multocida* B:2.

Samples of lungs, hearts, lymph nodes, kidneys, spleens, livers and edema fluid were taken aseptically for isolation of *P. multocida* B:2 onto SBA (Oxoid, UK) plates and incubated for 24 h at 37°C. *Pasteurella multocida* was identified based on the criteria and biochemical tests (Jablonski *et al*., 1996) while suspected colonies of *P. multocida* B:2 were confirmed by multiplex PCR using two primer sets; KMTI [KMT1T7-5'-ATC CGC TAT TTA CCC AGT GG-3' and KMT1SP6-5'-GCT GTA AAC GAA CTC GCC AC-3'] and 6b [KTT72-5'-AGG CTC GTT TTG ATT ATG AAG-3' and KTSP61-5'-ATC CGC TAA CAC ACT CTC-3'] (Zamri-Saad *et al*., 2006).

Immunohistochemistry: Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3 μm and subjected to immunohistochemistry (IHC) technique to detect the presence of bacteria in tissues. The sections were dewaxed by placing in oven at 60°C for 15 min and then in xylene for 5 min. After that, rehydration was done through graded alcohol from 100% to 70%, prior to quenching activity of endogenous peroxidase with 3% hydrogen peroxidase (H_2O_2) in methanol 0.05% v/v Tween 20 (PBS_T) and digested with 0.1% trypsin for 1 h at 37°C for antigen retrieval. The non-specific binding was blocked by incubation in 5% Bovine Serum Albumin (BSA) in PBS_T (v/v) at room temperature for 30 min. Rabbit anti-*P. multocida* B:2 antiserum, diluted 1:800 in PBS_T was added (3×5 min). Then, the anti-rabbit IgG conjugated with HRP, diluted 1:300 in PBS_T was added for 30 min at room temperature. Slides were washed in PBS_T (3×5 min) and incubated for at least 8 min or until colour change with 3,3'-diaminobenzidine (DAB) chromogen prior to being washed in water and counter stained with haematoxylin. Negative control slides were prepared using non-immunized rabbit serum.

Statistical analysis: Data of the serum IgG levels were expressed as Mean±SD. The different treatment groups were compared by analysis of variance with LSD All-pairwise comparisons test (Statistix 8) and were considered significant at $p<0.05$.

RESULTS

Serum IgG levels: Figure 1 shows the IgG patterns of the three buffalo groups; the vaccinated group 1, the commingled group 2 and the non-vaccinated group 3. At the beginning of the trial,

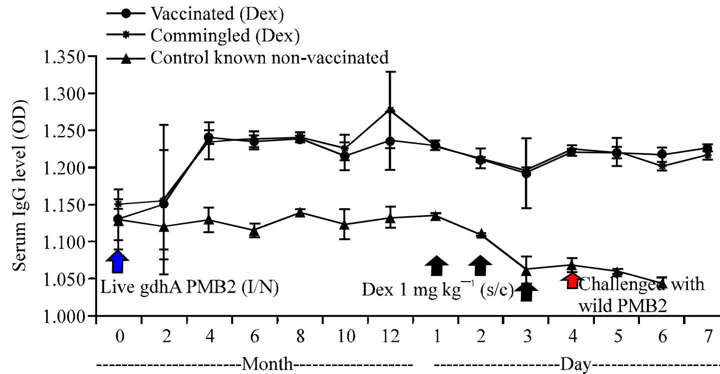

Fig. 1: Serum IgG profiles of field buffaloes 12 months post-vaccination with live gdhA derivative *P. multocida* B:2 (blue arrow). Dexamethasone was injected for three consecutives days (dark arrows) before all buffaloes were challenged with the wild-type *P. multocida* B:2 (red arrow)

the IgG levels showed non-significant ($p>0.05$) differences among the three groups. However, at 2 months post-vaccination and following booster, the serum antibody levels of group 1 had significantly ($p<0.05$) increased and remained high throughout the 12-month study period. Following dexamethasone injections, the serum IgG levels decreased insignificantly ($p>0.05$), particularly on the second and third days of treatment. Prior to challenge with wild type *P. multocida* B:2, the serum IgG increased significantly ($p<0.05$).

At 2 months post-vaccination, the serum IgG level of group 2 remained as pre-vaccinated level ($p>0.05$) before it significantly ($p<0.05$) increased at 4 months and remained high until the end of the 12-month study period. Prior to dexamethasone injections, the serum IgG level significantly ($p<0.05$) decreased and decreased further but insignificantly ($p>0.05$) on the second and third days post-dexamethasone injections. Prior to challenge on day 4, the serum IgG level increased insignificantly ($p>0.05$) but remained significantly ($p<0.05$) higher than the control non-vaccinated group (Fig. 1).

The serum IgG levels of control non-vaccinated buffaloes of group 3 remained significantly ($p<0.05$) low throughout the study period. On challenge day 4, the serum IgG level was significantly ($p<0.05$) lower than the levels prior to the dexamethasone injections.

Clinical observations: All buffaloes of groups 1 and 2 survived the challenge with wild-type *P. multocida* B:2. They remained asymptomatic with no significant ($p>0.5$) increase in rectal temperature. The appetite and rumination remained normal.

In contrast, all control non-vaccinated buffaloes developed moderate to severe classical signs of HS. These included dullness, depression, anorexia, salivation, lacrimation, dyspnoea and congested mucous membranes. Those buffaloes presented with severe clinical signs were killed humanely at 36, 43 and 48 h post-challenge.

Post-mortem findings: All control non-vaccinated buffaloes of group 3 that were killed between 36 and 48 h post-challenge showed moderate to severe lesions, including brisket and submandibular edema, generalized congestion, moderate hydrothorax and hydropericardium,

congested and occasionally haemorrhagic lymph nodes, congested kidneys and gastrointestinal tract. They also had acute pneumonia, particularly at the anterior lobes with generalised congestion of the lung.

Histological findings: Microscopic examinations of the organs of groups 1 and 2 showed mild congestion of the lung with occasional mild oedema and infiltration of few inflammatory cells. The control group 3 buffaloes showed moderate to severe pulmonary congestion with edema, extensive cellular infiltration and thickening of the interalveolar septa. The lumen of bronchi, bronchiole and alveoli were filled with exudate consisted of a mixture of fibrins, blood and neutrophils. There were also mild to moderate heamorrhage and congestion of the lymph nodes, spleen, liver and heart. Thrombi were observed in the lung, liver and kidney.

Bacteriology: *Pasteurella multocida* B:2 was successfully isolated from the lung tissue, lymph nodes, heart blood swabs, kidneys, spleen, liver and intestinal samples of the control non-vaccinated buffaloes. None of the samples taken from the survived groups 1 and 2 buffaloes yielded *P. multocida* B:2 (Table 1).

Table 1: Detection rate of *Pasteurella multocida* B:2 in organ samples of vaccinated, commingled and control non-vaccinated buffaloes challenged with wild-type *Pasteurella multocida* B:2

	No. of positive buffalo						
Group (n = 3)	Lung	Lymph node	Heart	Kidney	Spleen	Liver	Intestine
G1: Vaccinated	0	0	0	0	0	0	0
G2: Commingled	0	0	0	0	0	0	0
G3: Control	3	2	3	1	2	1	1

Immunoperoxidase: Immunohistochemical stain demonstrated positive reactions in the pneumocytes and bronchiolar epithelium of the affected lungs, closely associated with the blood vessels of hearts, liver, spleen and lymph nodes, kidneys and the intestinal tissue.

DISCUSSION

Infection by *P. multocida* B:2 has traditionally been associated with stressful situations, such as rainy season (De Alwis, 1993) that reduces the defence mechanism of the respiratory tract, leading to outbreaks of HS (Zamri-Saad *et al*., 1989; Zamri-Saad, 2005). These stresses increase the level of hydrocortisone and allow subsequent colonisation of bacteria, particularly in the respiratory tract (Zamri-Saad *et al*., 1991). Previous study by Rafidah *et al*. (2012) has concluded that the live gdhA derivative *P. multocida* B:2 administered intranasally was able to protect vaccinated and commingled field buffaloes against challenge by wild-type live *P. multocida* B:2. However, stress that reduces immunity may affect immune responses following vaccination. Therefore, this challenge trial was carried out to determine whether vaccination using live gdhA derivative *P. multocida* B:2 remained protective on buffaloes subjected to stress. The result has shown that experimental stress via dexamethasone injections did not significantly reduce the immune responses to vaccination using live vaccine. In fact, the stressed vaccinated and commingled buffaloes were protected against challenge by wild-type *P. multocida* B:2. On the other hand, the control non-vaccinated buffaloes showed significant reduction in the IgG levels following

dexamethasone injections leading to severe signs and lesions of HS following challenge with wild-type *P. multocida* B:2.

This challenge trial of stressed vaccinated and unvaccinated buffaloes is important in understanding the role of stress in HS. The findings of this experiment proved the fact that stress is only important in predisposing non-vaccinated buffaloes to HS where the vaccinated animals remain protected despite undergoing stressful condition. However, further research should be done to include field trials that observe the rates of HS outbreaks during stressful periods among vaccinated and unvaccinated buffaloes in endemic areas. Results of this experiment were partially in agreement to an existing assumption that outbreaks of HS is influenced by stress (De Alwis, 1999) when non-vaccinated buffaloes were severely affected by the challenge. On the other hand, the findings of this study confirmed our earlier observation that the live gdhA derivative *P. multocida* B:2 induced strong immunity (Rafidah *et al*., 2011) that remained protective even though animals were subjected to stress. Therefore, stress does not influence outbreaks of HS among vaccinated animals (Rafidah *et al*., 2010).

CONCLUSION

Intranasal vaccination with live gdhA derivative *P. multocida* B:2 induced strong immunity in exposed and in-contact buffaloes that protected the buffaloes against challenge by wild-type *P. multocida* B:2 even though they were under stress.

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