

# Acute Phase Proteins (Haptoglobin and Serum Amyloid A) and Pro-Inflammatory Cytokines (IL-12 and IL-10) in Cows Vaccinated with Prototype Killed *S. aureus* Mastitis Vaccine and Challenged with *S. aureus* Mastitis Infection.

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## Research article

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# Abstract

**Background** The economic downturn experienced by farmers and the fear of milk borne infection are of a greater public health concern. Haptoglobin, Serum Amyloid A, IL-12 and IL-10 in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine and challenged with *S. aureus* were evaluated. Bacterin concentration at  $10^8$  cfu /ml of the local isolate of *S. aureus* was adjuvanted with  $KAl(SO_4)_3$ . Six lactating Friesian cows were grouped into A= Negative control, B = Positive control and C = vaccine group. Group C was vaccinated intramuscularly with 2ml of the monovalent vaccine, groups A and B with physiologic normal saline. Groups B and C were later challenged with the live bacterium via intramammary route .

**Result** There was a significant increase in IL-10 concentration in vaccinated group post primary vaccination (PPV), booster phase (PB) and during the bacterial challenge phase. There was also a significantly increased IL-12 concentration in the vaccinated group at 24 hours, weeks 1 and 2 PPV. Haptoglobin at 12 and 24 hours PPV had a significant difference in group C. During the PB at 8 and 12 hours there was a significant difference in group C. During the bacterial challenged phase at 0, 3, 24 hours and day 7 PC there was a significant difference in group B. At 8 hours PC there was a significant difference in group C. For Serum Amyloid A, during PPV at 0, 3, 8, 12, 24 hours and weeks 1 and 2, the concentrations was significantly different in groups C. During PB at 0, 3, 8 and 12 hours PB there was a significant difference in groups C. During the bacterial challenge phase at 3, 8, 12, 24 hours, days 7 and 14 PC there was a significant difference in group B. At 0 hour PC there was a significant increase observed in group C.

**Conclusion** The developed prototype killed *S. aureus* mastitis vaccine using local isolates was able to stimulate acute phase proteins and pro-inflammatory cytokines. The pattern of responses PC indicated protection, thereby suggesting that vaccination can protect against mastitis infection in dairy cows.

## Background

Mastitis is a disease of economic importance in the dairy sector [1, 2, 3]. It is caused by coliform bacteria, staphylococci and streptococci with a range of clinical appearances such as fever, feed withdrawal and reduced milk production [4, 5, 6, 7]. Dairy cows exposed to mastitis do express a complex interaction between the cow's defence mechanism and the specific bacterial virulence factors like; toxins, polysaccharide, cell surface factors, secreted factors, gene, biofilm, capsule and membrane proteins [8, 9, 10]. These virulent factors hence trigger both the cellular and humoral immune responses [11, 12, 13]. Accordingly, cytokines responses have been found to play crucial role in the pathogenesis of most bacterial infection [14, 15, 16, 17].

Cytokines as immunological mediators are involved in the immune build up in inflammatory conditions of the udder [18, 19]. They are known for their cell signalling roles and anti-inflammatory pathways during

infection as they strike a balance between the cellular and humoral immunity [20, 21, 17]. An experimental study following *S. aureus* intramammary challenge in lactating cows indicated that IL-12 is a good marker in response to *S. aureus* challenge and vaccination [22]. In a more related study IL-10 concentration following *S. aureus* infection in cows also indicated an increased in its concentration hence regarded as a good indicator of immune activation in inflammatory condition [23]. Early cytokine transcriptional alterations could be an important yardstick in unravelling their role in the pathophysiology of *S. aureus* mastitis infection.

Acute phase proteins are produced in the liver, synthesised by the agranulocytes, epithelial cells and found in different concentrations based on the state of animal health [24, 25, 26]. During any form of tissue injury, acute phase responses (APR) are initiated [27, 28]. At this stage the APP either increases or reduces in concentration [29, 30, 31, 32] reflecting the degree of damages in the tissue [24]. In cases of clinical mastitis SAA and Hp have been indicated as APP biomarkers of inflammation in response to cytokine stimulation [33, 34, 35, 36, 37, 38]. APPs are considered a powerful diagnostic measure in field or experimental mastitis studies. The pathogenesis of mastitis have been reported to involve inflammatory reactions [39, 40, 41] which results into increased production of Acute-Phase Proteins (APP) [31, 32]. Recently, APPs are considered as important diagnostic biomarkers not only in veterinary diagnostics but also in human medicine [42, 43, 44].

In the present study, the responses of acute phase proteins (Haptoglobin and Serum Amyloid A) and pro-inflammatory cytokines (IL-12 and IL-10) in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine and challenged with *S. aureus* mastitis infection were studied. These biomarkers were opted to be studied in the present study to determine the efficacy of the developed prototype killed *S. aureus* mastitis vaccine.

## Results

### Interleukin 10 (IL-10)

The means of IL-10 concentrations of groups A, B and C are presented in Fig. 1. During the acute phase PPV at 0 hour the concentrations of IL-10 in groups A, B and C were  $3.00 \pm 0.038$  ng/ml,  $3.866 \pm 0.038$  ng/ml and  $5.08 \pm 0.038$  ng/ml respectively with a significant difference ( $P < 0.05$ ) in groups C having about 1 fold increased. The concentrations of IL-10 at 3 hours PV indicated a significant difference ( $P < 0.05$ ) in groups C (with about 1 fold increased) and was  $4.28 \pm 0.014$  ng/ml as compared to negative control which was  $3.38 \pm 0.014$  ng/ml. At 8, 24 hours and week 1 PPV there was no significant increase ( $P > 0.05$ ) between the groups. At 12 hours PPV there was a significant difference ( $P < 0.05$ ) in group C (with about 1 fold increased) and was  $4.79 \pm 0.05$  ng/ml as compared to negative control which was  $3.19 \pm 0.05$  ng/ml. At week 2 PPV there was a significant difference ( $P < 0.05$ ) in group C with 2 folds increased ( $5.40 \pm 0.04$  ng/ml) as compared to negative control ( $2.66 \pm 0.04$  ng/ml). During the vaccine booster phase at 3, 8, 12 and 24 hours PB there was a significant difference ( $P < 0.05$ ) in groups C ( $3.86 \pm 0.02$  ng/ml,  $10.77 \pm 0.03$  ng/ml,  $6.78 \pm 0.03$  ng/ml and  $4.06 \pm 0.02$  ng/ml) as compared to the negative

control ( $2.37 \pm 0.02$  ng/ml,  $5.35 \pm 0.03$  ng/ml,  $6.15 \pm 0.03$  ng/ml and  $2.08 \pm 0.02$  ng/ml) with 2, 2, 1 and 2 folds increased respectively. During the bacterial challenge phase at 0, 3 and 12 hours PC there was a significant difference ( $P < 0.05$ ) in group C ( $5.35 \pm 0.03$  ng/ml,  $12.16 \pm 0.03$  ng/ml,  $12.30 \pm 0.03$  ng/ml) as compared to the negative control ( $3.48 \pm 0.03$  ng/ml,  $9.46 \pm 0.03$  ng/ml,  $11.78 \pm 0.03$  ng/ml) with about 1, 1 and 1 folds increased respectively. At 24 hours PC there was a significant difference ( $P < 0.05$ ) in group B ( $15.66 \pm 0.05$  ng/ml) as compared to the negative control ( $5.85 \pm 0.05$  ng/ml) with 3 folds increased. There was no significant increase ( $P > 0.05$ ) at 8 hours, days 7 and 14 PC in all the treatment groups.

## FIGURE 1

### Interleukin 12

The means of IL-12 concentration of Groups A, B and C are presented in Fig. 2. During the acute phase PPV at 0, 3, 8 and 12 hours there was no significant difference ( $P > 0.05$ ) between the groups. The concentrations of IL-12 at 24 hours PPV indicated a significant difference ( $P < 0.05$ ) in groups C (with about 223 folds increased) and was  $422.5 \pm 58$  ng/ml as compared to negative control which was  $1.89 \pm 58$  ng/ml. At weeks 1 and 2 PPV there was a significant difference ( $P < 0.05$ ) in group C (with about 3 and 2 folds increased) which were  $531.6 \pm 0.2$  ng/ml and  $279.8 \pm 0.14$  ng/ml as compared to the negative control which was  $196 \pm 0.23$  ng/ml and  $169.8 \pm 0.14$  ng/ml respectively.

During the vaccine booster phase at 0, 3, 8, 12 and 24 hours PB there was a significant difference ( $P < 0.05$ ) in groups C ( $108.9 \pm 14.8$  ng/ml,  $135.9 \pm 8.6$  ng/ml,  $261.8 \pm 0.2$  ng/ml,  $473.8 \pm 0.11$  ng/ml,  $196.9 \pm 0.2$  ng/ml) as compared to the negative control ( $26.13 \pm 14.8$  ng/ml,  $17.9 \pm 8.6$  ng/ml,  $47.8 \pm 0.2$  ng/ml,  $151.8 \pm 0.11$  ng/ml,  $133 \pm 0.2$  ng/ml) with about 4, 8, 5, 3 and 1 folds increased respectively.

During the bacterial challenge phase at 12, 24 hours, days 7 and 14 PC there was a significant difference ( $P < 0.05$ ) in group C ( $447 \pm 28.7$  ng/ml,  $294.5 \pm 0.3$  ng/ml,  $137.8 \pm 0.21$  ng/ml,  $155.8 \pm 0.3$  ng/ml) as compared to the negative control ( $184.7 \pm 28.7$  ng/ml,  $180.7 \pm 0.3$  ng/ml,  $26.7 \pm 0.21$  ng/ml,  $28.6 \pm 0.3$  ng/ml) with about 2, 2, 5 and 5 folds increased respectively. At 3 and 8 hours PC there was a significant difference ( $P < 0.05$ ) in group B ( $809.9 \pm 0.3$  ng/ml and  $378.7 \pm 0.2$  ng/ml) as compared to the negative control ( $10.51 \pm 0.3$  ng/ml and  $162.9 \pm 0.2$  ng/ml) with an IL-12 surge of about 80 and 2 folds increased respectively.

## FIGURE 2

### Haptoglobin (Hp)

The means of Hp concentrations of groups A, B and C are presented in Fig. 3. During the acute phase PPV at 0 hour the concentrations of Hp in groups A, B and C were  $25381 \pm 19617$  ng/ml,  $26217 \pm 19617$  ng/ml and  $25158 \pm 19617$  ng/ml respectively with no significant difference ( $P > 0.05$ ) between the groups. At 3 and 8 hours PPV there was no significant difference ( $P > 0.05$ ) between the groups. At 12 and 24 hours PPV there was a significant difference ( $P < 0.05$ ) in group C (with about 4 and 3 folds increased respectively) and were  $21794 \pm 1601$  ng/ml and  $65958 \pm 2795$  ng/ml as compared to the negative control

which were  $5236 \pm 1601$  ng/ml and  $19842 \pm 2795$  ng/ml respectively. At weeks 1 and 2 PPV there was no significant increased ( $P > 0.05$ ) in groups B and C.

During the vaccine booster phase at 0, 3 and 24 hours PB there was no significant difference ( $P > 0.05$ ) between the groups. At 8 and 12 hours PB there was a significant difference ( $P < 0.05$ ) in group C (with about 10 and 13 folds increased respectively) and were  $149307 \pm 3372$  ng/ml and  $201804 \pm 3710$  ng/ml as compared to the negative control which were  $14418 \pm 3372$  ng/ml and  $15062 \pm 3710$  ng/ml respectively.

During the bacterial challenge phase at 0, 3, 24 hours and day 7 PC there was a significant difference ( $P < 0.05$ ) in group B (with about 17, 83, 80 and 24 fold increased respectively) and were ( $25588 \pm 513$  ng/ml,  $28673.4 \pm 205$  ng/ml,  $101089 \pm 6751$  ng/ml and  $41228 \pm 4147$  ng/ml respectively) as compared to the negative control group which were  $1463 \pm 513$  ng/ml,  $342.7 \pm 205$  ng/ml,  $1264 \pm 6751$  ng/ml and  $1714 \pm 4147$  ng/ml respectively. At 8 hours PC there was a significant difference ( $P < 0.05$ ) observed in group C (with about 35 fold increased) and was  $58790 \pm 395$  ng/ml as compared to the negative control group which was  $1650 \pm 395$  ng/ml. At 12 hours and day 14 PC there was no significant increase ( $P > 0.05$ ) observed between the groups.

### FIGURE 3

#### Serum Amyloid A (SAA)

The means of SAA concentrations of Groups A, B and C are presented in Fig. 4. During the acute phase PPV at 0, 3, 8, 12, 24 hours and weeks 1 and 2 PPV the concentrations of SAA was significantly difference ( $P > 0.05$ ) in groups C (with about 5, 6, 3, 4, 9, 5 and 16 folds increased respectively) and were  $115.9 \pm 2.4$   $\mu$ g/L,  $670.56 \pm 23.6$   $\mu$ g/L,  $1266 \pm 4.5$   $\mu$ g/L,  $285.5 \pm 15.8$   $\mu$ g/L,  $264.6 \pm 20.5$   $\mu$ g/L,  $425.7 \pm 3.4$   $\mu$ g/L and  $219.5 \pm 1.1$   $\mu$ g/L as compared to the negative control group which were  $24.8 \pm 2.4$   $\mu$ g/L,  $113.7 \pm 23.6$   $\mu$ g/L,  $470.6 \pm 4.5$   $\mu$ g/L,  $76.1 \pm 15.8$   $\mu$ g/L,  $30.1 \pm 20.5$   $\mu$ g/L,  $94.1 \pm 3.4$   $\mu$ g/L and  $14.5 \pm 1.5$   $\mu$ g/L respectively.

During the vaccine booster phase at 0, 3, 8 and 12 hours PB there was a significant difference ( $P < 0.05$ ) in groups C (with about 29, 11, 8 and 8 folds increased respectively) and were  $378.9 \pm 0.7$   $\mu$ g/L,  $397.7 \pm 0.8$   $\mu$ g/L,  $455.4 \pm 1.3$   $\mu$ g/L and  $489.9 \pm 0.8$   $\mu$ g/L as compared to the negative control which were  $12.9 \pm 0.7$   $\mu$ g/L,  $35.5 \pm 0.8$   $\mu$ g/L,  $46.5 \pm 1.3$   $\mu$ g/L and  $64.4 \pm 0.8$   $\mu$ g/L respectively. At 24 hours PB there was no significant increased ( $P > 0.05$ ) between the groups.

During the bacterial challenge phase at 3, 8, 12, 24 hours, days 7 and 14 PC there was a significant difference ( $P < 0.05$ ) in group B (with about 4, 11, 3, 6, 8 and 1 folds increased respectively) and were  $1320.5 \pm 1.9$   $\mu$ g/L,  $4222 \pm 0.6$   $\mu$ g/L,  $1288.9 \pm 1.2$   $\mu$ g/L,  $2311 \pm 0.9$   $\mu$ g/L,  $3244 \pm 25.7$   $\mu$ g/L and  $555.8 \pm 25.6$   $\mu$ g/L as compared to the negative control group which were  $373 \pm 1.9$   $\mu$ g/L,  $376.4 \pm 0.6$   $\mu$ g/L,  $371.8 \pm 1.2$   $\mu$ g/L,  $375.1 \pm 0.9$   $\mu$ g/L,  $372.1 \pm 25.7$   $\mu$ g/L and  $373.3 \pm 25.6$   $\mu$ g/L respectively. At 0 hour PC there was

a significant increased ( $P < 0.05$ ) observed in group C with about 1 fold increased ( $331.1 \pm 0.3 \mu\text{g/L}$ ) as compared to the negative control ( $287.3 \pm 0.3 \mu\text{g/L}$ )

FIGURE 4

## Discussion

The potency of immune response to *S. aureus* mastitis in dairy cows is defined by the concentrations of lymphocyte, leucocytes and cytokines released during its invasion of the mammary gland [47, 48, 49, 50, 51]. Studies have indicated that *S. aureus* enterotoxin A (SEA), B (SEB), C (SEC) and toxic shock syndrome toxin-1 (TSST-1) usually act as superantigens (SAg) by way of activating T-lymphocytes, mononuclear cells and also initiate the release of IL-1, 2, 4, 6, 10 and 12 [52, 53, 54, 55]. SEA, SEB, SEC and TSST-1 have been shown to be potential inducers of polyclonal activation of bovine T-cells, CD4+, CD8+, and monocytes with release of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [56, 57]. Changes in concentration of cytokine synthesis are regarded as a crucial factor in scoring the inflammatory responses to *S. aureus* infection.

To the very best of our knowledge this present work is the new study that demonstrates the effect of laboratory-prepared killed *S. aureus* vaccine from local Malaysian isolate on Friesian dairy cows. The current study had indicated that there was a significant increase in IL-10 concentration in vaccinated group at 0, 3, 12 hours and week 2 post primary vaccination (PPV) with about 1, 1, 1 and 2 folds increased respectively. There was also a significant increase in IL-10 concentration in vaccinated group C at 3, 8, 12 and 24 hours during the vaccine booster phase (PB) with about 2, 2, 1 and 2 folds increased respectively. During the bacterial challenge phase at 0, 3 and 12 hours PC there was also a significant increase in IL-10 in vaccinated group with about 1, 1 and 1 folds increased respectively. Though at 24 hours PC there was a significant difference in IL-10 recorded in positive control group with about 3 folds increased. There was also a significantly increased IL-12 concentration in the vaccinated group at 24 hours, weeks 1 and 2 PPV with about 2.23, 3 and 2 folds increased respectively. During the vaccine booster phase at 0, 3, 8, 12 and 24 hours PB there was a significant increase of IL-12 in the vaccinated group with about 4, 8, 5, 3 and 1 folds increased respectively. The bacterial challenge phase at 12, 24 hours, days 7 and 14 PC recorded a significant increase in IL-12 concentration in the vaccinated group with about 2, 2, 5 and 5 folds increased respectively. Although, at 3 and 8 hours PC there was a significant increase in IL-12 noted in the positive control group with a surge of about 80 and 2 folds increased respectively which return to 0 fold afterwards at 12 hours PV. The extended increase in IL-10 and IL-12 in the vaccinated group across the phases of PPV, PB and PC in the present study is an indication suggesting the potency of the prototype killed vaccine in suppressing the effect of *S. aureus* intramammary challenge in the Friesian dairy cows. The result of the present study is in harmony with the study reported on cytokines as markers in *S. aureus* mastitis of bovine mammary gland which indicated that the suppression of these bacteria PPV or PC is usually accompanied by an elevated IL-10 and IL-12 concentration [58]. There are similarities between the findings of the present study and that of D'andrea, (1993) where a different model and an in-vitro studies indicated that a negative correlation existed between IL-10 and IL-12 concentration with an explanation

that IL-10 plays a down regulatory role on IL-12 which can be why it limits the duration of IL-12 in most bacterial infection [59]. This may suggest why IL-12 concentration was downregulated by IL-10 at 0, 3 and 8 hours PPV and at 0 hours PC in the current study. There are studies where a positive strong correlation had been reported between IL-10 and IL-12 during bacterial infection [22]. Bernthal,(2011), in his study described on the protective roles of cytokines in *S. aureus* infection indicated that the increase in the concentration of most cytokines is a positive sign of prompt defence response against *S. aureus*infection [60].

The findings of the present study further supports the reports of [61], in a study conducted on the characterisation of cytokines in mammary glands infected with *S. aureus* which related that most cytokines generally pick up increasingly up to about 6 hrs PC (as seen in IL-12 in the present study) and gets to its peak by 12 hrs PC, but a gradual decline was noticed in their concentrations 24hrs to about 48hrs PC (as observed in IL-10 the present study) [61]. Though Wang, (2015) in the same study also maintained that at 24 hrs to 48 hrs PC, elevated levels of IL-10 can be sustained but not all the time which will eventually fade away by 48 hrs PC. The findings of the current study are consistent with the study of [62] in an investigation on cytokines as markers in *S. aureus* mastitis infection of bovine mammary gland where he indicated that there exist an emergence of IL-12 at 7hrs PC ( as seen in IL-12 in the current study) and 24hrs PC (as seen in IL-12 and IL-10 in the current study) which later declined 32hrs PC [62].

In a more related study, the excessive production of IL-10 PC or PPV had been suggested to be associated with the recruitment of more CD8 + T suppressor cells [52], altering pathophysiology of the bovine mammary gland and early recruitment of neutrophils [63]. The shift from Th2 cells production to Th1 helper cell production as a result of IL-10 elevation in *S. aureus* infection had been confirmed and documented as a fundamental phenomenon in immune response [64]. The results of the current findings matches those observed by Hesse et al., 2000 in a study on bacteria being potent inducers of IL-10 production, where they indicated an elevated increase in IL-10 following *S. aureus* infection in cows, [23]. The same findings in the association of IL-10 elevation following *S. aureus* challenge was concluded by Bannerman et al., 2004 in their study on the ability of *S. aureus* to elicit differential innate immune responses following intramammary infection [22]. In accordance with the present results, previous study by Armstrong, (1996) on alveolar macrophages and peripheral blood monocytes also reported that generally high levels of IL-10 were observed in challenge quarters during experimental mastitis infection (Armstrong et al., 1996).

IL-12 (produced by macrophages) plays the special role of mediating between the innate and specific immune response [65] thereby bears the name maintainer of antibody production. The expression of IL-12 have also been detected in dairy cows during acute phases PC indicating their important role in regulation of the immune responses of bovine mammary gland in *S. aureus* infection [62]. Riollet, (2001) had also reported that increased concentrations of IL-12 is a norm observed in serum samples obtained from dairy of cows challenged with *S. aureus* [65]. Previous studies have reported that intramammary challenge with *S. aureus* elicited both localised and systemic response in the vaccinated cows which also indicated that cytokines are potent inducers of acute phase responses during natural or experimental

bacterial challenge. This was obtainable in the report of Bannerman, (2004) in an experimental study on *S. aureus* challenge in lactating cows which indicated that IL-12 were seen at 32 h PC which remained elevated through 40hrs PC [22]. The increase in IL-12 concentration observed in positive control group in the present study at 3 hrs PC which was about 80 folds increase suggested that such an increase is directly associated with the deterioration of udder health due to *S. aureus* challenge, this is seen in cases where the titre values of cytokines increased greatly with increased inflammation of the mammary glands [66] as seen in the positive control group.

An increased concentration of cytokines is considered strong evidence in its role in inducing the migration of leukocytes and production of antibody [61]. However, contrary opinions have been indicated by Hagiwara, where they clearly stipulated that there was no relationship between cytokine concentration and stages of mammary inflammation in mastitis cows [67]. Taylor, (1997) studied the immunomediative role of increased cytokines from mastitic cows and stipulated that the causative agent of mastitis does not relatively determine alteration in cytokine response [68]. There are several possible explanations for the discrepancies which could be attributed to different animal model used in the *S. aureus* experimental mammary challenge, gram status of the bacteria (positive or negative), types of cytokine estimated, the specificity of assay used, individual animal heterogeneity and functional impairment of white blood cells in infected udder.

Based on the data obtained and analysed from the current study, we can hypothesis that our vaccine provided protection against *S. aureus* mastitis in Friesian cows. This explains why the IL-12 and IL-10 levels were significantly higher in vaccinated group than in the positive control group as the major role of IL-12 is associated with regulating Th-1 and Th-2 lymphocytes, mediating between the innate and acquired immunity [69, 70] and its enhancing role in immune responses in the mammary gland during *S. aureus* infection. While IL-10 is associated with the immune cell signalling and T- cell shift production against *S. aureus* challenge in the Friesian cows.

Recently, the applications of APPs biomarkers as indicators of inflammation of the mammary gland have gained a wider acceptance in the assessment of udder health. Acute phase proteins constitute part of innate immune components whose concentration varies with respect to stimuli of external or internal origin [71]. APPs concentration such as SAA and Hp increases during early phase of reaction of cows to bacterial challenge or infection [72, 73]. These two APPs aid in determining the progress of inflammatory conditions thereby acting as a valuable diagnostic and prognostic tool in cases of mastitis in dairy cows [74, 41].

To the very best of our knowledge, this current study is the new information on the responses of SAA and Hp to locally developed killed *S. aureus* vaccine of Malaysian isolate on lactating Friesian cows. The current study had indicated that there was a significant increase in Hp concentrations of vaccinated group at 12 and 24 hours PPV with about 4 and 3 folds increased respectively. During the vaccine booster phase there was also a significant increase in the Hp concentration of the vaccinated group at 8 and 12 hours PB with about 10 and 13 folds increased respectively. During the bacterial challenge phase there

was a significant increase in Hp concentration of the positive control group at 0, 3, 24 hours and day 7 PC with about 17, 83, 80 and 24 fold increased respectively. Interestingly, at about 8 hours PC there was a significant increase observed in the Hp concentration vaccinated group with about 35 fold increased. The current study had also indicated a significant increase in SAA concentration of vaccinated group at 0, 3, 8, 12, 24 hours and weeks 1 and 2 PPV with about 5, 6, 3, 4, 9, 5 and 16 folds increased respectively. During the vaccine booster phase there was also a significant increase in SAA concentration of vaccinated group at 0, 3, 8 and 12 hours PB with about 29, 11, 8 and 8 folds increased respectively. During the bacterial challenge phase there was a significant increase in SAA concentration of positive control group at 3, 8, 12, 24 hours, days 7 and 14 PC with about 4, 11, 3, 6, 8 and 1 folds increased respectively. The elevated levels of Hp and SAA of vaccinated group PPV and PB in the present study are a prominent and sensitive indication that the prototype killed *S. aureus* vaccine has the potential to curtail inflammatory tendencies of *S. aureus* challenge. The reduced SAA and Hp concentration in the vaccinated group and increased concentrations in the positive control group PC is quite an indication that the prototype killed *S. aureus* vaccine had played a vital role of immune modulation and bacterial opsonisation in the vaccinated group.

The findings of the current study are consistent with the reports of [72], who studied the acute phase proteins in serum from dairy cows with clinical case of mastitis, where they concluded that SAA and Hp have both been identified as a major acute phase protein in cattle associated with mastitis infection and that their elevation and detection in serum samples is always a clear indication of acute phase response (APR) to opsonisation, infection or challenge. In a more detailed report by [73] who conducted a study on the early pathogenesis and inflammatory responses in experimental bovine mastitis due to gram positive bacteria stipulated that increased in SAA and Hp is a tool of measuring the severity of mastitis infection in both experimentally and naturally challenged cows in dairy farms.

In accordance with present results, previous study by [74] had also reported that SAA and Hp in serum of bovine mastitic cases increases during response to inflammation, thereby suggesting that the demonstration of these APPs in the serum is a key diagnostic value in differentiating mastitis cows from non-mastitis ones. Increased levels of SAA and Hp have been associated with the occurrence of acute or sub-acute mastitis in a similar study conducted by [75] where the results were in harmony with the present study thereby indicating that increased APPs is associated to sub-clinical and clinical forms of mastitis.

There are similarities between the response of SAA and Hp in the current study and those described by [76, 77] in a study conducted on APPs as a diagnostic tool for bovine sub-clinical mastitis, where the report indicated that SAA and Hp were on the increased during post infection period thereby advocating that both APPs are always on the rise in response to inflammatory disease such as mastitis in cows. The findings of the present study also corroborates with the reports of [78] who carried out a study on Hp and SAA in serum during acute and chronic experimentally induced *S. aureus* mastitis where they concluded that cows challenged with *S. aureus* developed a high concentration of SAA in serum during an acute

phase of the challenge and this is in harmony with findings observed in SAA response in the present study.

Furthermore, a study on the acute phase response in naturally occurring coliform mastitis related that increased levels of both SAA and Hp were observed during an experimental challenge of dairy cows [79] which tends to be in harmony with the findings in the present study. The observed increased SAA in the current study also agrees with study of [80] who measured the acute phase protein concentrations in serum samples from healthy cows, cows with clinical mastitis and cows with extra mammary inflammatory conditions where they reported that the SAA concentration in bovine serum with clinical mastitis had an elevation indicative of its response to bacterial invasion.

This simultaneous elevation in serum concentrations of both SAA and Hp as seen in the current work is in agreement with observations made in several studies on APP in dairy cows. Eckersall in their study on the acute phase proteins in serum from dairy cows with clinical case of mastitis had indicated a significant positive strong correlation between Hp and SAA [72]. In contrast to our findings, however, only serum Hp concentration elevated in mastitis cows as was reported by [81], whereas both SAA and Hp levels have increased extensively PC in our findings. A study on the local immunization and impacts on the response of dairy cows to mastitis was conducted by [82], where their study concluded that only serum Hp was a better marker in assessing impact of local immunization PC in dairy cows. This is in contrary to other studies and the present study where both SAA and Hp have proven to be potential biomarkers of inflammatory tendencies caused by *S. aureus* in dairy cows. In another study by Pyörälä, they conducted a research on the acute phase proteins in naturally occurring bovine mastitis caused by different pathogens and they concluded that Hp is never a better tool to be used as bovine mastitis marker as SAA proved to be more reliable and sensitive [83].

A possible explanation for the discrepancies in the findings of different authors as it relates to SAA and Hp reliability as markers of bovine mastitis could be attributed to different geographical locations where such studies were conducted, samples used for the detection of APPs, type of APP assayed and the fact that lower APPs in serum can be as a result of leakages via the (disrupted) blood milk barrier into the milk during mastitis [80]. It also seems possible that the inconsistency in these results from different authors might be partly explained by the different methods used in measuring these variables, variation in duration and severity of mastitis in experimentally or naturally infected cows and possibly due to different animal models used in the study of mastitis infection [31].

A very important and relevant point outlined by the present study was the fact that the vaccinated Friesian cows showed evidence of lower degree of Hp and SAA responses in PC group as compared to the positive control group. Friesian cows in the positive control group had evidence of increased Hp and SAA indicating the existence of bacterial proliferation in the mammary gland of the positive group more than the vaccinated group. Based on these findings in the present study, we can hypothesise that the prototype killed *S. aureus* mastitis vaccine conferred immunity against *S. aureus* challenge mastitis

infection in vaccinated lactating Friesian cows. Where lower concentrations of APPs were measured in the vaccinated group as compared to the positive control group PC.

## **Conclusion**

In conclusion, IL-12, IL-10, SAA and Hp have the potential to be used as biomarkers to evaluate the vaccine efficacy. Therefore, in this study it can be concluded that the developed prototype killed *S. aureus* mastitis vaccine using local isolates was able to stimulate responses of acute phase proteins and pro-inflammatory cytokines and the pattern of responses post challenged with mastitis infection showed evidence of protection pattern. It can be concluded that mastitis infection in dairy cows can be protected via vaccination.

## **Materials And Method**

### **Ethical approval**

The laid guidelines of the Institutional Animal Care and use committee (IACUC) of the Universiti Putra Malaysia was strictly adhered to and the experimental trial was approved by IACUC with approval code as UPM/IACUC/AUP- R072/ 2016. To this effect a written informed consent letter was obtained to use the animals for the present study.

### **Sources of Experimental animals**

The experimental lactating Friesian dairy cows were sourced from the Faculty of Veterinary Medicine, University Putra Malaysia Farm.

### **Bacterial Segregation/Identification**

Mastitis positive milk samples were smeared on blood agar (OXOID® England) and subjected to gram staining. Catalase test and analytical profile indexing (API) were further used for bacterial profiling. The *S. aureus* corresponds fully with strain HC1340 with Accession CP012011.1.

# Experimental Design

Six healthy lactating Friesian dairy cows weighing about 300-350kg body weight between the ages of 5 – 7 years were selected randomly for the purpose of this study. The cows were divided into 3 main groups with 2 animals each. Group A was the Negative control, Group B the Positive control and Group C is the  $10^8$  cfu/ml of the killed bacteria vaccine group. Groups A and B were inoculated intramammary (IM) with 2ml of physiologic normal saline while Group C was vaccinated with  $10^8$  cfu/ml of the killed bacteria vaccine IM. Blood samples were collected prior to the experiment and at days 1(0hr, 3hr, 8hr,12hr and 24h post primary vaccination (PV)), 7, 14, 21(0hr, 3hr, 8hr,12hr and 24h Post booster (PB)), 28(0hr, 3hr, 8hr,12hr and 24h Post challenge (PC) and 35 and 42PV.

## Euthanasia/ Animal Slaughter Post Experiment

Experimental animals were not anaesthetized but rather slaughtered post experiment by the institution based certified veterinary animal slaughter officer working on the farm. The carcass were properly discarded by burying.

## Prototype Killed Mastitis Vaccine Preparation

The *S. aureus* isolates were grown in Brain heart infusion but with a little modification as described by [45,46], autoclaved at  $121^{\circ}\text{C}$  for 15 minutes to achieved sterility and incubated at  $37^{\circ}\text{C}$  for 24hrs. The broth was however killed using 0.5% formalin at  $37^{\circ}\text{C}$  for 24hrs. Aluminium potassium sulfate adjuvant was suspended in normal saline to achieve 1% (Mohi-ud-din *et al.*, 2014). An adjustment of  $10^8$  cfu/ml of the killed bacteria was finally prepared. The vaccine was sub-cultured on a Mannitol agar, MacConkey agar, Sabouraud dextrose agar and blood agar to ascertain effective killing and non-contamination of the vaccine by bacteria and fungi [45]. There was no evidence of growth on the entire agar used which was an indicative of the killed vaccine sterility.

## Enzyme linked immunosorbent assay for IL-10 and IL-12

Enzyme linked immunosorbent assay for Bovine IL-10/IL-12 was performed using a competitive inhibition enzyme immunoassay kit from the CUSABIO BIOTECH CO., LTD, China. A 96-well plates were

pre-coated with goat-anti-rabbit antibody. Samples were added to the wells with an antibody specific for IL-10/IL-12 and Horseradish Peroxidase (HRP) conjugated IL-10. A Blank well was set without any solution followed by addition of 50µl of Standard and Sample per well. Then 50µl of HRP-conjugate was added to each well (not to Blank well), 50µl antibody was further added to each well. This was then incubated for 1 hour at 37°C followed by aspiration and washing using a Wash Buffer (200µl). The plate was then blotted and 50µl of Substrate A and 50µl of Substrate B were added to each and incubated for 15 minutes at 37°C. Finally, 50µl of Stop Solution was added to each well and optical density of each well was determined within 10 minutes using a microplate reader set to 450 nm.

### **Bovine Serum Amyloid A (SAA) ELISA Assay**

Enzyme linked immunosorbent assay for Bovine SAA was performed using a commercially available CUSABIO ELISA kit from CUSABIO BIOTECH CO., LTD, China. The assay employed the competitive inhibition enzyme immunoassay technique where the 96-well plates were pre-coated with goat-anti-rabbit antibody. Samples were added to the wells with an antibody specific for SAA and Horseradish Peroxidase (HRP) conjugated SAA. A blank well was set without any solution followed by addition of 50µl of Standard and Sample per well. Then 50µl of HRP-conjugate was added to each well (not to blank well), 50µl antibody was further added to each well. This was then incubated for 1 hour at 37 °C followed by aspiration and washing using a Wash Buffer (200µl). The plate was then blotted and 50µl of Substrate A and 50µl of Substrate B were added to each and incubated for 15 minutes at 37 C. Finally, 50µl of Stop Solution was added to each well and optical density of each well was determined within 10 minutes using a microplate reader set to 450 nm. The average of the duplicate readings for each standard and sample were calculated and subtracted from the average optical density of blank. A standard curve was then created and the concentration read from the standard curve was however multiplied by the dilution factor of the samples.

### **Bovine Serum Haptoglobin (Hp) Assay**

Enzyme linked immunosorbent assay for Bovine Hp was performed using a commercially available CUSABIO ELISA kit from CUSABIO BIOTECH CO., LTD, China. The assay employed the competitive inhibition enzyme immunoassay technique where the 96-well plates were pre-coated with goat-anti-rabbit antibody. Samples were added to the wells with an antibody specific for Hp and Horseradish Peroxidase (HRP) conjugated Hp. A blank well was set without any solution followed by addition of 50µl of Standard and Sample per well. Then 50µl of HRP-conjugate was added to each well (not to blank well), 50µl antibody was further added to each well. This was then incubated for 1 hour at 37 °C followed by aspiration and washing using a Wash Buffer (200µl). The plate was then blotted and 50µl of Substrate A and 50µl of Substrate B were added to each and incubated for 15 minutes at 37 °C. Finally, 50µl of Stop Solution was added to each well and optical density of each well was determined within 10 minutes using a microplate reader set to 450 nm. The average of the duplicate readings for each standard and sample were calculated and subtracted from the average optical density of blank. A standard curve was then created and the concentration read from the standard curve was however multiplied by the dilution factor of the samples.

## **Statistics**

JMP version 11 software (SAS Institute Incorporation, Cary NC) was used for the analysis with significant at  $p \leq 0.05$ . ANOVA and Dennett's tests were applied.

## **Abbreviations**

Hp

Haptoglobin

SAA

Serum Amyloid A

IL-12

Interleukin 12

IL-10

Interleukin 10

PPV

Post primary vaccination

PB

booster phase

PC

Post challenge

APP

Acute phase proteins

APR

Acute phase responses

IACUC

Institutional Animal Care and use committee

CMT

California Mastitis test

API

Analytical profile indexing

IM

Intramammary

HRP

Horseradish Peroxidase

## **Declarations**

### **Ethics declaration and consent to participate**

The protocol was approved by IACUC of Universiti Putra Malaysia with a caption approval number UPM/IACUC/AUP- R072/ 2016.

A written informed consent letter was obtained to use the animals for the present study.

### **Consent to Publish**

All members of the author list have attested their consent for the article to be published.

### **Availability of data and materials**

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

## Competing interest

There is no conflict of interest declared by the authors.

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## Authors contribution

1. F Jesse conceptualised the research, A. Lila together with A. W. Haron and Z. Zakaria assisted in the research frame work. I. U. Hambali and K. Bhutto carried out the research and M. Jefri assisted practically.

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## Figures

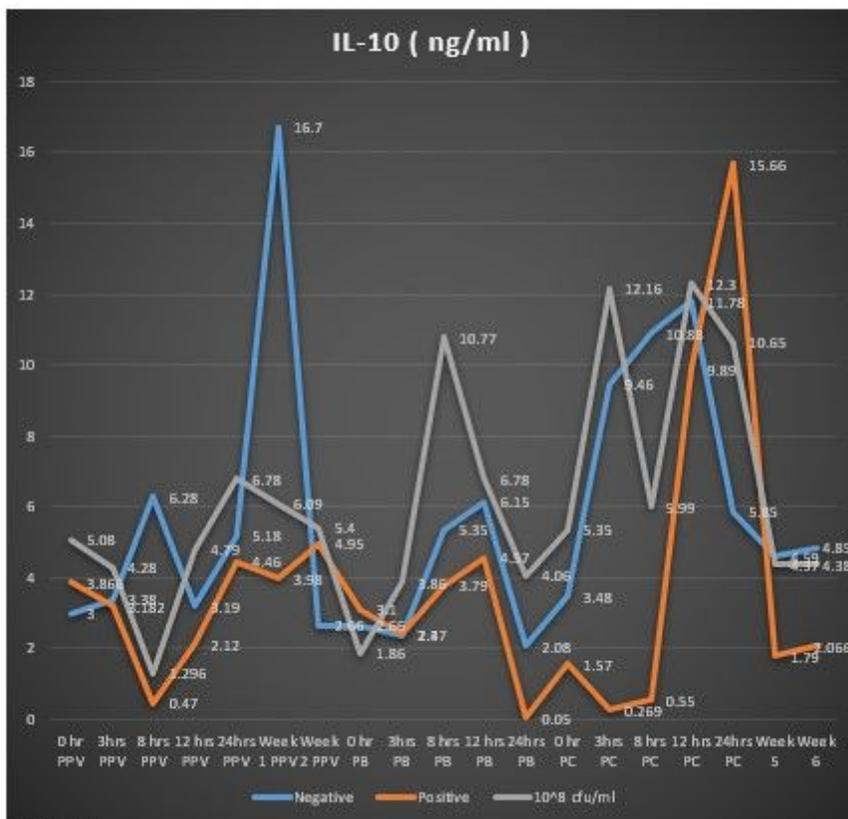


FIGURE 1

### Figure 1

Periodic concentrations of IL-10 in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of 10<sup>8</sup> cfu/ml. PPV = Post Primary Vaccination, PB = Post Booster, PC = Post Challenge, 0 hr = 0-1 hour.

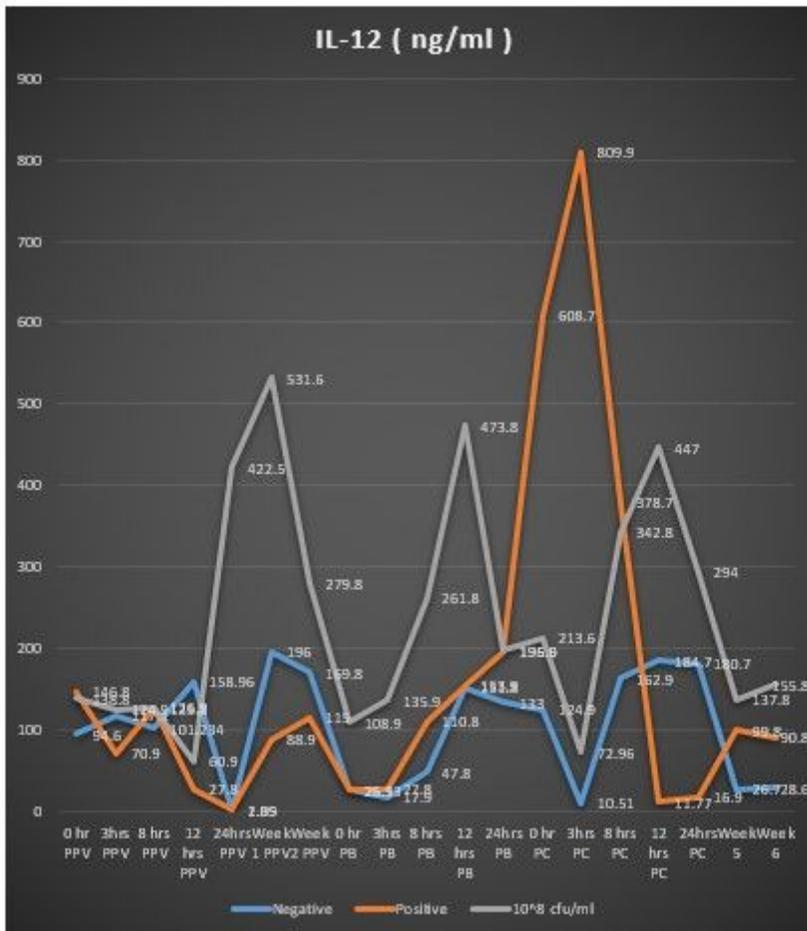


FIGURE 2

Figure 2

Periodic concentrations of IL-12 in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of 10<sup>8</sup> cfu/ml. PPV = Post Primary Vaccination, PB = Post Booster, PC = Post Challenge, 0 hr = 0-1 hour.

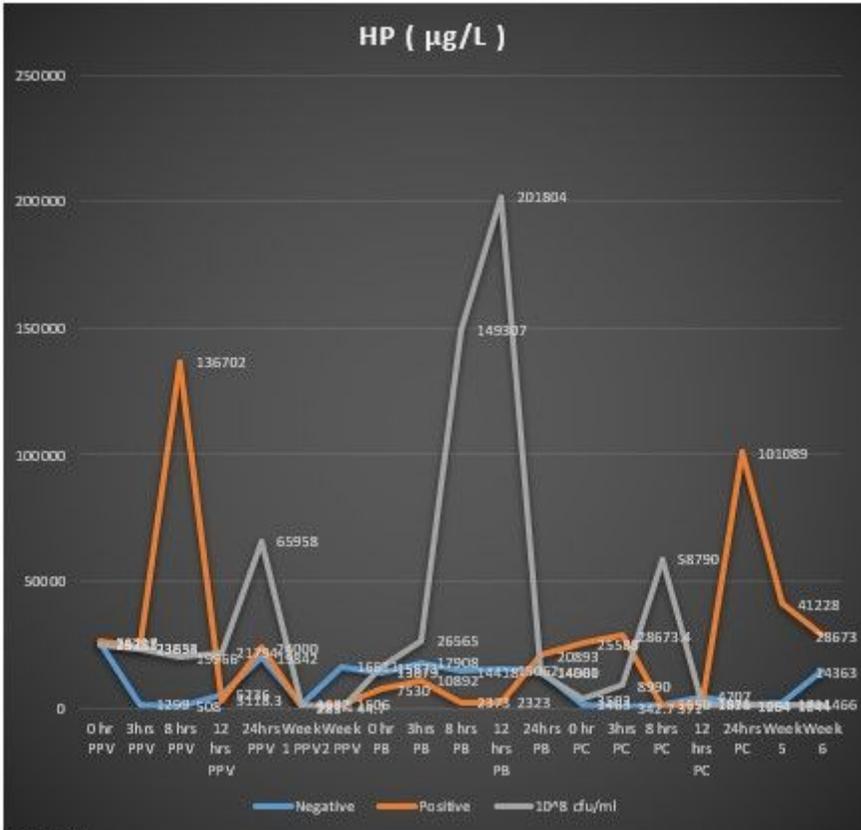


FIGURE 3

Figure 3

Periodic concentrations of Hp in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of 108 cfu/ml. PPV = Post Primary Vaccination, PB = Post Booster, PC = Post Challenge, 0 hr = 0-1 hour.

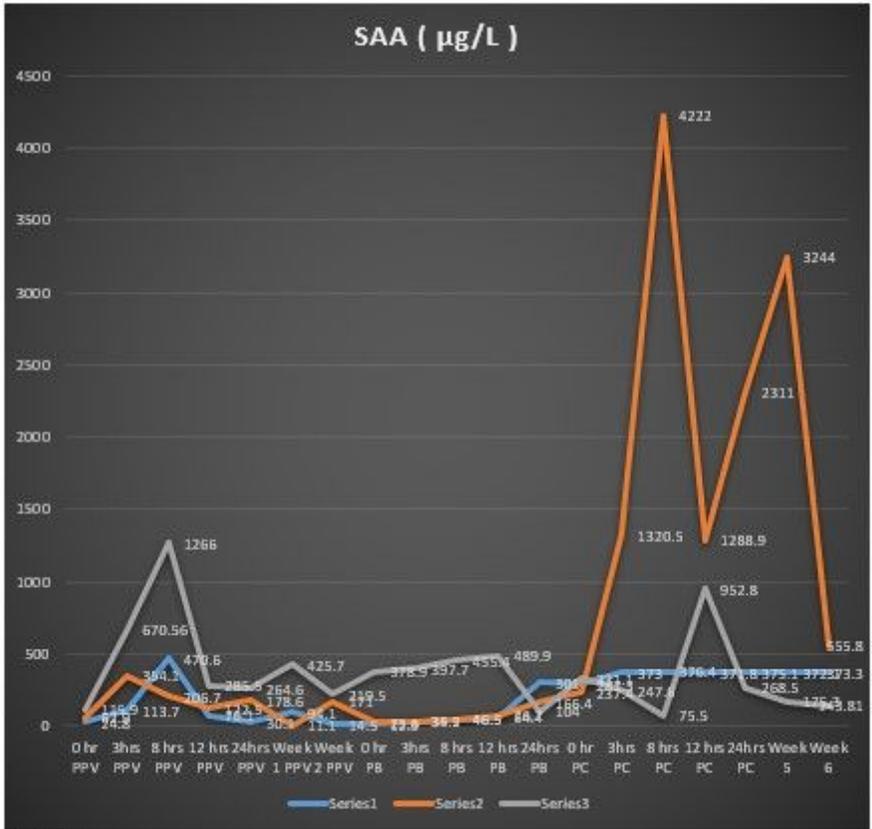


FIGURE 4

Figure 4

Periodic concentrations of SAA in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of 108 cfu/ml. PPV = Post Primary Vaccination, PB = Post Booster, PC = Post Challenge, 0 hr = 0-1 hour.

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