UNIVERSITI PUTRA MALAYSIA

FIELD TRIALS OF LIVE gdhA DERIVATIVE PASTEURELLA MULTOCIDA B:2 VACCINE AGAINST HAEMORRHAGIC SEPTICAEMIA IN BUFFALOES

RAFIDAH BINTI OTHMAN

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DOCTOR OF PHILOSOPHY

UNIVERSITI PUTRA MALAYSIA

2013

Dedicated to

My husband Ir. Moh Zakir Kamunri

and

My dear children

Muhammad Akmal Moh Zakir

Nurul Sabrina Moh Zakir

Muhammad Azri Moh Zakir

Muhammad Adam Moh Zakir

They have given me strength to make this fulfillment and effort in endorsing my dream for my higher studies

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

FIELD TRIALS OF LIVE *gdhA* DERIVATIVE *PASTEURELLA MULTOCIDA* B:2 VACCINE AGAINST HAEMORRHAGIC SEPTICAEMIA IN BUFFALOES

By

RAFIDAH BINTI OTHMAN

March 2013

Chairman: Professor Mohd Zamri Saad, DVM, PhD
Faculty: Veterinary Medicine

Haemorrhagic septicaemia (HS) is an infectious disease mainly affecting cattle and buffalo caused by *P. multocida* B:2. Vaccination is the best method to prevent HS in cattle and buffaloes. The main objective of this work was to study the field immunoprotective efficacy of the newly constructed live *gdhA* derivative of *P. multocida* B:2 to the exposed and in-contact susceptible buffaloes.

A retrospective analysis of records of outbreaks of HS in cattle and buffaloes was carried out to study the pattern of the disease in Sabah, Malaysia. A total of 45 outbreaks involving 1,774 susceptible animals were reported for

the past 16 years between 1994 and 2009. Outbreaks ranged between 1 and 8 per year, involving 4 of 6 regions in Sabah and occurred in all months except April but with higher frequencies in dry months of June, July and September. The most affected region was Beaufort while the least was Kota Kinabalu, while buffaloes were found to be more frequently involved than cattle. Tawau and Sandakan regions could be considered as HS-free zones while Beaufort, Kudat, Keningau and Kota Kinabalu as endemic zones.

A study was conducted on the herd immunity in field buffaloes following initial intranasal exposures to the live *gdhA* derivative *P. multocida* B:2 that was given twice at two weeks apart to 30% of the three groups buffaloes. Following vaccination, herd antibody levels in both the areas gradually but insignificantly ($p>0.05$) increased to peak values by the 6th month and then gradually decline until month 10. Following booster dose at 10th month, the antibodies declined to levels similar to those of unvaccinated animal at 12 to 14 months. Nevertheless, when compared with the control unvaccinated herd, the immune status of both vaccinated herds remained significantly ($p<0.05$) high throughout the 22-month study period, except for the months 12 to 14. It was concluded that field vaccination using *gdhA* derivative *P.*

multocida B:2 was able to maintain the herd immunity for 10 months before a booster dose can be considered.

Efficacy of HS vaccine containing live *gdhA* derivative *P. multocida* B:2 was tested in 60 field buffaloes that were divided into three groups; exposed (Group 1), commingled (Group 2) and control unexposed (Group 3). Buffaloes of group 1 were exposed intranasally to 5 mL vaccine containing 10^6 CFU/mL of the live *gdhA* derivative *P. multocida* B:2, twice at two weeks apart. Twelve months after the first vaccination, three buffaloes from each group were challenged subcutaneously with 10^9 CFU/mL of live wild-type *P. multocida* B:2. All buffaloes of groups 1 and 2 survived with mild, transient symptoms while all control unvaccinated buffaloes developed severe signs of HS and were killed humanely between 28h and 38h post-challenge with signs and lesions typical of HS. The *gdhA* derivative vaccine successfully induced systemic immunity and spread the vaccinal strain to the in-contact animals, this vaccine effectively protect both exposed and in-contact buffaloes against challenge with the virulent parent strain. Control unexposed buffaloes succumbed to the infection showed severe microscopic and ultrastructural lesions typical of HS with the average total microscopic lesion scoring was 2.13±0.19, it was significantly ($p<0.05$) higher than those

of vaccinated and commingled buffaloes with average score of 0.06±0.26 and 0.63±0.29, respectively.

The effect of stress using dexamethasone on protective efficacy of the live *gdhA* derivative *P. multocida* B:2 against challenge by wild-type *P. multocida* B:2 was studied. Nine buffaloes were selected and divided into 3 groups; exposed (Group 1), commingled (Group 2) and control unexposed (Group 3). Buffaloes of Group 1 were exposed intranasally to 10^6 CFU/mL of the *gdhA* derivative *P. multocida* B:2, twice at two weeks apart. At the end of 12-month period, all buffaloes were injected intramuscularly with dexamethasone at the dose rate of 1 mg/kg body weight for 3 consecutive days. At the end of the 3-day dexamethasone treatment, all buffaloes were challenged subcutaneously with 10^9 CFU/mL 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 but Groups 1 and 2 showed insignificant ($P>0.05$) reduction. Following challenge, all control Group 3 succumbed to the infection while buffaloes of Groups 1 and 2 survived the challenge. 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.

As a conclusion, vaccination plays a major role and is the only practical approach to control HS. Vaccination should be concentrated within the endemic areas or 'hot spots' where HS outbreaks have been reported within the last 3 years. The vaccination must cover at least 70% of the cattle and buffalo populations. This study revealed that intranasal live attenuated *gdhA* derivative *P. multocida* B:2 vaccine was able to provide protection between 8 and 10 months. The annual use of intranasal live attenuated *gdhA* derivative *P. multocida* B:2 that permit self-vaccination among free-roaming buffalo within endemic or hot spot areas is recommended to increase the vaccination coverage. This, of course, needs to be followed by sero-surveillance.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

UJIAN LAPANGAN VAKSIN HIDUP *gdhA* DERIVATIF *Pasteurella multocida* B:2 TERHADAP PENYAKIT HAWAR BERDARAH PADA KERBAU

Oleh

RAFIDAH BINTI OTHMAN

Mac 2013

Pengerusi: Professor Mohd Zamri Saad, DVM, PhD
Fakulti: Perubatan Veterinar

Hawar berdarah (HS) merupakan penyakit berjangkit yang banyak menyerang lembu dan kerbau disebabkan oleh *P. multocida* B: 2. Pemvaksinan merupakan cara terbaik mencegah HS pada lembu dan kerbau. Kajian ini bertujuan untuk mengkaji keberkesanan perlindungan imun bagi *gdhA* derivatif *P. multocida* B:2 hidup terhadap kelompok kerbau yang terdedah secara intranasal dan kelompok kerbau yang bercampur dengan kerbau yang telah didedahkan kepada vaksin itu.

Analisis retrospektif wabak HS pada lembu dan kerbau telah dijalankan bagi mengkaji pola penyakit ini di Sabah, Malaysia. Sebanyak 45 wabak di

kalangan 1,774 haiwan rentan telah dilaporkan bagi tempoh 16 tahun antara 1994 dan 2009. Setiap tahun sebanyak satu hingga lapan wabak telah direkodkan pada 4 daripada 6 kawasan kajian, wabak berlaku hampir setiap bulan dalam sepanjang tahun, kecuali pada bulan April. Frekuensi wabak lebih tinggi direkodkan pada musim kemarau iaitu bulan Jun, Julai dan September. Kawasan paling terjejas adalah Beaufort dan paling kurang berlaku wabak ialah Kota Kinabalu manakala kerbau lebih kerap terlibat daripada lembu. Tawau dan Sandakan adalah zon bebas-HS, manakala Beaufort, Kudat, Keningau dan Kota Kinabalu adalah zon endemik.

Satu kajian mengenai imuniti dikalangan gerompok kerbau dilapangan selepas diberikan 5 mL inokulum 10^6 CFU/mL *gdhA* derivatif *P. multocida* B: 2 hidup secara intranasal sebanyak dua kali dalam selang dua minggu ke atas 30% daripada tiga kumpulan kerbau. Dos penggalak telah diberikan 10 bulan kemudian. Selepas pemvaksinan, aras antibodi gerompok di kedua-dua kawasan kajian menunjukkan peningkatan secara beransur-ansur yang tidak signinfikan ($p<0.05$) kepada nilai puncak pada bulan ke-6 , kemudian mula beransur-ansur merosot pada bulan ke-10. Selepas pemberian dos booster pada bulan ke-10, aras antibodi telah menurun ke tahap yang sama

dengan kerbau-kerbau yang tidak divaksin pada bulan ke-12 hingga ke-14. Walaubagaimanapun, jika dibandingkan dengan gerompok kawalan yang tidak diberi vaksin, status immun kedua-dua gerompok yang diberi vaksin kekal berada di aras yang tinggi secara signifikan ($p<0.05$) sepanjang tempoh 22-bulan kecuali pada bulan ke-12 dan ke-14. Kajian ini mendapati pemvaksinan menggunakan *gdhA* derivatif *P. multocida* B:2 dapat mengekalkan immune gerompok untuk tempoh 10 bulan sebelum dos penggalak diperlukan.

Keberkesanan vaksin intranasal *gdhA* derivatif *P. multocida* B:2 hidup telah diuji terhadap 60 ekor kerbau dilapangan yang dibahagikan kepada tiga kumpulan; didedahkan (Kumpulan 1), bercampur (Kumpulan 2) dan kawalan tidak terdedah (Kumpulan 3). Kerbau kumpulan 1 telah didedahkan sebanyak 5 mL 10^6 CFU/mL *gdhA* derivatif *P. multocida* B:2 hidup melalui intranasal, dua kali dalam selang dua minggu. Dua belas bulan selepas pemvaksinan, tiga kerbau dari setiap kumpulan telah dipindahkan ke rumah eksperimen dan dicabar melalui subkutaneus dengan 10^9 CFU/mL *P. multocida* B:2 hidup jenis liar. Semua kerbau kumpulan 1 dan 2 telah terselamat dengan menunjukkan gejala ringan,

manakala semua kerbau yang tidak terdedah kepada vaksin, menunjukkan gejala HS yang teruk dan telah dibunuh secara berperikemanusiaan antara 28h dan 38h selepas-cabaran menunjukkan tanda-tanda klinikal dan lesi yang biasa diperhatikan pada kes HS. Data-data ini menunjukkan strain *gdhA* derivatif berjaya mendorong imuniti sistemik dalam kumpulan terdedah dan menyebarkan strain vaksin itu kepada kerbau dalam kumpulan yang bercampur, vaksin ini berkesan melindungi kedua-dua kumpulan kerbau yang terdedah dan bercampur terhadap cabaran oleh strain virulen yang sama. Kajian ini mendapati, kumpulan kerbau kawalan yang tidak terdedah menunjukkan lesi mikroskopik dan ultrastruktur yang teruk dengan purata jumlah skor lesi microskopik 2.13 ± 0.19 adalah signifikan ($p < 0.05$) lebih tinggi berbanding kumpulan kerbau terdedah (0.06 ± 0.26) dan kumpulan kerbau bercampur (0.63 ± 0.29).

Kajian kesan imunosupresif oleh deksametason keatas kerbau yang telah divaksin terhadap cabaran oleh *P. multocida* B: 2 jenis liar dilakukan ke atas sembilan ekor kerbau yang dibahagikan kepada 3 kumpulan; terdedah(Kumpulan 1), bercampur(Kumpulan 2) dan kawalan tidak terdedah(Kumpulan 3). Kerbau Kumpulan 1 telah didedahkan kepada 10^6

CFU/mL *gdhA* derivatif *P. multocida* B: 2 hidup melalui intranasal, dua kali dalam selang 2 minggu. Selepas pendedahan bagi tempoh 12 bulan, semua kerbau diberikan suntikan deksametason pada kadar dos 1 mg/kg berat badan secara intramuskular (i.m) selama 3 hari berturut-turut. Selepas tiga hari rawatan deksametason, semua kerbau telah dicabar dengan 10^9 CFU/mL *P. multocida* B: 2 jenis liar secara subkutaneus (s.c). Terdapat peningkatan yang signifikan ($P<0.05$) bagi aras IgG dalam Kumpulan 1 dan 2 berikutan pendedahan intranasal. Rawatan dekametason mengakibatkan pengurangan yang signifikan ($P< 0.05$) bagi aras IgG yang berlaku dengan cepat dalam Kumpulan kawalan 3, sebaliknya bagi Kumpulan 1 dan 2 ianya menunjukkan pengurangan yang tidak signifikan ($P> 0.05$). Berikutan cabaran, semua kerbau kelompok kawalan (Kumpulan 3) telah mati akibat jangkitan manakala kerbau dalam Kumpulan 1 dan 2 dapat bertahan dan terselamat. Suntikan deksametason tidak mengurangkan keberkesanan terhadap pelindungan yang dihasilkan oleh *gdhA* derivatif *P. multocida* B:2 hidup yang dilemahkan, tetapi telah menyebabkan kecenderungan jangkitan yang signifikan kepada kerbau yang tidak terdedah kepada vaksin ini.

Kesimpulannya, pemvaksinan memainkan peranan utama dan pendekatan paling praktikal untuk mengawal HS. Pemvaksinasan seharusnya tertumpu

di kawasan endemik atau 'kawasan panas' dimana wabak HS telah dilaporkan dalam tempoh 3 tahun yang lalu. Pemvaksinan hendaklah meliputi sekurang-kurangnya 70% daripada populasi lembu dan kerbau. Kajian ini mendapati vaksin *gdhA* derivative *P. multocida* B:2 hidup yang diberikan secara intranasal memberi perlindungan selama 8 dan 10 bulan. Maka, disyorkan agar penggunaan vaksin hidup intranasal *gdhA* derivatif *P.multocida* B: 2 dilakukan secara tahunan, dimana vaksin ini mampu dipindahkan dan akan bertindak sebagai 'pemvaksinan-sendiri' di kalangan kerbau yang bebas merayau di dalam kawasan endemik atau panas untuk meningkatkan liputan vaksinasi. Kaedah ini hendaklah diikuti dengan pemantauan serologi.

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Lastlty I would like to pray thanks to Almighty Allah S.W.T who blessed me to achieve the success.

I certify that a Thesis Examination Committee has met on 07 March 2013 to conduct the final examination of Rafidah binti Othman on her thesis entitled "Field Trials of Live gdhA Derivative Pasteurella multocida B:2 Vaccine Against Haemorrhagic Septicaemia in Buffaloes" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Abd. Wahid bin Haron, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahman bin Omar, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Latiffah binti Hassan, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Khurshi Muhammad, PhD
Professor
Department of Microbiology
University of Veterinary & Animal Science
Pakistan
(External Examiner)

NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 23 May 2013

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement fot the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Mohd Zamri Saad, DVM, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Nasip Eli, DVM, MVSc
Deputy Director
Department of Veterinary Services and Animal Industry, Sabah
Ministry of Agriculture and Food Industry, Sabah
(Member)

Abd. Aziz Saharee, DVM, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia
Date:

DECLARATION

I declare that the thesis is based on my original work except for quotations and citation, which have been dully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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RAFIDAH BINTI OTHMAN

Date:

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CHAPTER 1

INTRODUCTION

Haemorrhagic septicaemia (HS) is a disease that occurs in Southern Europe, Africa, Near and Middle East countries and throughout Southeast Asia (Joseph, 1979; Bain et al., 1982; De Alwis 1992). HS occurs mostly as outbreaks during periods of environmental stress. It is a long-held belief that haemorrhagic septicaemia occurs during the monsoon season (Bain et al., 1982). HS causes a great economic loss in Asia, where buffaloes are reported to be particularly susceptible (Bain et al., 1982; De Alwis, 1990) and younger animals are reported to succumb to HS more readily than older animals (Bain et al, 1982).

Vaccination has been used to control the disease. Although vaccination programs against HS have been conducted in all endemic areas in many affected Asian countries, it appears not to influence the occurrence of the disease (Saharee, 2006). This is because the vaccination rate in the endemic areas was found to be generally low. In cattle, the vaccination coverage is

between 5% and 31% and between 4% and 22% of the population in the endemic and non-endemic areas, respectively. In buffaloes, the vaccination percentage was even lower at between 3% and 11% (Saharee and Salim, 1991). These figures are far below the recommended minimum vaccination coverage of 50% (Bain et al., 1982) and 70% (Neramitmansook, 1993; Syamsudin, 1993) of the population to control HS outbreaks.

Although injectable vaccines such as the oil adjuvant vaccine have been shown to stimulate prominent and prolonged immunity, they are difficult to be administered since livestock production system in many developing Asian countries uses either extensive or semi-extensive type of management that involved little animal handling. In Malaysia, Saharee and Salim (1991) concluded that the free-ranging type of buffalo management leads to difficulties in gathering animals for vaccine injection, thus vaccination coverage is as low as 17%. Similar low vaccination coverage when oil adjuvant vaccine is used was reported in Indonesia (Syamsudin, 1993). Disease outbreaks are often reported in the unvaccinated extensive animals. Similarly in India, Kundu (1993) reported that the injectable oil adjuvant vaccine is used mainly in organized farm where animals are easier restrain. Since most of the

free-ranging livestock belong to smallholder and villagers, their support in HS vaccination program is deemed essential in Thailand (Neramitmansook, 1993). DeAlwis (1993) faced similar problem in vaccination against HS in Sri Lanka since the disease is poorly managed in free-roaming herds. Therefore, HS is likely to be seen in these herds due to low vaccination coverage.

A live attenuated vaccine is a vaccine that contains live organism that has been attenuated or reduced in its virulence. Live attenuated vaccines have the advantage of a natural route of entry into the host and are able to stimulate better immune responses than the killed vaccines. One of the advantages of live attenuated vaccines is the use of natural route of entry into the host. Intranasal administration not only stimulates the mucosal immunity of the exposed host, but also may transmit the organism to the in-contact host and eventually stimulate the mucosal immunity of the in-contact host (Zamri-Saad et al., 2006). Sarah et al. (2006) have identified the *gdhA* housekeeping gene of *Pasteurella multocida* B:2. They eventually sequenced and cloned the gene before disrupting the gene to create an attenuated, avirulent *P. multocida* B:2. The *gdhA* gene was disrupted by insertion of a kanamycin resistance cassette by allelic replacement (Sarah, 2005). Since a housekeeping gene was disrupted,

the *gdhA* derivative *P. multocida* B:2 was found to be able to survive for a period of 14 days, thus, avoids possible reversion to the wild type (Sarah, 2007). Furthermore, the *gdhA* derivative of *P. multocida* B:2 were efficiently removed from the blood, spleen and liver and readily taken up by alveolar macrophages and neutrophils (Trang et al., 2008).

Initial experiment using a mouse experimental model by Sarah (2007) revealed that mice infected intraperitoneally with live *gdhA* derivative *P.multocida* B:2 were survived throughout the 5-day study period, as compared to mice infected with wild-type *P.multocida* B:2 did not survive and were dead in less than 24 hours.. Whereas, previous study in controlled situation by Khin (2009) revealed that intranasal exposures to live *gdhA* derivative *P. multocida* B:2 to calves successfully stimulated the mucosal immunity of the respiratory tract and also the systemic immunity by the increased size of bronchus associated lymphoid tissue (BALT), the number of lymphocytes, the IgA levels in the lung and significant increase in the serum IgG levels. In fact, adherence of wild-type challenge *P.multocida* B:2 onto the cells of nasal mucosa and lungs were prevented in the calves that were exposed to live *gdhA* derivative *P.multocida* B:2 (Trang et al. 2008). Therefore, it was also able preventing establishment of

infection by wild-type *P.multocida* B:2 in the exposed and commingled calves (Khin, 2005; Trang et al. 2008)

Therefore, the objectives of this study were;

1. To perform retrospective analysis of previous outbreaks of HS in Sabah
2. To determine the antibody response of buffalo following intranasal vaccination with the live *gdhA* derivative HS vaccine in the endemic and non-endemic field situations
3. To determine the field protective efficacy of the live *gdhA* derivative HS vaccine in buffalo following challenge with wild-type *P. multocida* B:2
4. To study the effect of dexamethasone treatment on the field antibody response by buffalo vaccinated with live *gdhA* derivative HS vaccine

The hypotheses included:

1. There was strong correlation between previous outbreaks of HS in Sabah with the vaccination status, environmental factors and susceptible animals density within the study area
2. Intranasal exposures to live *gdhA* derivative *P. multocida* B:2 are able to stimulate the mucosal immunity of the respiratory tract and at the

same time induce local and systemic immunities both in exposed and in-contact buffaloes leading to higher vaccination coverage

3. The immune response that are induced following intranasal exposures is able to give protection to the stressed and unstressed buffaloes against challenge by wild-type *P.multocida* B:2

The published manuscripts in this thesis could be integrated as described below:

1. Manuscript 1: Analysis of HS outbreaks in cattle and buffalo in Malaysia (Most data had been published in Online J.Vet. Res., 14 (2): 325-333, 2010). This published manuscript describes the outbreak pattern of HS in Sabah during 16-year period between 1994 and 2009. This article provides information on various important factors especially on vaccination issues that contribute to the outbreaks of HS. It helps clarify the main research topic for a need to study an alternative and effective vaccine and vaccination strategy as control and preventive HS in the near future

2. Manuscript 2: Herd immunity in buffaloes after intranasal live *gdhA* derivative *P. multocida* B:2 (Most data had been published in Online J.Vet. Res., 15 (3): 283-290, 2011). This published manuscript reports on the herd immunity status via antibody response profiles in field buffaloes following intranasal exposures to live *gdhA* derivative *P. multocida* B:2 in HS-endemic and non-endemic area. This study revealed that field vaccination using *gdhA* derivative *P. multocida* B:2 increased the herd immunity for 8 to 10 months before a booster dose was required.

3. Manuscript 3: Efficacy of intranasal vaccination of field buffalo against HS with a live *gdhA* derivative *P. multocida* B:2 (Most data had been published in Veterinary Record, 171 (7): 175(2012). This published manuscript determines the efficacy of a live *gdhA* derivative *P. multocida* B:2 that were administered intranasally in protecting exposed and in-contact field buffaloes against challenge by wild-type live *P. multocida* B:2. The data from this study showed that the *gdhA* mutant strain, given intranasal as two doses 2 weeks apart, successfully induced systemic immunity in exposed and also leads to spread of vaccine strain to the in-contact animals, where it acted as an effective live vaccine to protect

both exposed and in-contact buffaloes against challenge with the virulent parent strain. Where, the exposed and in-contact buffaloes did not show any typical clinical signs of HS, there were no prominent gross, microscopic and ultrastructure lesions being observed and also negative findings for bacterial re-isolation and immunoperoxidase reaction

4. Manuscript 4: Effect of dexamethasone on protective efficacy of live *gdhA* derivative *P. multocida* B:2 vaccine (Most data had been published in the Asian Journal of Veterinary and Animal Advances, 8(3): 548-554 (2013). This accepted manuscript for publication determined the protective efficacy of the live *gdhA* derivative *P. multocida* B:2 against challenge with the wild type *P. multocida* B:2 among the dexamethasone induced immunosuppressived buffaloes. The findings from this study concluded that 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.

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