# Tropical study on Clinical and Host Cell Responses of Acute Phase Proteins, Pro-Inflammatory Cytokines and Antibodies' in Lactating Dairy Cows Vaccinated and Challenged with S. aureus

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# Abstract

In the tropical region of Malaysia (the study area), the climate has a mean annual temperature of 25.4°C with the hottest months of the year being April, May and June. On an average, December is 83.0% the most humid (MMD, 2017). It was confirmed by Cunha et al. (2008); that dairy cows raised in tropical conditions had less milk production as the somatic cell count (SCC) increased in mastitis cases. In humid tropics, Insua et al. (2008) also reported mastitis prevalence of about 42.0 %. The reduction in milk production due to cases of clinical mastitis is the component with the largest weight (42.2%) in the total economic impact of mastitis due to the large percentage of cows with SCCs. Therefore, the intensification of dairying, especially under tropical conditions, presents disease problems with mastitis as an important occurrence. And dairy farmers in the study area have reported cases of reduced dairy production and evidence of mastitis in dairy cows. This paper aims to provide an understanding of the effect developed killed vaccine on IgG, IgM, IgA, interleukin-10, interleukin-12, haptoglobin and serum amyloid A concentration on vaccinated and challenged dairy cows raised under tropic condition. Six lactating dairy cows were grouped into 3 treatment groups. Group A and B were inoculated intramuscularly with 2ml of normal saline while Group C received $10^8$ CFU/ml of the killed bacteria followed by a booster. The cows in group B and C were inoculated with 2ml of $10^6$ CFU live *S. aureus* bacterin in each mammary gland quarter while treatment group A was inoculated with 2ml of normal saline a week post booster. Blood samples were periodically collected. There was significant difference in the rectal temperatures, heart, and respiratory rates among the treatment groups. Results of the current study however, revealed that antibodies titres of immunoglobulin M, G and A were significantly ($p<0.05$) elevated throughout the post-primary vaccination, post-booster vaccination and post challenge phases. In addition, there were significant differences ($p<0.05$) in the interleukin-10, interleukin-12, haptoglobin and serum amyloid A concentration at different phases between treatment groups.

In conclusion, this study revealed that the prototype killed *S. aureus* vaccine could induce significant clinical and humoral immune responses needed for improving dairy production in tropical climates such as that of Malaysia. This was deduced from the reaction of pro-inflammatory cytokines and acute phase proteins biomarkers.


# Simple Summary

In the tropical region of Malaysia (the study area), the climate is mostly humid with dairy cows producing less as the somatic cell count (SCC) increases in mastitis cases. The intensification of dairying, especially under tropical conditions, presents with mastitis. And dairy farmers in the study area have reported cases of reduced dairy production and evidence of mastitis in dairy cows. The immunological, proinflammatory cytokine and acute phase protein responses of dairy cows following intramammary injection of a prototype killed bacterin vaccine was investigated. Our study revealed increased temperature, elevated respiration, heart rate and swelling of mammary gland in treatment groups. Also,

primary, and secondary vaccinations both resulted in significant responses in antibody, proinflammatory cytokines and acute phase protein.

# 1. Introduction

Mastitis is the inflammation of the mammary gland that occurs as a result of the infiltration of white blood cells into the mammary gland during the response to bacterial invasion of the teat canal in cows and other species [1,2,3]. The disease condition is found in most dairy farms [4] and characterised by inflamed and red udder, enlarged supramammary lymph nodes, distended teat, reduced milk production, lowered milk quality and loss of mammary integrity [5,6,7]. This disease condition is associated with considerable economic losses to the dairy farmers worldwide and of serious public health importance [8] mainly due to contamination and condemnation of dairy products [3,7], cost of antibiotic treatment and associated decreased reproductive performances of affected cows [9,10]. Due to the sub-clinical nature of mastitis, control is usually difficult and hence prevalence in dairy animals is high [11].

In order able to achieve improvement in the food production chain, safety and herd health immunity should be addressed [12,13,14]. 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) [15,16]. This major concern had suggested for better prevention, control and surveillance strategies on dairy infections such as mastitis and 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 [17,18]. Public health concerns regarding the menace of bovine mastitis are the antibiotic residues in milk [19,20]. These residues are a consequence of uncontrolled extended usage of antibiotics in treating mastitis [21,22]. Antibiotic residues have been reported to be a major source of severe reactions in humans due to allergy to antibiotics [23]. 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.

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 [24]. Vaccines have been prepared using whole organisms, which are either attenuated bacteria or viruses that are live but have been altered to reduce their virulence or pathogens that have been inactivated and effectively killed through exposure to either heat or chemical agents like formaldehyde [25]. 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 [26].

Generally, mastitis has a global distribution and it is endemic in most developed and developing nations of the world [21,27]. For instance, the prevalence of mastitis in dairy farms had been reported in different states in Malaysia [5,28]. Malaysia has a sizeable cattle population of breeding cows, with an estimate drawn from the existing cattle population of about 0.7 million as of 2015 [29]. But apart from cows, other

mastitis cases had also been reported in goat within Malaysia [30,31]. Therefore, the present study was designed to evaluate the clinical responses as well as the pro-inflammatory cytokines, acute phase proteins and antibodies changes in lactating Friesian cows vaccinated and challenged with *S. aureus* killed vaccine.

## 2. Materials And Methods

# 2.1 Study Approval

This study was performed based on the guidelines of the care and use of experimental animals provided by the Institutional Animal Care and use committee (IACUC). The procedures were approved by Universiti Putra Malaysia (UPM/IACUC/AUP- R072/2016).

# 2.1 Bacterial Isolation and Identification

*S. aureus* was isolated from CMT positive milk samples collected from cows showing signs of mastitis in the study area. Combination of bacterial culture, phenotypic identification, chemical tests, and sequencing techniques were used for confirmation of *S. aureus* in the Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM.

# 2.3 Preparation of prototype killed vaccine

A broth of 260ml was prepared using 9.26g of brain heart infusion and distilled water as described by [32], but with a little modification. The broth was then autoclaved at $121^0$C for 15 minutes to achieve sterility. The sterility was further double-checked by incubating the broth at $37^0$C for 24hrs and sub-culturing it on blood agar. There was no evidence of bacterial growth thereafter. Then, 10ml of the total mixture was seeded with the same colony types of *S. aureus* and incubated at $37^0$C for 24hrs [33,34]. The germinated growth in the 10ml broth was further seeded into the 250ml broth and further incubated at $37^0$C for 24hrs. A tenfold serial dilution was prepared using 9ml of distilled water and initial 1ml of the broth to calculate the coliform forming unit of the bacterin per ml (CFU/ml) of the original stock [33]. A sub-culture on blood agar was prepared from 0.5ml of serially diluted mixtures of dilution factor numbers seven, eight and nine respectively and incubated at $37^0$C for 24hrs for subsequent colony count. The 250ml of broth was however formalised using 1.25ml of 0.5% alcohol and incubated at $37^0$C for 24hrs to achieve absolute bacterial killing. The adjuvant was prepared by suspending the aluminium potassium sulphate in normal saline to achieve the final concentration of 1% of the aluminium adjuvant [35]. The inactivation procedure was considered a success as there was no bacterial growth and haemolysis observed on the blood agar after streaking and incubation at $37^0$ C for 24 hours. Mixing of aluminium potassium sulphate (adjuvant) to a final concentration of 1% (0.4g as to 40ml of 20 doses) with the killed bacterin as required by standards [35] was done and afterward, the vaccine was ready for sterility and safety test at a dose of 2ml/cow.

# 2.4 Dairy cattle management

A total of 6 lactating Friesian cows weighing about 300 to 350kg and age from 5 to 7-year-old were selected randomly from dairy farms within Malaysia for this study. Screening tests were carried out on the individual animal to ascertain they are in a sound health condition. The animals were milked, and the milk samples were screened to ensure animals were free from clinical mastitis. Selected animals were kept in a well-ventilated shelter that provides a 3 x $3m^2$ of the shaded concrete surface for about 2 weeks for acclimatisation. Feed and water were provided *ad libitum*.

# 2.5 Experimental design

The cows were randomly divided into 3 treatment groups consisting of 2 cows per treatment group. Group A, B and C were categorised as Negative control, Positive control, and Vaccinated group respectively. Both group A and group B cattle were inoculated intramuscularly (IM) with 2ml of normal saline while Group C was vaccinated IM with $10^8$ CFU/ml of the killed bacteria vaccine. Booster doses were administered for all treatment groups on day 21 post-vaccination. Intra mammary challenge was then performed on day 7 post-booster vaccination where both treatment groups B and C were inoculated with 2ml of $10^6$ live *S. aureus* bacterin into each mammary gland quarter whereas treatment group A was inoculated with 2ml of normal saline in each mammary gland quarter. During the post-primary vaccination (PPV), post-booster vaccination (PB) and post-challenge (PC) period, all treatment groups were observed for clinical responses such as temperature, pulse, respiration, and udder condition. The cardinal signs of mastitis which included red colouration of the udder, enlargement of the gland, hot on touch, painful upon palpation and loss of function were recorded throughout the study period. Besides, blood samples were collected at pre-determined after PV (0 hour, 3 hours, 8 hours, 12 hours, 24 hours, day 7, day 14, day 21), PB (0 hour, 3 hours, 8 hours, 12 hours, 24 hours) and PC (0 hour, 3 hours, 8 hours, 12 hours, 24 hours, day 35, day 42). The collected blood samples were analysed for pro-inflammatory cytokines responses [interleukin (IL)-10 and IL-12], acute phase proteins (APPs) concentrations [haptoglobin (Hp) and serum amyloid A (SAA)] and antibody titres [immunoglobulins (Ig) M, IgG and IgA] through the use of enzyme-linked immunosorbent assay (ELISA) kits.

# 2.6 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA kits for bovine IL-10, IL-12, Hp, SAA, IgM, IgG and IgA purchased from CUSABIO BIOTECH CO., LTD, China were used to determine the serum concentrations of those immunoglobulins, cytokines and acute phase proteins. The serum samples were first diluted using the provided diluent solution at a 1:10000 ratio. 100µl of the standards and the samples were pipetted in duplicates into predesigned wells. The microtiter plate was then incubated at room temperature for 60 minutes. All the content of the microplate was aspirated out after the incubation. Each well was filled with the wash solution and was aspirated. The wash was repeated for a total of four washes. 100 µl of the Enzyme-Antibody conjugate was added to each well. The microtiter plate was then incubated at room temperature in the dark for 30 minutes. The wells were then washed as described in steps above.100 µl of the TMB Substrate solution was pipetted into each well. The microtiter plate was then left at room temperature in the dark for another 10 minutes. 100 µl of the Stop solution was then added to each well after the incubation. The absorbance of the plate was read at 450nm using Labomed EMR-500 ELISA microplate reader. A curve of the absorbance of the

standards against their known concentrations was plotted and the different concentrations of those biomarkers were determined using the curve.

## 2.7 Statistical analysis

All data collected were analyzed using Statistical Analysis Software (SAS) version 9.4. One-way analysis of variance (ANOVA) and Duncan's multiple comparison post-hoc tests were used to compare means between treatment groups. The data were considered significant at $p < 0.05$.

# 3. Results

## 3.1 Interleukin 10

The mean concentrations of IL-10 for treatment groups A, B and C are presented in Fig. 1. During PPV at 0 hour, the concentrations of IL-10 in treatment groups of A, B and C were 3.00 ± 0.038ng/ml, 3.866 ± 0.038ng/ml and 5.08 ± 0.038ng/ml respectively with a significant difference ($p < 0.05$) where group C showed 1 fold increase in the concentration. The concentrations of IL-10 at 3 hours of PPV indicated a significant increased ($p < 0.05$) in groups C with about 1-fold increased and the mean concentration was 4.28 ± 0.014ng/ml compared to the negative control. At 8 hours, 24 hours and week 1 of PPV, there were no significant increases ($p > 0.05$) between the groups. At 12 hours of PPV, there was a significant difference ($p < 0.05$) in group C with 1-fold increased the mean concentration was 4.79 ± 0.05ng/ml. At week 2 of PPV, there was a significant difference ($p < 0.05$) in group C with 2 folds increased (5.40 ± 0.04ng/ml) as compared to the negative control (2.66 ± 0.04ng/ml).

At 3, 8, 12 and 24 hours of PB, there was a significant increase ($p < 0.05$) in the concentration of group C with mean concentrations of 3.86 ± 0.02ng/ml, 10.77 ± 0.03ng/ml, 6.78 ± 0.03ng/ml and 4.06 ± 0.02 ng/ml respectively compared to the negative control with 2, 2, 1 and 2 folds increased respectively.

During PC, there was a significant increase ($p < 0.05$) in group C at 0, 3 and 12 hours with a mean concentration of 5.35 ± 0.03ng/ml, 12.16 ± 0.03ng/ml, 12.30 ± 0.03ng/ml as compared to the negative control with about 1, 1 and 1 folds increased respectively. At 24 hours of PC, there was a significant difference ($p < 0.05$) in group B (15.66 ± 0.05ng/ml) as compared to the negative control (5.85 ± 0.05ng/ml) with 3 folds increased. There was no significant increase ($p > 0.05$) at 8 hours, day 7 and day 14 of PC in all the groups.

## 3.2 Interleukin 12

The mean concentrations of IL-12 for treatment groups A, B and C are presented in Fig. 2. During the PV at 0, 3, 8 and 12 hours, there was no significant difference ($p > 0.05$) between the groups. The concentration of IL-12 at 24 hours of PV indicated a significant increase in concentration ($p < 0.05$) for group C with about 223 folds increased and the mean concentration was at 422.5 ± 58ng/ml compared to the negative control. On week 1 and 2 of PV, there were significant increases ($p < 0.05$) for group C with

about 3 and 2 folds increased and the mean concentrations were 531.6 ± 0.2ng/ml and 279.8 ± 0.14ng/ml respectively compared to the negative control. At 0, 3, 8, 12 and 24 hours of PB, there were significant differences ($p < 0.05$) in groups C with mean concentrations of 108.9 ± 14.8ng/ml, 135.9 ± 8.6ng/ml, 261.8 ± 0.2ng/ml, 473.8 ± 0.11ng/ml and 196.9 ± 0.2 ng/ml respectively as compared to the negative control with about 4, 8, 5, 3 and 1 folds increased respectively. During PC at 12, 24 hours, days 7 and 14, there were significant increases ($p < 0.05$) in group C with the mean concentrations of 447 ± 28.7ng/ml, 294.5 ± 0.3ng/ml, 137.8 ± 0.21ng/ml and 155.8 ± 0.3ng/ml respectively compared to the negative control with about 2, 2, 5 and 5 folds increased respectively. At 3 and 8 hours of PC, there were significant differences ($p < 0.05$) in group B (809.9 ± 0.3ng/ml and 378.7 ± 0.2ng/ml) compared to the negative control with about 80 and 2 folds increased respectively.

# 3.3 Haptoglobin

The mean concentration of Hp for treatment groups A, B and C are presented in Fig. 3. During PPV at 0 hour, the mean concentrations of Hp in treatment groups of A, B and C were 25381 ± 19617ng/ml, 26217 ± 19617ng/ml and 25158 ± 19617ng/ml, respectively. At 3 and 8 hours, there were no significant differences ($p > 0.05$) between the groups. At 12 and 24 hours, there were significant increases ($p < 0.05$) in group C with about 4 and 3 folds increased respectively with mean concentrations of 21794 ± 1601ng/ml and 65958 ± 2795ng/ml respectively compared to the negative control group. On week 1 and 2 of PPV, there were no significant increases ($p > 0.05$) observed in treatment groups of B and C. During PB, there were no significant differences in mean concentrations observed ($p > 0.05$) between the groups at 0, 3 and 24 hours. At 8 and 12 hours of PB, there were significant differences of mean concentrations ($p < 0.05$) in treatment group C with about 10 and 13 folds increased at 149307 ± 3372ng/ml and 201804 ± 3710ng/ml respectively compared to the negative control. During the PC phase, at 0, 3, 24 hours and day 7 of PC, there were significant differences of mean concentrations ($p < 0.05$) in treatment group B with about 17, 83, 80 and 24 fold increased respectively and the concentrations were 25588 ± 513ng/ml, 28673.4 ± 205ng/ml, 101089 ± 6751ng/ml and 41228 ± 4147ng/ml respectively compared to the negative control group. At 8 hours of PC, there was a significant difference ($p < 0.05$) observed in group C with about 35-fold increased and the mean concentration was 58790 ± 395ng/ml compared to the negative control group. At 12 hours and day 14 of PC, there were no significant increases ($p > 0.05$) observed between the groups.

# 3.4 Serum Amyloid A

The mean concentration of SAA for treatment groups A, B and C are presented in Fig. 4. During PPV at 0, 3, 8, 12, 24 hours and weeks 1 and 2, the concentrations of SAA were significantly different ($p > 0.05$) in treatment group C with about 5, 6, 3, 4, 9, 5 and 16 folds increased respectively and the mean concentrations were 115.9 ± 2.4µg/L, 670.56 ± 23.6µg/L, 1266 ± 4.5µg/L, 285.5 ± 15.8µg/L, 264.6 ± 20.5µg/L, 425.7 ± 3.4µg/L and 219.5 ± 1.1µg/L respectively compared to the negative control group. During PB at 0, 3, 8 and 12 hours, there were significant differences ($p < 0.05$) observed in the treatment group of C with about 29, 11, 8 and 8 folds increased respectively and the mean concentrations were

378.9 ± 0.7µg/L, 397.7 ± 0.8µg/L, 455.4 ± 1.3µg/L and 489.9 ± 0.8 µg/L respectively compared to the negative control. At 24 hours of PB, there was no significant increase ($p > 0.05$) observed between the groups. During the PC phase at 3, 8, 12, 24 hours, days 7 and 14, there were significant differences ($p < 0.05$) observed in group B with about 4, 11, 3, 6, 8 and 1 folds increased respectively and the mean concentrations were 1320.5 ± 1.9µg/L, 4222 ± 0.6µg/L, 1288.9 ± 1.2µg/L, 2311 ± 0.9µg/L, 3244 ± 25.7µg/L and 555.8 ± 25.6µg/L respectively compared to the negative control group. At 0 hour of PC, there was a significant increase ($p < 0.05$) observed in group C with about 1 fold increased (331.1 ± 0.3µg/L) compared to the negative control.

# 3.5 Immunoglobulin M

The mean concentration of IgM for treatment groups A, B and C are presented in Fig. 5. During PPV at 0, 3, 8, 12, 24 hours and weeks 1 and 2, the concentrations of IgM were significantly higher ($p < 0.05$) in the treatment group of C with about 3, 13, 6, 1, 3, 8, and 6 folds increased respectively and the mean concentrations were 215.2 ± 2.7µg/ml, 388 ± 3.1µg/ml, 357 ± 0.7µg/ml, 465.3 ± 2.7µg/ml, 508.3 ± 0.3µg/ml, 210 ± 0.5µg/ml and 158.3 ± 1.1µg/ml respectively compared to the negative control group. During PB at 8, 12 and 24 hours, there were significant increases observed ($p < 0.05$) in the treatment group of C with about 4, 17 and 2 folds increased respectively and the concentration were 1276.3 ± 1.8µg/ml, 1217 ± 0.8µg/ml and 380 ± 1.7µg/ml respectively compared to the negative control. At 0 and 3 hours of PB, there were no significant increases observed ($p > 0.05$) between the groups. During PC at 0, 3, 12, 24 hours, day 7 and 14, there were significant increases ($p < 0.05$) observed in group C with about 3, 11, 5, 14, 11 and 35 folds increased respectively and the mean concentrations were 344.3 ± 0.3µg/ml, 392.7 ± 1.6µg/ml, 89.3 ± 0.4µg/ml, 839.1 ± 0.8µg/ml, 330.7 ± 5.4µg/ml and 641.1 ± 0.7µg/ml respectively compared to the negative control group. At 8 hours of PC, there was a significant increase ($p < 0.05$) observed in group B with about 7 folds increased (404.5 ± 3.7 µg/ml) as compared to the negative control (55 ± 3.7µg/ml).

# 3.6 Immunoglobulin G

The mean concentration of IgG for treatment groups of A, B and C are presented in Fig. 6. During the PPV period at 12, 24 hours and week 1 and 2, the concentrations of IgG were significantly higher ($P < 0.05$) in the treatment group of C with about 5, 2, 10 and 6 folds increased observed respectively and the mean concentration were 574.8 ± 4.4µg/ml, 519 ± 1.9µg/ml, 2055 ± 33µg/ml and 2006 ± 4.0µg/ml respectively compared to the negative control group. During PB at 0, 3, 8, 12 and 24 hours, there were significant differences ($p < 0.05$) observed in group C with about 2, 14, 3, 24, and 10 folds increased respectively and the mean concentration were 491.5 ± 14.6µg/ml, 2168 ± 98 µg/ml, 611.8 ± 10.3µg/ml, 342.3 ± 24 µg/ml and 649.9 ± 3µg/ml respectively compared to the negative control. At 0, 24 hours, day 7 and 14 of PC, there were significant increases ($p < 0.05$) observed in the treatment group of C with about 2, 20, 12 and 6 folds increased respectively and the mean concentrations were 646.6 ± 21.1µg/ml, 246 ± 36µg/ml, 448.9 ± 1.7µg/ml and 2056 ± 25µg/ml respectively compared to the negative control group.

## 3.7 Immunoglobulin A

The means concentrations of IgA for treatment groups of A, B and C are presented in Fig. 7. During PPV phase at 3, 8, 12 hours and week 1 and 2, the concentrations of IgA were significantly higher ($P < 0.05$) in the treatment group of C with about 1, 3, 1, 3 and 5 folds increased respectively and the mean concentrations were 2984.2 ± 2.8 µg/ml, 2786 ± 0.8 µg/ml, 2811 ± 25 µg/ml, 7029 ± 21 µg/ml and 24125 ± 76µg/ml respectively compared to the negative control group. During PB at 0, 3, 8, 12 and 24 hours, there were significant increases ($p < 0.05$) observed in the treatment group of C with about 8, 45, 2, 37 and 24 folds increased respectively and the mean concentrations were 16310 ± 179 µg/ml, 11400 ± 283 µg/ml, 5563.4 ± 334 µg/ml, 22969 ± 27µg/ml and 19919 ± 15µg/ml respectively compared to the negative control. At 0, 3, 8, 24 hours, day 7 and 14 of PC phase, there were significant increases ($p < 0.05$) in concentrations observed in group C with about 6, 6, 2, 15, 23 and 3 folds increased respectively and the mean concentrations were 25438 ± 40µg/ml, 8547.8 ± 124µg/ml, 6125 ± 87µg/ml, 13419 ± 181µg/ml, 2888 ± 7µg/ml, 3807 ± 26µg/ml respectively compared to the negative control group.

# 4. Discussion

Mastitis is a common fatal disease in lactating dairy animals [11,36,37]. Exotoxins produced by *S. aureus* induce swelling of the mitochondria and cytoplasm [38] thereby forming spores on cell surfaces and releasing ions eventually leading to cell lysis [39]. Studies have indicated that experimental and natural *S. aureus* infection of the mammary gland induces pyrexia [36,40,41,42], which eventually eliminates or reduces the bacterial load from the infected gland with the aid of innate or acquired immune response [40,42]. The induced pyrexia has been indicated to have a correlation with other physiologic parameters like the respiratory rates, heart rates [36] swollen and red udder [12], pain on palpation [43] and reduced milk secretion [12] resulting to a downturn in financial gains of dairy farmers [44]. These physiologic parameters are however considered useful in the prognosis of mastitis in dairy cows [45]. This clinical finding observed in this study corroborates with the findings from other researchers above and explained that the killed *S. aureus* vaccine able to stimulate and alter the physiological responses of the vaccinated and challenged cows with mastitis infections.

The potency of the immune response to *S. aureus* mastitis in dairy cows is defined by the concentrations of lymphocytes, leucocytes and cytokines released during its invasion of the mammary gland [46,47,48,49,50]. 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 and12 [51,52,53,54], 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 the release of interferon-c (IFN-c) and tumour necrosis factor-a (TNF-a) [55,56]. Changes in the concentration of cytokine synthesis are regarded as a crucial factor in scoring the inflammatory responses t o *S. aureus* infection. To the very best of our knowledge, this present work is the first study to demonstrate the effect of laboratory-prepared killed *S. aureus* vaccine from local isolate on Friesian dairy cows.

The current study had indicated that there were significantly increased observed in IL-10 and IL-12 concentrations in the vaccinated group. These increased observed in the present study showed that the potency of the killed vaccine was able to suppress the effect of *S. aureus* intramammary challenge. The result of the present study is in harmony with the study reported on cytokines as markers in *S. aureus* mastitis of the bovine mammary gland which indicated that the suppression of this bacterium is usually accompanied by an elevated IL-10 and IL-12 concentrations [57]. There are similarities between the findings of the present study and that of [58], where a different model and *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 [58]. [59] also stated that the protective roles of cytokines during *S. aureus* infection where the increase in concentrations of cytokines is a positive sign towards prompting positive defence responses against *S. aureus* infection. The findings of the present study also corroborate with the study of [50], who described that a study on characterisation of cytokines involved during mammary glands infected with *S. aureus* revealed a similar pattern of increment of concentrations of IL-10 and IL-12. The findings of the current study are consistent with the study of [60], where their study investigated the cytokines as markers during *S. aureus* mastitis infection and showed a similar pattern of increment. Therefore, pro-inflammatory cytokines particularly IL-12 and IL-10 have the potential to be used as biomarkers for vaccine efficacy trials.

Applications of APPs biomarkers as indicators of inflammation of the mammary gland have gained a wider acceptance in the assessment of udder health. APPs constitute part of innate immune components whose concentration varies with respect to stimuli of external or internal origin [11]. APPs concentration such as SAA and Hp increases during the early phase of the reaction of cows to bacterial challenge or infection [61,62]. 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 [63,64]. The findings of responses Hp and SAA in this current study can be considered as new information toward the efficacy of killed *S. aureus* vaccine.

The current study had indicated that there was a significant increase in Hp and SAA concentrations of the vaccinated group and the findings of the current study were consistent with the reports of [65], who concluded that SAA and Hp as the major APP 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 [66], the increase in SAA and Hp are tools of measuring the severity of mastitis infection in both experimentally and naturally challenged cows in dairy farms. In accordance with present results, a previous study by [67], had also reported that SAA and Hp in the serum of bovine mastitis cases increase during the 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.

The findings of our present study also corroborates with previous report by [68], who found 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. Nevertheless, [69], stated that serum Hp was a better marker in assessing the impact of local immunization PC in dairy cows. This is 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. 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 APPs 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 [70]. An important and relevant observation observed in this present study where the vaccinated cows showed evidence of a lower degree of Hp and SAA responses during PC compared to the positive control group. Based on these findings in the present study, we can state that the APPs could be used as a biomarker to evaluate the efficacy of the vaccine and the severity of the mammary infection.

This present study was conducted to investigate the humoral immune responses to killed *S. aureus* vaccine. The current study had demonstrated that the total IgM, IgG and IgA concentrations of the vaccinated group were significantly high compared to other treatment groups. The elevated levels of total IgM and IgG observed in the present study is in harmony with [71]. The researcher indicated that serum IgM and IgG were greater in cows during post-vaccination and during challenged in the vaccinated group than in the control group. In another related study conducted by [72], the study concluded that serum IgG and IgM were significantly high during post-vaccination in the vaccinated group than in the control group indicative of vaccine–induced opsonisation in the treatment group. Total IgG elevation in vaccinated group post-vaccination and challenge as observed in the vaccinated group of the current study corroborate with the report of [73], who proved that IgG antibodies increased during post-vaccination or challenge period. In another related experimental study on the generation of a novel *S. aureus* ghost vaccine and examination of its immunogenicity against the virulent challenge, the IgG titer increased robustly at weeks 2, 4, 6 post-vaccination [74].

The current finding was further supported by the report of [75], who stipulated that *S. aureus* stimulates greater immune response and protects against the field bacterin. The study concluded that immunoglobulins should be able to confer immunity to the udder [76]. The result of the present study corroborates with the reports of [77], who stipulated that IgA increased during mammary gland infection, in the vaccinated group but maintain at a low level in negative groups or healthy state. Another consistent finding involving the elevated IgA in mastitis infection had also been reported by [78]. The evidenced from the robustly increased titres of IgM, IgG and IgA in this study showed that the killed mastitis vaccine confers protection.

# 5. Conclusions

In conclusion, pro-inflammatory cytokines particularly IL-12 and IL-10, as well as APPs particularly Hp and SAA, have the potential to be used as biomarkers indicators for vaccine efficacy together with clinical

and immune response parameters. Therefore, the killed *S. aureus* vaccine developed in this study showed some degree of protection against mastitis infection.

# Declarations

**Supplementary Materials:** N/A

**Author Contributions:** FFAJ: conceptualization, funding acquisition, supervision, review & editing; IUH: methodology; data curation; formal analysis, writing the original draft; CELT, ZZ, AWH & MLMA: supervision, review & editing; KRB, MJN participated in data curation, formal analysis, writing and editing of the original drafts. All other authors have contributed equally in the critical revisions leading to the final draft of this manuscript.

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**Conflicts of Interest:** The researchers have no potential conflict of interest.

**Data availability statement**

Data will be available upon request.

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# Figures

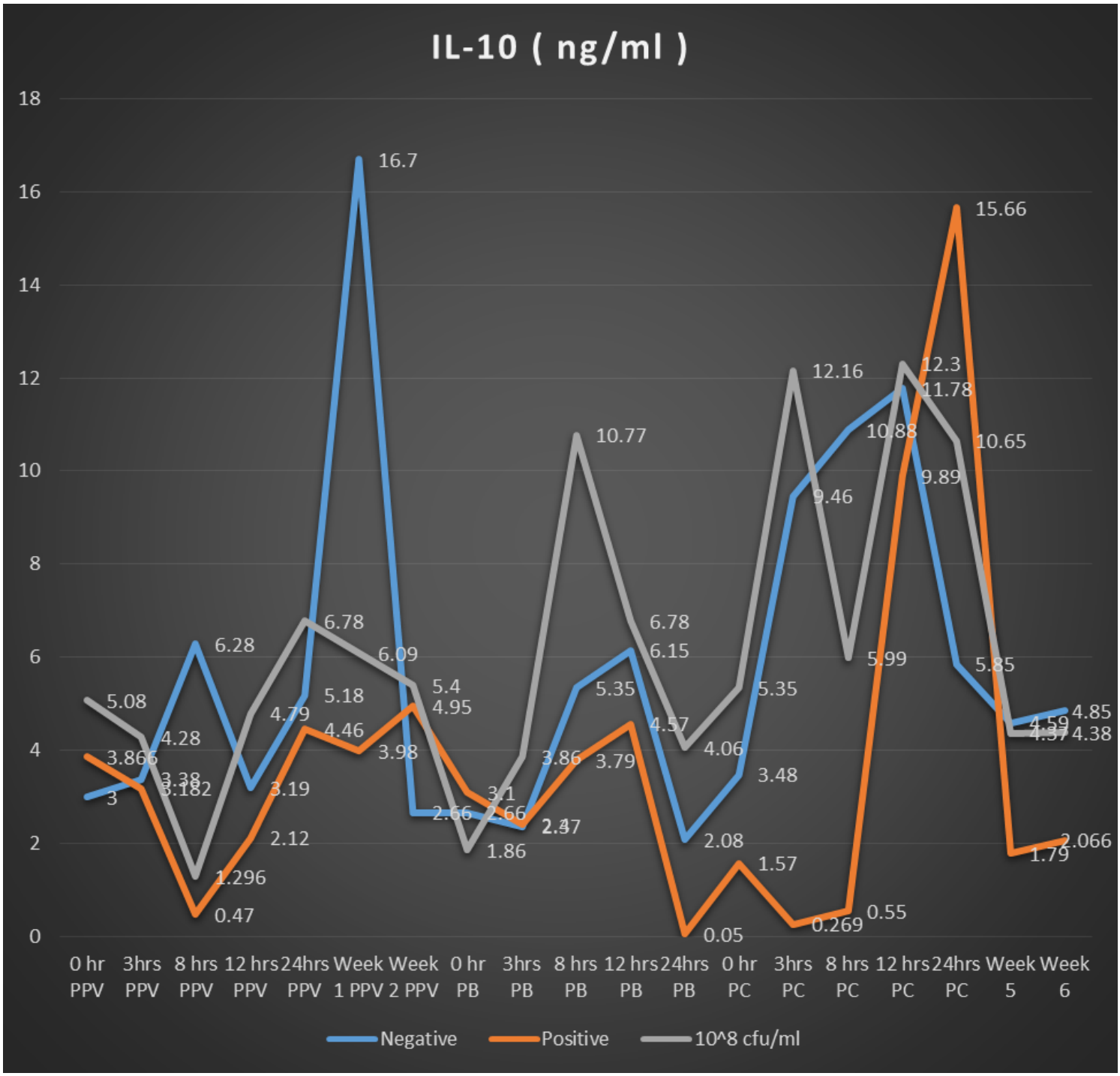
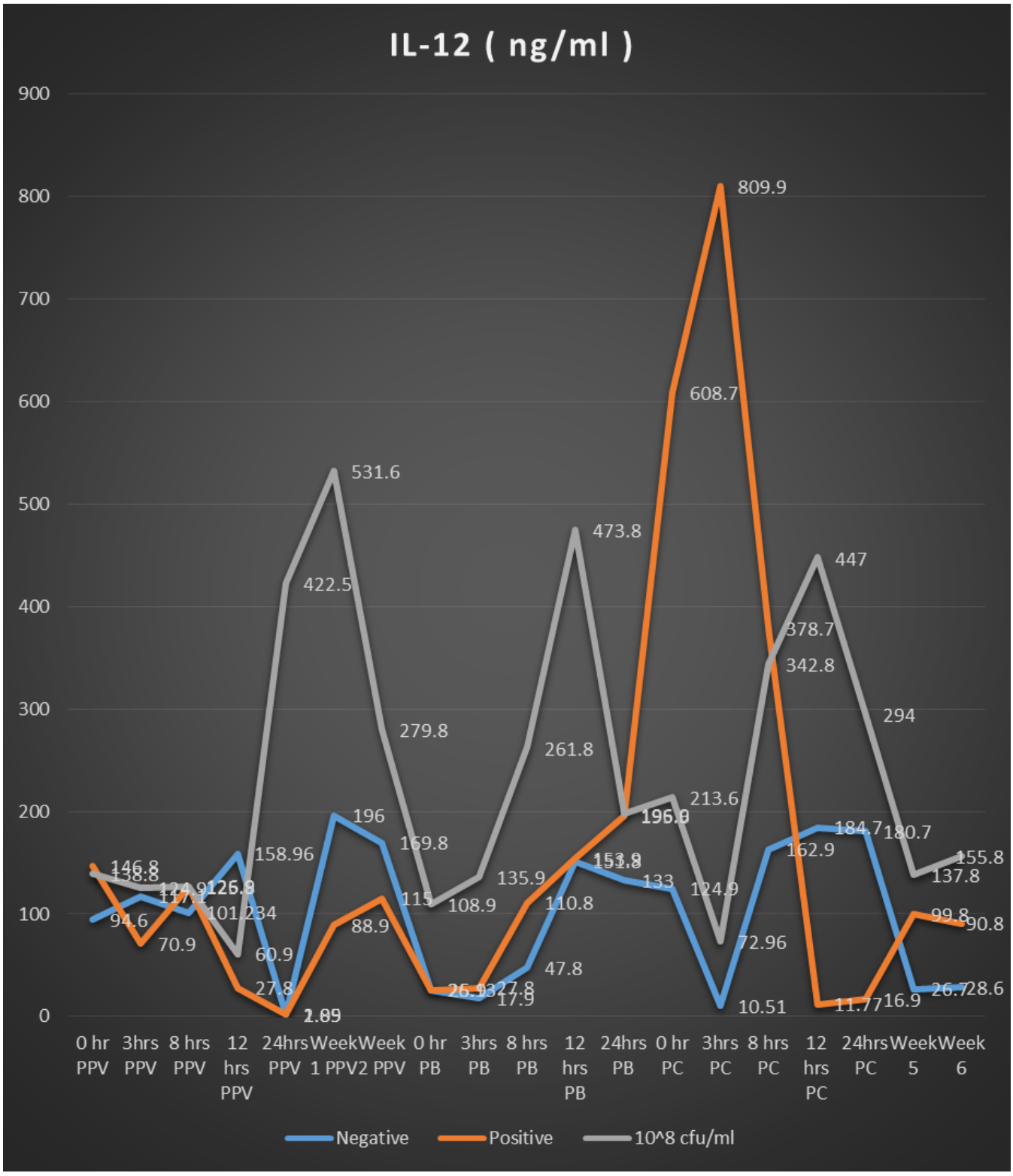



Figure 1

Periodic concentrations of IL-10 in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.




## Figure 2

**Periodic concentrations of IL-12 in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.**

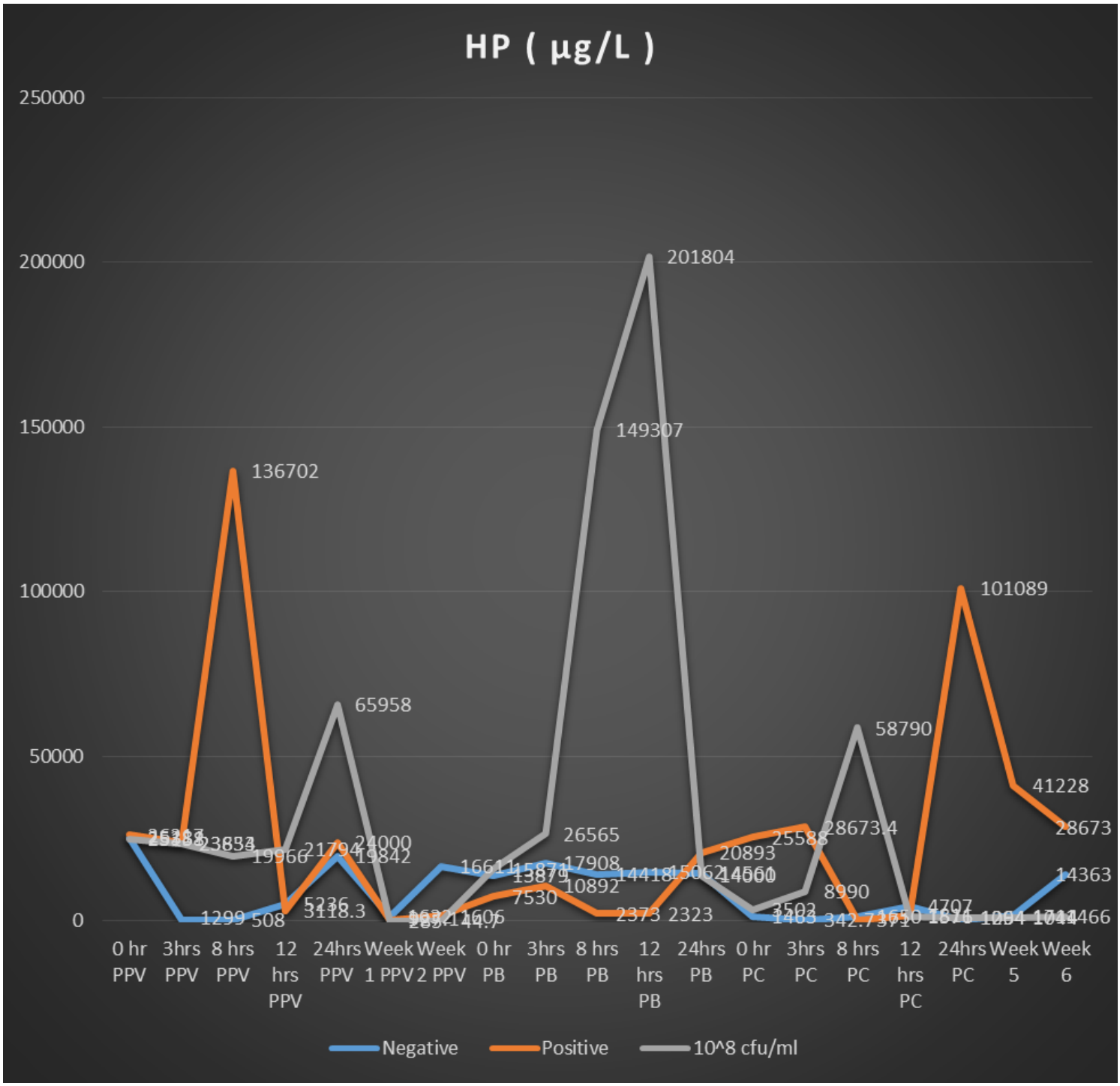



Figure 3

Periodic concentrations of Hp in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.

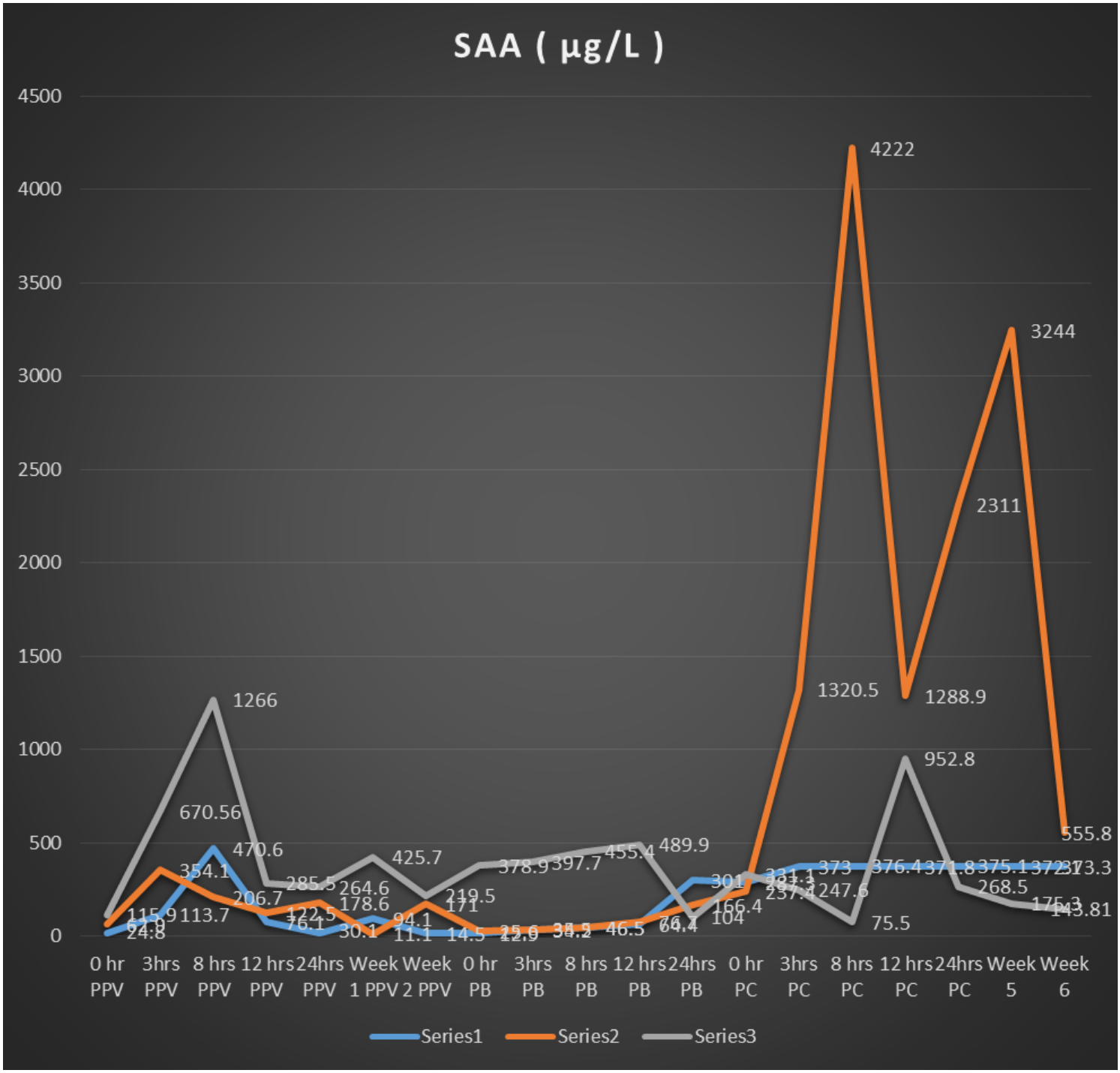



Figure 4

Periodic concentrations of SAA in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.

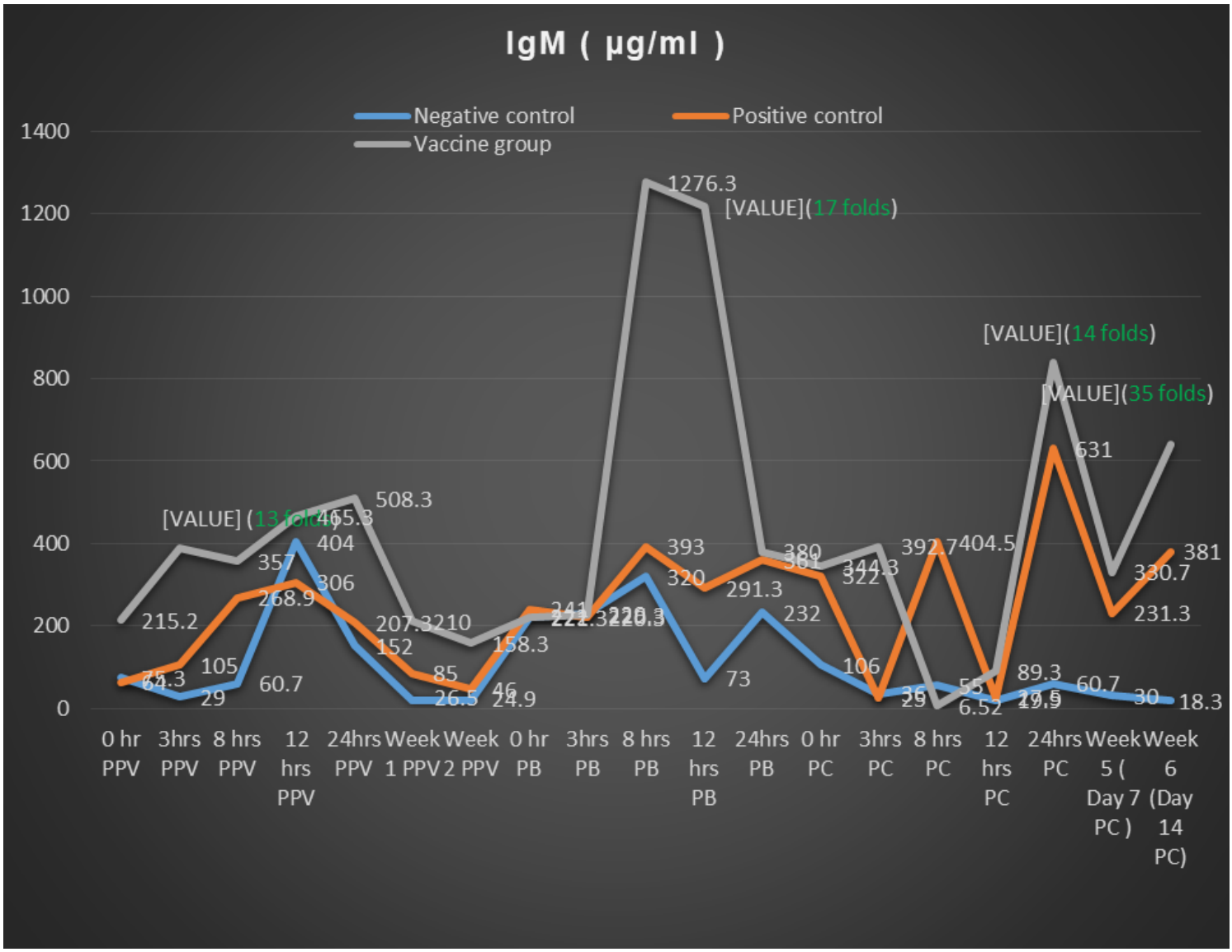



Figure 5

Periodic concentrations of IgM in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.

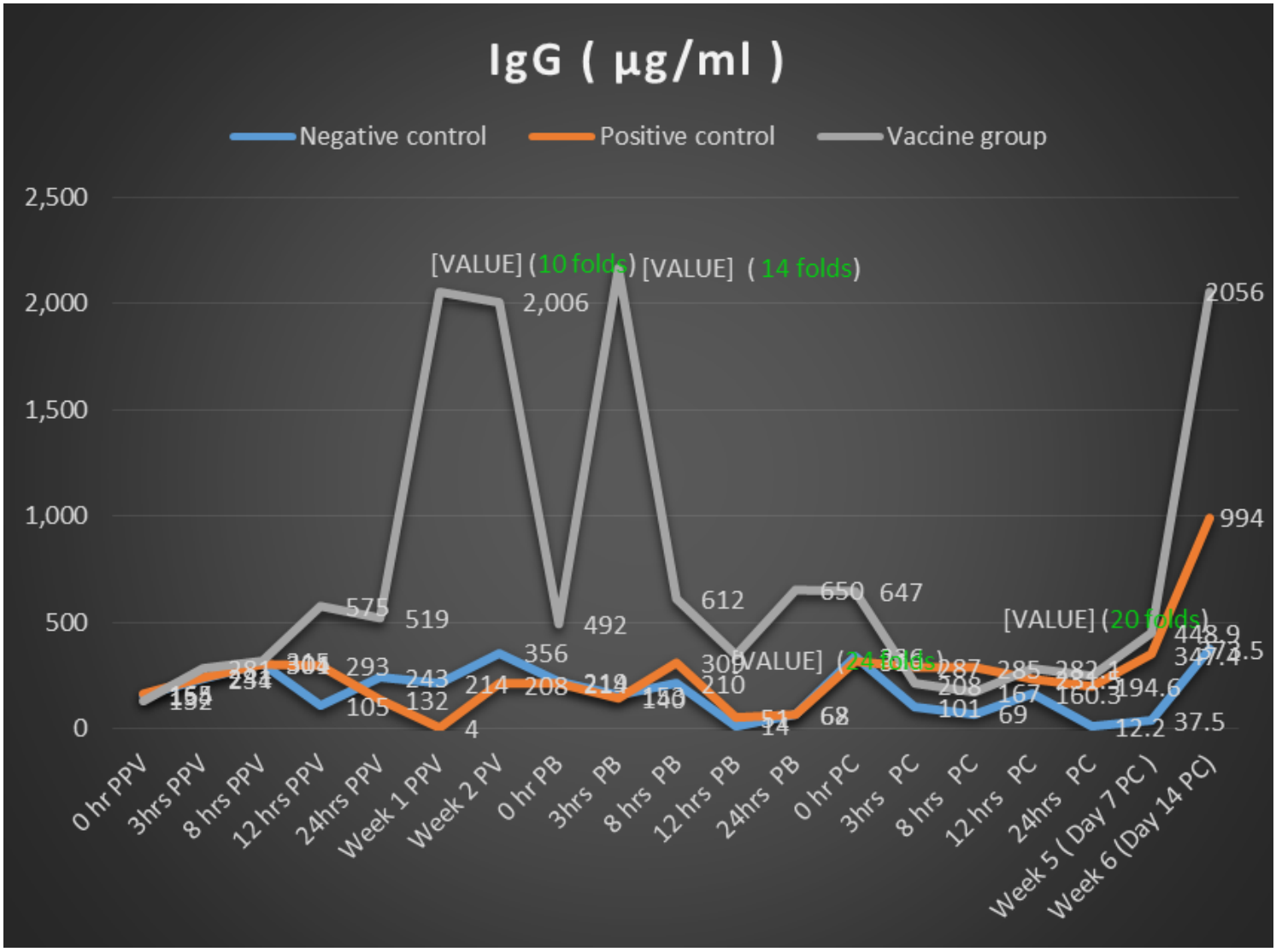



Figure 6

Periodic concentrations of IgG in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.

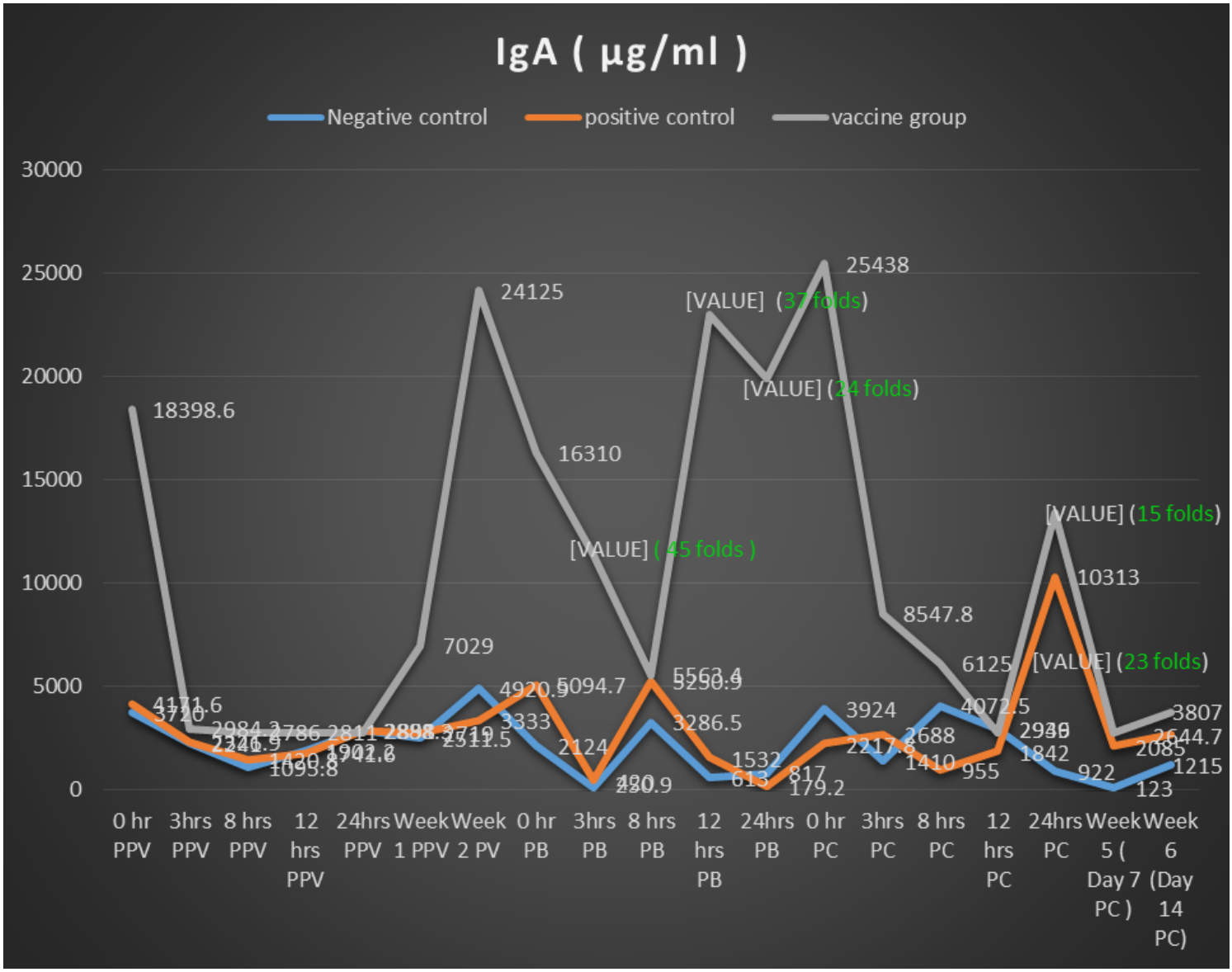



Figure 7

Periodic concentrations of IgA in lactating Friesian cows vaccinated with prototype killed *S. aureus* mastitis vaccine of $10^8$ CFU/ml. PPV: Post-primary vaccination; PB: Post-booster; PC: Post-challenge; 0 hr: 0-1 hour.