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DEVELOPMENT OF PROTOTYPE KILLED VACCINE AGAINST Staphylococcus aureus MASTITIS IN DAIRY COWS

DR IDRIS UMAR HAMBALI

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By

DR IDRIS UMAR HAMBALI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia
In Fulfillment of the Requirements for the Degree of Doctor of Philosophy

November 2018

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DEDICATION

I dedicate this work to my beloved unreplacable parents Alhaji Umar Hambali and Hajjia Meimunat Umar, thank you for your unconditional love, support, encouragement and prayers. I am so lucky to have you as my parent. I love you. May ALLAH continue to reward and protect you, AMEEN.

To my lovely younger brothers; Engineer(s) Hambali, Shuaibu and Ahmad Abulfathi, my lovely younger sister Saratu Umar: thank you for all your support during my absence. I really appreciate your kind gestures. Words alone cannot express my happiness. Indeed you've justified the saying that blood is heavily thicker than water. I love you all. May ALLAH continue to reward and protect you, AMEEN.

To my lovely wife Quraibah Idris Umar Hambali and my daughters Hajjia Meimunat Idris and Hajjia Fatima Idris : thank you for believing in me, thank you for your prayers, understanding, patience and perseverance throughout the course of my PhD study. I love you all. May ALLAH continue to reward and protect you, AMEEN.

To my teachers, instructors, mentors: I will like to say a big thank you to you all.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfillment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF PROTOTYPE KILLED VACCINE AGAINST *Staphylococcus aureus* MASTITIS IN DAIRY COWS

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DR IDRIS UMAR HAMBALI

November 2018

Chairman : Associate Professor Faez Firdaus Jesse Abdullah, PhD
Faculty : Veterinary Medicine

Mastitis is the inflammation of the udder in dairy cows and other species which is caused by bacteria, virus, fungi, toxins, physical and other chemical factors. Despite the continued use of antibiotics in treating mastitis in dairy cows, there are reports of occurrence of mastitis in dairy farms in Malaysia due to antibiotic failures. Therefore, the present study opined to develop a prototype killed mastitis vaccine using local Malaysian isolate of S. *aureus* with a view that it may assist in reducing the burden of mastitis among cows in Malaysia.

The current study was therefore designed and experimented on heifer and lactating Friesian cows models. Killed vaccine was developed using the Malaysian local isolate of S. *aureus* and adjuvated with Aluminium potassium sulfate. A preliminary proof of concept study using the heifer cows was carried out where four different concentrations of the vaccines were prepared to contain 106, 107, 108 and 109 cfu/ml of S. *aureus* so as to evaluate the best concentration in terms of evoking immune response in cows. Thirty heifer cows were grouped into 5; group A (control), group B (106 cfu/ml), group C (107 cfu/ml), group D (108 cfu/ml) and group E (109 cfu/ml). The experimental animals were vaccinated intramuscularly with 2 ml of the prepared vaccine and observed for acute and chronic responses post vaccination at 0, 3, 24 hours and at weeks 1, 2, 3 and 4 post vaccination.

The vaccination with killed S. *aureus* vaccine in heifer cows was observed to induce significant immune response more in group D (108 cfu/ml) as compared to other groups. The preliminary study assessed the periodic effect of the developed prototype vaccine groups on both the vital signs and immune regulators. The vital signs

examined were rectal temperature, heart rate and respiratory rates, while the immune parameters examined were IL-10, SAA, IgM and IgG.

In the present study SAA was significantly different in groups D and E post vaccination (PV). The IL-10 concentrations indicated a statistical significant difference in group D and C PV. IgM in serum of the heifer at PV indicated a statistical significant increase in Groups B and C. Serum IgG at PV indicated a statistical significant difference in Group E and D. The IgG concentrations of Group D tend to be significant from its onset to the end of the experiment. This degree of potency evoked by group D suggested that this vaccine group (108 cfu/ml) could confer immunity compared to other vaccine groups and hence can be considered as capable of evoking immunity against S. *aureus* challenge in cows.

This vaccine group 108 cfu/ml, became the vaccine candidate of choice for the next phase of trial on lactating Friesian dairy cows. In this trial, six lactating Friesian cows were grouped into three groups with two cows each in group C (108 cfu/ml vaccine), group B (positive control) and group A (negative control). During primary vaccination, booster vaccination and S. *aureus* challenge phases of the experimental trial, variables like clinical manifestation of mastitis, cytokine, APPs, antibodies and tissue histopathology were evaluated. The experimental animals were vaccinated intramuscularly with 2 ml of the prepared vaccine and observed for acute and chronic responses at post primary vaccination (PPV) (at 0, 3, 8, 12, 24 hours and at weeks 1, 2 PPV), at post booster vaccination phase (PB) (at 0, 3, 8, 12, 24 hours PB) and at post S. *aureus* challenge phases (PC) (at 0, 3, 8, 12, 24 hours and at weeks 1, 2 PC).

The rectal temperature of the vaccinated lactating Frisian cows was significantly increased following primary vaccination and booster doses as well as slightly following bacterial challenge, but, the rectal temperature of the positive control group was found to be non-significant following primary vaccination and booster doses but significantly increased following bacterial challenge.

The heart rates of lactating Friesian cows in the vaccinated group were increased significantly post vaccination. The heart rates in the positive control group were found to have increased significantly post challenge with S. *aureus*. The increased heart rate post vaccination and challenge could be a compensatory mechanism to cope with the increased rectal temperature.

In this present vaccine trial on lactating Friesian cows, palpation revealed the enlargement of the teat, mammary gland and supramammary lymph nodes especially from the positive control group following S. *aureus* challenge at weeks 1 and 2 post challenge. The absence of these enlargements in the vaccinated group following challenge with S. aureus suggested a good prognosis of the efficacy of the killed S. aureus vaccine against mastitis in cows. The killed S. *aureus* vaccine was unable to

confer a 100% immunity to the vaccinated group as one out of the eight quarters from the vaccinated group was swollen at week 1 post challenge which later reduced in size and resumed milking with no pain at week 2 post challenge.

There was evidence of significant increase in IL-10 concentration in vaccinated group PPV, PB and PC. There was also a significant increase in IL-12 concentration in the vaccinated group PPV, PB and PC. The findings further demonstrated that IgM concentrations of the vaccinated group was significantly high during the PPV, PB and P. The IgG concentrations of the vaccinated group was high during the acute phase PB and PC. IgA was also assayed from the vaccinated groups which was higher during the PB and PC.

Grossly, the positive control group developed a significant inflammatory sign in the teat and mammary gland following challenge with S. *aureus*. Mammary gland incision revealed that the parenchyma of the positive control group had a clotted thick mastitic milk and inflammatory products blocking the milk duct.

Histopathological study of the mammary gland, teat, GALT, spleen and thymus in the positive group indicated inflammatory cell infiltration, congestion, degeneration, traces of oedema.

This current study provided a dependable vaccine candidate against S. *aureus* mastitis and the detailed involvement of the vital signs, SAA, Hp, IL-10, IL-12, IgM, IgG, IgA, mammary gland, supramammary lymph node, spleen, thymus and GALT. Based on these findings, it can therefore be concluded that the study had further demonstrated the efficacy of the prototype S. *aureus* vaccine against S. *aureus* mastitis in cows.

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

PEMBANGUNAN VAKSIN PROTOTAIP YANG DIMATIKAN TERHADAP *Staphylococcus aureus* BAGI MENGAWAL PENYAKIT RADANG MAMARI LEMBU TENUSU

Oleh

DR IDRIS UMAR HAMBALI

November 2018

Pengerusi : Profesor Madya Faez Firdaus Jesse Bin Abdullah, PhD
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Penyakit radang mamari dalam lembu tenusu disebabkan oleh bakteria, virus, kulat, toksin, kecederaan fizikal, kimia dan faktor lain. Penggunaan antibiotik dalam merawat penyakit radang mamari pada lembu tenusu, diteruskan tetapi kes penyakit ini amat kerap di Malaysia akibat kegagalan antibiotik berfungsi akibat kerintangan antibiotik. Dengan itu, kajian ini berpendapat suatu keperluan untuk membangunkan vaksin prototaip yang dimatikan menggunakan isolat *S. aureus* tempatan bagi mengurangkan kerkerapan kes penyakit ini di kalangan lembu di Malaysia.

Dengan itu, sebuah kajian telah direka dan ujikaji telah di jalankan pada model lembu dara dan lembu tenusu Friesian. Vaksin yang dimatikan telah dibangunkan menggunakan isolat S. aurues tempatan Malaysia dan diadukan dengan aluminium kalium sulfat. Sebuah bukti awal daripada konsep kajian yang telan dijalankan menggunakan lembu lembu dara di mana empat kepekatan vaksin yang berbeza mengandungi 106, 107, 108 dan 109 cfu / ml *S. aureus* telah digunakan untuk menilai kepekatan terbaik dari segi menilai keberkesanan keimunan dalam lembu. Tiga puluh lembu lembu dara telah dibahagikan kepada 5 kumpulan; kumpulan A (kawalan), kumpulan B (106 cfu / ml), kumpulan C (107 cfu / ml), kumpulan D (108 cfu / ml) dan kumpulan E (109 cfu / ml). Kesemua lembu ini telah diberi vaksin secara intramuskular pada kadar 2ml daripada vaksin yang telah disediakan dan respon diperhatikan gagi tindakbalas akut pasca vaksinasi pada jam ke 0, 3, 24 dan pada minggu 1, 2, 3 dan 4 pasca vaksinasi.

Vaksinasi dengan vaksin *S. aureus* yang dibunuh dalam lembu dara telah memperlihatkan suatu tindak balas imun yang signifikan dalam kumpulan D (108 cfu / ml) berbanding dengan kumpulan lain dimana kedua-dua tanda-tanda klinikal penting dan pengawal selia imun terdapat keputusan yang positif. Tanda-tanda kilinkal penting yang diperiksa adalah suhu rektum, kadar denyutan jantung dan kadar pernafasan, manakala permain keimunan yang diperiksa adalah IL-10, SAA, IgM dan IgG.

Dalam kajian ini, keperkatan SAA menunjukkan perbezaan yang katara dalam Kumpulan D dan E pasca vaksinasi. Kepekatan IL-10 pada pasca vaksinasi menunjukkan perbezaan yang ketara bagi Kumpulan D dan C.

Kepekatan IgM pasca vaksinasi menunjukkan peningkatan ketara dalam Kumpulan B dan C. Keperkatan IgG pasca vaksinasi ia menunjukkan perbezaan ketara dalam Kumpulan E dan D. Kepekatan IgG Kumpulan D cenderung menjadi signifikan dari permulaan hingga akhir percubaan. Tahap potensi yang ditimbulkan oleh kumpulan D mencadangkan bahawa kumpulan vaksin ini (108 cfu / ml) dapat memberikan imuniti berbanding dengan kumpulan vaksin lain dan dengan itu dapat dianggap sebagai mampu menimbulkan kekebalan terhadap cabaran S. *aureus* dalam sapi.

Kumpulan vaksin 108 cfu / ml ini dipili, menjadi calon vaksin untuk fasa percubaan berikatiya pada lembu tenusu Friesian. Dalam percubaan ini, enam ekor lembu yang menyusu, tilah dikelompokkan kepada tiga kumpulan dengan dua ekor lembu masing-masing dimana kumpulan C (108 cfu / ml vaksin), kumpulan B (kawalan positif) dan kumpulan A (kawalan negatif). Semasa suntikan utama, suntikan penggalak dan fasa ujian S. aureus percubaan eksperimen, pembolehubah seperti manifestasi klinikal mastitis, sitokin, APP, antibodi dan histopatologi tisu telah dinilai. Haiwan eksperimen telah diberi vaksin secara intramuskular dengan 2 ml vaksin yang disediakan dan diperhatikan untuk tanggapan akut di pasca vaksinasi utama (PPV) (pada 0, 3, 8, 12, 24 jam dan pada minggu 1, 2 PPV), pada suntikan pemangkasan pos fasa (PB) (pada 0, 3, 8, 12, 24 jam PB) dan pada fasa cabaran S. aureus pasang (PC) (pada 0, 3, 8, 12, 24 jam dan pada minggu 1, 2 PC).

Berkenaan dengan kesan vaksinasi *S. aureus* dan cabaran pada suhu rektum, RR, HR, teat, kelenjar susu, nodus limfa supramammary dan GALT, disimpulkan bahawa vaksin itu menimbulkan kekebalan terhadap *S. aureus*. Ini adalah jelas dari fasa percubaan dimana suhu rektum bagi lembu Frisian yang telah disuntik dengan vaksin telah meningkat dengan ketara berikutan dos vaksinasi dan dorongan utama serta sedikit mengikuti cabaran bakteria, tetapi, suhu rektum dalan kumpulan kawalan positif didapati meningkat dengan ketara selepas cabaran bakteria. Kadar denyutan jantung lembu Friesian dalam kumpulan yang divaksinasi telah meningkat dengan ketara selepas vaksinasi. Kadar denyutan jantung dalam kumpulan kawalan positif didapati meningkat dengan ketara apabila dicabar dengan *S. aureus*.

Palpasi mamari mendedahkan pembesaran kelenjar, kelenjar susu dan kelenjar getah bening supramammary terutama daripada kumpulan kawalan positif berikutan cabaran *S. aureus* pada minggu 1 dan 2 pasca cabaran. Ketiadaan lesi abnormal dalam kumpulan yang divaksinasi berikutan cabaran dengan *S. aureus* mencadangkan satu prognosis yang baik tentang keberkesanan vaksin *S. aureus* yang terbunuh terhadap penyakit radang mamari dalam lembu tenusu. Daripada kajian ini didapati , vaksin S. aureus yang terbunuh tidak dapat memberikan imuniti 100% kepada kumpulan yang divaksinasi dimana satu daripada lapan kelanjar mamari dari pada kumpulan yang divaksin didapati mehunjukkan simptom keradangan pada minggu pertama pasca cabaran diman bengkak mamari telah berkurangan dalan saiz dan didapati proses pemerahan susu dapat dilaksana tanpa kesakitan pada minggu kedua pasca cabarah.

Terdapat bukti peningkatan ketara dalam kepekatan IL-10 dalam kumpulan PPV, PB dan PC yang divaksinasi. Terdapat juga peningkatan ketara dalam kepekatan IL-12 dalam kumpulan PPV, PB dan PC yang divaksinasi. Penemuan selanjutnya menunjukkan bahawa kepekatan IgM kumpulan yang divaksin adalah tinggi tinggi semasa PPV, PB dan P. Konsentrasi IgG kumpulan yang divaksin adalah tinggi semasa fasa akut PB dan PC. IgA juga diuji dari kumpulan vaksin yang lebih tinggi semasa PB dan PC.

Sekali lagi, kumpulan kawalan positif telah menghasilkan tanda radang yang signifikan dalam kelenjar susu dan mamalia berikutan cabaran dengan *S. aureus*. Penyakit kelenjar mamma mendedahkan bahawa parenchyma kumpulan kawalan positif mempunyai susu mastitis tebal dan produk keradangan yang menyekat saluran susu. Nod limfa supramammary kumpulan kawalan positif dalam kajian ini secara signifikan diperbesarkan berbanding dengan kumpulan yang divaksin. Kajian histopatologi kelenjar susu, teat, GALT, limpa dan timus dalam kumpulan positif menunjukkan penyusupan sel keradangan, kesesakan, degenerasi, jejak edema.

Kajian semasa ini menyediakan calon vaksin yang boleh dipercayai membukan respon S. Aureus baik terhadap dan penglibatan terperinci tanda-tanda vital, SAA, Hp, IL-10, IL-12, IgM, IgG, IgA, kelenjar susu, nodus limfa supramamari, limpa, timus dan GALT telah dikaji. Berdasarkan penemuan-penemuan ini, dapat disimpulkan bahawa kajian ini telah menunjukkan keberkesanan vaksin prototaip S. aureus yang dihasilkan dapat membukan perlindugan cabran penyakit radang inamani dakan lembu tenusu.

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

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^{o}C	Degree celsius
APP	Acute phase proteins
CFU	Colony forming unit
ELISA	Enzyme Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
Hp	Haptoglobin
I.V	Intravenous
IACUC	Institutional Animal Care and Use Committee
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
µg	Microgram
mg	Milligram
ng	Nanogram
ml	Milliliter
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
SAA	Serum Amyloid A
TPU	Taman Pertanian Universiti
UPM	Universiti Putra Malaysia
VLSU	Veterinary Laboratories Service
PPV	Post primary vaccination
PB	Post booster
PC	Post challenge

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Veterinary public health is a module of public health that aims at protecting and improving animal and human health through the utilisation of applied scientific tools (Groot and Van't Hooft, 2016; Issa *et al.*, 2016; Chen *et al.*, 2018). Conventionally, to achieve such improvement, a greater concern is placed on food production chain, its safety and herd health immunity (Santman-Berends *et al.*, 2016; Villa *et al.*, 2018; Shahudin *et al.*, 2018). The "farm to cup" concept as seen in dairy farming is concerned with the safety of dairy products from farms to homes, supermarkets and finally to consumers (animal and human) (Sudhanthiramani *et al.*, 2015; Tolosa *et al.*, 2016). This major concern had suggested for a better prevention, control and surveillance strategies on dairy infections like mastitis and on herd health management. The emergence of antibiotic resistance in eluding treatment hence antibiotic failures had suggested the need for exploiting vaccination in curtailing the menace of mastitis in dairy farms (Ferdous *et al.*, 2016; Xue *et al.*, 2016).

Public health concerns regarding the menace of bovine mastitis are the antibiotic residues in milk (Chowdhury *et al.*, 2015; Groot and Van't Hooft, 2016). These residues are a consequence of uncontrolled extended usage of antibiotics in treating mastitis (Jamali *et al.*, 2014; Kuipers *et al.*, 2016). Antibiotic residues have been reported to be a major source of severe reactions in humans due to allergy to antibiotics (Bendary *et al.*, 2016). The emphasis of public health on bovine mastitis is born out of the fact that same causal organisms have been isolated and implicated in human cases involving endocarditis, toxic shock syndrome, necrotising Pneumonia, skin infections, Q-fever, Brucellosis, gastroenteritis, epidemic diarrhoea in infants and food poisoning (Pexara *et al.*, 2012; Ro, 2016). Therefore, a need becomes requisite to assure public health safety and improve herd health immunity by way of developing preventive and control measures through vaccination for mastitis.

Mastitis also known as mammitis is a condition that occurs as a result of the infiltration of white blood cells into the mammary gland during response to bacterial invasion of the teat canal in cows and other species (Kumar *et al.*, 2016; Abdalhamed *et al.*, 2018; Harjanti *et al.*, 2018). Simply it is the inflammation of the mammary gland (Notcovich *et al.*, 2018; Ottalwar *et al.*, 2018). The disease condition is found in most dairy farms (Ferronatto *et al.*, 2018) and characterised by inflamed and red udder, enlarged supramammary lymph nodes, distended teat, reduced milk production, lowered milk quality and loss of mammary integrity (Marimuthu *et al.*, 2014; McDougall *et al.*, 2018; Mishra *et al.*, 2018). Milk-secreting tissues and various ducts in the mammary glands are damaged due to toxins released by Eslami *et al.*, 2015; Hoque *et al.*, 2018; Luoreng *et al.*, 2018). This disease condition is associated with considerable economic

losses to the dairy farmers worldwide and of a serious public health importance (Yadav, 2018) mainly due to contamination and condemnation of dairy products (Mishra et al., 2018; Harjanti et al., 2018), cost of antibiotic treatment and associated decreased reproductive performances of affected cows (Abdisa, 2018; Mellado *et al.*, 2018). Due to the sub-clinical nature of mastitis, control is usually difficult and hence prevalence in dairy animals is high (Hussein *et al.*, 2018).

Prevention of mastitis using immunological tools and the development of vaccines to control mastitis as an alternative trend has recorded huge attention and trials in recent times (Denis *et al.*, 2009). Vaccines have been prepared using whole organisms, which are either attenuated bacteria or viruses that are live but have been altered weak to reduce their virulence, or pathogens that have been inactivated and effectively killed through exposure to either heat or chemical agents like formaldehyde (Jones, 2015). The use of whole organisms to elicit immune response introduces the potential risk of infections arising from a reversion to its virulent form in live pathogen vaccines ,however, formalin-killed whole-cell vaccines have recorded tremendous successes with no fear of virulence reversion in most preventive and control cases (Mani *et al.*, 2016). Commercial vaccines targeted at mastitis caused by *S. aureus* are currently available in the United States (Denis et al., 2009). There are two *S. aureus* vaccines commercially available and are marketed as Somato-Staph® and Lysigin®. These vaccines were reported to have low efficacy, moderate potential of reducing severity of mastitis and a limitation of non-resumption of milk secretion in mastitis cows (Mata, 2013). In view of the difficulties and challenges of controlling mastitis in dairy cows, the quest for the development of an efficient vaccine becomes a necessity as there is no commercial mastitis vaccine developed in Malaysia. Therefore, the present study opined to develop a prototype killed mastitis vaccine using local Malaysian isolate of *S. aureus.*

Mastitis has a global distribution and it is endemic in most developed and developing nations of the world (Jamali *et al.*, 2018; Matos *et al.*, 2018). The prevalence of mastitis in dairy farms had been reported in different states in Malaysia. In Johor, 81.7% was reported (Othman and Bahaman, 2005), 68% in Selangor, 55% also in Selangor (Marimuthu *et al.*, 2014). Malaysia has a sizeable cattle population of breeding cows, with an estimate drawn from the existing cattle population of about 0.7 million as at 2015 (Ariff *et al.*, 2015). But apart from cows, other mastitis cases had also been reported in goat within Malaysia (Zubaidah *et al.*, 2005; Jesse *et al.*, 2016).

The two forms of mastitis are basically the clinical or subclinical mastitis (Harjanti et al., 2018) and can be divided into two categories namely; contagious pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* species) and the coliforms or environmental pathogens which include *Escherichia coli* and *Klebsiella* species (Wu *et al.*, 2016; Misra *et al.*, 2018). Subclinical mastitis is the most common form of mastitis among dairy cattle with a prevalence of about 40-50 times higher than the clinical mastitis (Marimuthu et al., 2014; Mishra et al., 2018).

Clinical responses are usually observed during immunization in dairy cows. These clinical responses include alteration in rectal temperature, heart rate, respiratory rates and hotness of the udder (Shittu *et al.*, 2012) . The severity of these clinical responses depends on age, disease condition of host, nutritional status, type of pathogenic microrganisms, severity and duration of response to immunization in animals (Azevedo *et al.*, 2016). Temperature, heart rate and respiratory rate are basic tools used in assessing response to infection or disease conditions in animals. Cows are generally homoeothermic and requires to maintain a temperature of about 38.8°C +/- 0.5 °C, RR is about 10-30 breath/minute and HR is about 40-90 beats/minute (Sartori *et al.*, 2002). Several findings have reported that increased temperature in cows was observed following immunization to mastitis infection (Herry *et al.*, 2017; Rainard *et al.*, 2015; Hajdu *et al.*, 2010). Other reports have also indicated that increased heart rate is a clinical sign observed in the response of immune to cases of mastitis in cows (Mehrzad *et al.*, 2001). Kemp, 2015 indicated that respiratory rates increases in cows during immune response to mastitis (Kemp *et al.*, 2008; Vels *et al.*, 2009). There is paucity of data on the clinical responses of dairy cows to *S. aureus* mastitis vaccination in Malaysia. This present study therefore examined the clinical parameters (temperature, heart rate and respiratory rate) of Frisian dairy cows in response to killed *S. aureus* vaccination.

Acute phase proteins (APPs) are blood molecules produced by hepatocytes, lymphocytes, monocyte and fibroblasts that change in concentration in animals subjected to external/internal alterations such as infection and inflammation (Eckersall *et al.*, 2001; Pyorala, 2003; Murata *et al.*, 2004). An acute inflammation is the local response to tissue injury or infection, large number of changes occur in the physiological system that last for one to two days before the system returns to normal at 4-7 days provided there is no further stimulation, this response is called acute phase reaction or acute phase response (APR) (Manimaran *et al.*, 2016). The APR which is characterized by fever and increased number of peripheral white blood cells coupled with change in the protein molecules in the plasma (Tian *et al.*, 2016) thereby serving as useful indicators of inflammatory conditions and diseases. When it is an increase, the APPs are called positive APPs while negative APPs refers to those APPs that decrease in concentration after an insult during APR (Murata et al., 2004). Acute phase proteins such as serum amyloid A, haptoglobin have been identified as markers of mammary inflammation in cows because they are produced by the liver in response to pro-inflammatory cytokines (Hirvonen *et al.*, 1999; Gronlund *et al.*, 2005; Gerardi *et al.*, 2009). The initial or periodic measurement of these APPs can be of prognostic value in cases of mastitis (Eckersall et al., 2001; Bhat et al., 2018). The understanding of the kinetics of APPs response to mastitis in dairy cows is key for a better diagnosis and treatment of the infection. The present study was therefore designed to evaluate the concentrations of Serum Amyloid A and Haptoglobin in vaccinated Frisian dairy cows post intramammary *S. aureus* challenge.

Cytokines are proteins that plays a vital role in cell signalling (Fietta *et al.*, 2015; Mortha and Burrows, 2018), they are produced by B lymphocytes, T lymphocytes, mast cells and macrophages (Zheng *et al.*, 2016; Göbel *et al.*, 2018) . Cytokines

include chemokines, interleukins, tumour necrosis factor and interferons (Packialakshmi *et al.*, 2016; Wang *et al.*, 2016). They strike a balance between the humoral and cell mediated immunity (Baia *et al.*, 2016; Mortha and Burrows, 2018). They are crucial in ruminant responses to inflammation, sepsis, trauma and cancer (Lin *et al.*, 2016). They are classified as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IL-18, interferon(IFN), transforming growth factor- TGF-β1 , TGF-β2 , TGF-β3 (Fietta et al., 2015). Intramammary challenge with *S. aureus* have been reported to elicit both localised and systemic response in the challenged cows. An experimental study following *S. aureus* intramammary challenge in lactating cows indicated that IL-12 is a good marker in responding to challenge and vaccination (Bannerman *et al.*, 2004). In a more related study IL-10 concentration following *S. aureus* infection in cows also indicated an increase hence a good indicator of immune activation in inflammatory condition (Hessle *et al.*, 2000). Interleukin 10 and 12 are popular for their roles in enhancing immune response in mammary gland infection such as mastitis, therefore understanding their modulation is key in estimating immune build up in vaccine trials. There tend to be a shallow data on the response of IL-12 and IL-10 in vaccinated Frisian cows post *S. aureus* challenge. The present study was therefore designed to evaluate the concentrations of IL-10 and IL-12 in vaccinated Friesian dairy cows post intramammary *S. aureus* challenge.

In studies involving mammary challenge with *S. aureus*, increase in immunoglobulin concentrations have been reported post vaccination and challenge (Furukawa et al., 2018). These immunoglobulins include IgM, IgG and IgA (Herr *et al.*, 2011; Gogoi-Tiwari *et al.*, 2015). They are derived from blood serum or locally produced by cells of the lymphocyte-plasma cell series situated close to the glandular epithelium (Sordillo and Streicher, 2002; Camussone *et al.*, 2013). IgG and IgA are a product of class switching of IgM and IgD on plasma cells differentiating from B-cells (Herr et al., 2011). *S. aureus* invades the udder via the teat and stimulates immune mediators by triggering macrophages, phagocytes, antigen presenting cells, B and T cells (Rainard and Riollet, 2006; Roussel *et al.*, 2015). In a similar studies, a persisting local production of antibody is induced by infusion of bacterial antigen into the mammary gland of ruminants, the IgG, IgM and IgA cells in the mammary gland elevated exponentially post antigenic stimulation, indicating that these markers can be of diagnostic value in inflammatory conditions of the udder (Sordillo *et al.*, 1997; Herr et al., 2011; Espinosa-Martos *et al.*, 2016). In cases where antibiotic therapy fails such as in bovine mastitis, vaccination is usually the next line of action. The efficacy of any vaccine is defined by its ability to evoke maximum IgM, IgG and IgA productions. There is no vaccine developed in Malaysia to curtail the menace of mastitis in dairy cows. Therefore, the present study evaluated the concentrations of IgM, IgG and IgA in vaccinated Frisian cows in an attempt to determine the efficacy of the prototype *S. aureus* vaccine.

Cellular changes due to the presence of *S. aureus* in the udder by natural or artificial infection will result into inflammation of the bovine mammary gland (Mehrza et al., 2005; Kuroishi *et al.*, 2003). The same study by Mehrza also reported that polymorphonuclear neutrophils contains proteases which hydrolysis gelatin, collagen,

haemoglobin and mammary gland membrane proteins thereby damaging the mammary epithelium and resulting in the decline of milk production. These cellular changes also result into oedema, epithelial damage, congestion, haemorrhage, necrosis and lymphocytic infiltration of the mammary gland (Trinidad *et al.*, 1990; Benites *et al.*, 2002; Medan *et al.*, 2002; Monks *et al.*, 2002). The histopathology of the mammary gland comprises of the lymphoid organs and nodules involved in the immune build up (Matos *et al.*, 2018; Salguero, 2018). These organs and tissues of the immune system include the thymus, spleen, lymph node and aggregates (Kirby and Bockman, 2018; Ruehl-Fehlert *et al.*, 2018). Histological studies in experimental mastitis indicated that lesions are confined to the teat, milk ducts, alveoli and lymphoid organ (Zhao *et al.*, 2003; Burvenich *et al.*, 2003; Zhao and Lacasse, 2008). There is a very rare data on the cellular changes encountered in the teat, milk ducts, alveoli, lymphoid organ and GALT in *S. aureus* infection in dairy cows. The present study was therefore designed to evaluate the cellular effects of intramammary infection with *S. aureus* following vaccination with a prototype killed *S. aureus* mastitis vaccine in lactating dairy cows.

The synergistic roles and links between these key players of the innate and acquired immune network to *S. aureus* needs to be further studied and clearly understood in order to pin down a potential vaccine candidate for immune build up against mastitis in dairy cows. This present study evaluated various immune responses to *S. aureus* vaccine and challenge in lactating Friesian cows so as to have a better understanding of the immune mechanism

1.2 Problem Statement

Despite continued use of antibiotics in treating mastitis in dairy cows, there are reports of occurrence of mastitis in dairy farms in Malaysia due to antibiotic failures. Coupled with a sharp rise in the market demand of milk due to the hikes in human population and per capita milk consumption in Malaysia. This poses a public health threat to human and animal consumers as mastitic milk can serve as a potential vehicle for the transmission of zoonotic pathogen. Commercial vaccines targeted at mastitis caused by *S. aureus* are rare and only available in the United States, UK and some part of Europe. This therefore, suggested a need for a better and efficient approach to prevent and control mastitis by developing a vaccine. This aims at protecting consumers and minimising economic downturn experienced by dairy farmers as a result of reduced milk production, lowered quality of milk yield and reproductive inefficiency by mastitis cows in Malaysia.

1.3 Hypothesis

1. Null hypothesis : There will be no periodic changes in rectal temperature, respiratory rate, heart rate, IL-10, SAA, IgM and IgG concentrations in experimental beef cows post vaccination using prototype killed *S. aureus* mastitis vaccine.
   Alternative hypothesis : There will be periodic changes in rectal temperature, respiratory rate, heart rate, IL-10, SAA, IgM and IgG concentrations in experimental beef cows post vaccination using prototype killed *S. aureus* mastitis vaccine.
2. Null hypothesis : There will be no periodic changes in rectal temperature, respiratory rate, heart rate and clinical signs in vaccinated lactating Friesian cows pre and post *S. aureus* challenge.
   Alternative hypothesis : There will be periodic changes in rectal temperature, respiratory rate, heart rate with minimal clinical signs in vaccinated lactating Friesian cows pre and post *S. aureus* challenge.
3. Null hypothesis : There will be no periodic alterations in the concentrations of IL-10, IL-12, SAA, Hp, IgM, IgG and IgA in vaccinated lactating Friesian cows pre and post *S. aureus* challenge .
   Alternate hypothesis : There will be periodic alterations in the concentrations of IL-10, IL-12, SAA, Hp, IgM, IgG and IgA in vaccinated lactating Friesian cows pre and post *S. aureus* challenge.
4. Null hypothesis : There will be no severe cellular changes in the mammary gland, teat, supramammary lymph node and GALT in vaccinated lactating Friesian cows post *S. aureus* challenge.
   Alternate hypothesis: There will be less severe cellular changes in the mammary gland, teat, supramammary lymph node and GALT in vaccinated lactating Friesian cows post *S. aureus* challenge.
5. Null hypothesis: There will be no reduction in viable *S. aureus* isolate from the mammary gland, teat, supramammary lymph node and GALT in vaccinated lactating Friesian cows post *S. aureus* challenge.
   Alternate hypothesis: There will be reduction in viable *S. aureus* isolate from the mammary gland, teat, supramammary lymph node and GALT in vaccinated lactating Friesian cows post *S. aureus* challenge.

1.4 Objectives of the study

The objectives of the study were:

1. To develop *S. aureus* killed vaccine of four different concentrations from local Malaysian isolate and establish the best vaccine concentration with a higher degree of potency on beef cows.
2. To compare the clinical responses between infection by *S. aureus* and the vaccinated lactating Friesian cows.
3. To detect the acute phase proteins, cytokine and antibody responses (Hp, SAA, IL-10, IL-12, IgM, IgG and IgA respectively) between infection by *S. aureus* and the vaccinated lactating Friesian cows.
4. To evaluate the cellular changes in the mammary gland, teat, supramammary lymph node and GALT between infection by *S. aureus* and the vaccinated lactating Friesian cows.
5. To isolate and identify *S. aureus* from mammary tissues, teat, supramammary lymph nodes and GALT using phenotypic and biochemical tests between infection by *S. aureus* and the vaccinated lactating Friesian cows.

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