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Research Article

Investigation of Serum and Milk Acute Phase Proteins Level in Goats with Experimentally Infected *Staphylococcus aureus* Subclinical Mastitis

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Abstract | Mastitis is an inflammatory condition of the mammary gland which is usually caused by a bacterial infection. This study aims to determine acute phase protein (APP) and its association with somatic cell count (SCC) during experimentally induced subclinical mastitis in goats. Thirty lactating goats were divided into two groups (n=15 per group) challenged either by intramammary infusion of 1 x 10^3 cfu/mL *Staphylococcus aureus* (*S. aureus*) or phosphate-buffered saline (control). The haptoglobin (Hp), serum amyloid A (SAA) and α1-acid glycoprotein (AGP) levels in serum and milk were measured at pre-and post-infection (6, 24, 48 and 72 h) using the ELISA method. The SCC was determined by the direct microscopic method. Results revealed a time-dependent change of Hp, SAA and AGP in the infected group compared to the control. The APP levels in the milk were significantly higher ($p<0.05$) than those in the serum. A significant good correlation between milk Hp concentration and SCC was observed ($r_s = 0.73$, $p<0.05$). However, there were no associations between milk SAA and AGP concentrations with SCC respectively. In conclusion, altered expression of the serum and milk APPs was seen during *S. aureus*-induced subclinical mastitis infection in goats, which unlock the potentials of APP evaluation as alternative tools for early diagnosis of subclinical mastitis in goats. Also, there was a significant relationship between milk Hp and SCC, thus reflecting the intensity of the inflammatory response to mammary tissue.

Keywords | Acute phase proteins, Subclinical mastitis, Serum, Milk, Goats.

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INTRODUCTION

Mastitis is an inflammation of the mammary gland and is often caused by a bacterial infection. Mastitis in ruminants can be categorized into clinical and subclinical cases. The clinical mastitis may produce clinical signs that include swelling, heat, pain and oedema on the mammary gland and abnormal milk production such as watery, clots and presence of blood in milk. Subclinical mastitis is more common and of particular concern in farmers and veterinarians since no visible changes in the milk or udder can be identified. The subclinical infection has also been linked to decreased milk yield causing significant production loss. The diagnosis of subclinical mastitis is based on direct measurement of the somatic cell count (SCC) or performing a California Mastitis Test (CMT), which are generally used as an indicator for udder health (Gelasakis et al., 2016). The application of automatic SCC meas-

urement using the Fossomatic method and DeLaval cell counter could yield a rapid and repeatable result, however, these devices are relatively expensive and complex to use (Gonzalo, 2017). Similarly, CMT often has poor sensitivity on detecting subclinical mastitis and the yielded result could be difficult to interpret (Viguier et al., 2009). Early detection of subclinical mastitis is crucial to prevent the source of infection for herd mates, prevent transmission of zoonotic pathogens to human and minimize economic losses (Kabir et al., 2019).

Acute phase protein (APP) has been proposed as a reliable and rapid indicator of inflammation in ruminants (Eckersall et al., 2001). Acute phase response (APR) is an innate host defence to traumatic injury, inflammation and infection to reestablish homeostasis and promote healing (Chen et al., 2017). A prominent event in the APR is the change in the concentration of APP. During the early phase of inflammation and injury, the APP concentration can be increased (positive APP) or decreased (negative APP) by at least 25% (Eckersall and Conner, 1988). These APPs are mainly synthesized by the liver hepatocytes (Zhou et al., 2016). However, APPs are also can be locally produced by adipose tissue, lung, ovary, uterus, testis, digestive tract and mammary gland (Ceciliani et al., 2012; Thomas et al., 2018).

Animals exhibit different activities of APP depending on the disease and its causal agent (Eckersall and Bell, 2010). Haptoglobin (Hp) and serum amyloid A (SAA) are the major APPs described in goats (Heller and John, 2015), while α1-acid glycoprotein (AGP) is considered moderate (Gonzalez et al., 2008; Tothova et al., 2014; Iliev and Georgieva, 2018). Previous studies have reported changes in the serum Hp, SAA and fibrinogen in goats with gangrenous mastitis (El-Deep, 2013). In addition, serum levels of APP have been evaluated in goats with experimentally induced *S. aureus* mastitis (Fasulkov et al., 2014; Sadiq et al., 2019). However, none of these studies has investigated the levels of APP in the milk from goats with mastitis. Moreover, until today there is no information on the changes in APP levels in goats with subclinical mastitis. Therefore, the present study aimed to investigate the serum and milk acute phase proteins (Hp, SAA and AGP) levels and their association with SCC in goats during experimentally induced *S. aureus* subclinical mastitis. We hypothesized that experimental *S. aureus* subclinical infection would produce significant changes in the APP of lactating goats.

MATERIAL AND METHODS

Ethical Approval

Ethical approval was obtained from the Universiti Sultan Zainal Abidin (UniSZA) Animal and Plant Ethics Committee (UAPREC) Reff: UAPREC/17/005/UniSZA.0/3/374-3 (32).

Animals

Thirty mixed-breed lactating goats of similar age (mean 2.5 years, range 2-3 years) within the 1st parity at mid-lactation phase were used in the study. The animals were housed in an animal research enclosure, had *ad libitum* access to clean water and were fed twice daily with freshly cut Napier grass supplemented with commercial concentrate. The study was carried out at the UniSZA Pasir Akar Farm, Besut, Terengganu.

Prior to the experiment, rectal temperature, respiratory and pulse rates of each animal were recorded. Physical examination on the udder and teats were conducted to determine the udder health status. CMT method as described by Lucia et al. (2017) was conducted to determine the onset of subclinical mastitis. Approximately 10 mL milk from each udder half were sampled and examined for microbiological analysis. Milk SCC was determined with goats having milk SCC < 100,000 cells/mL were selected for the experiment (Moraes et al., 2013).

Intramammary challenge

Goats were experimentally infected with the *S. aureus* (ATCC 700699) strain (Ariffin et al., 2020). The freeze-dried bacteria were suspended in distilled water and inoculated onto nutrient agar at 37°C for 24 h. A single colony bacterium was transferred in phosphate-buffered saline (PBS). The bacterial suspension turbidity was adjusted to 0.5 McFarland standard to achieve an infective dose of 1 x 10^3 cfu/mL. Before inoculation, udder and teat ends were cleaned with a paper towel and disinfected with 70% ethanol. 1 mL of *S. aureus* inoculum was administered intramammary into the teat streak canal of 15 goats using a sterile 1 mL plastic syringe. 15 goats used as control were infused with 1 mL of sterile PBS using the same procedure as described previously.

Blood and milk sampling

Blood and milk samples were collected from both groups at pre-and post-challenge (6, 24, 48 and 72 h). 10 mL of jugular blood sample was collected by venipuncture and stored into a plain vacutainer tube to allow the serum to be separated. Serum samples obtained were stored at -20° C for further analysis. 10 mL milk samples were collected aseptically by hand-stripping and stored in a sterile polystyrene tube. Potassium dichromate was added as a preservative (Eckersall et al., 2006). 10 µl aliquots of the preserved milk were used for direct microscopic somatic cell count (DMSCC), and the remaining milk was centrifuged at 4000 g at 4°C for 15 min. The fat layer was discarded and

the skimmed milk was divided into 4 x 0.5 mL aliquots and stored at -20° C for further analysis.

Bacteriological Analysis

To determine the total bacterial count of *S. aureus* in milk, 100 µl of milk samples were plated onto Mannitol Salt Agar (MSA) (Oxoid Ltd, Hampshire, UK) and incubated at 37°C for 48 h. The presumptive *S. aureus* colonies were identified as golden-yellow colonies and calculated using a cell counter. The bacterial loads of *S. aureus* were presented as $\log_{10}$ cfu/mL.

Direct Microscopic Somatic Cell Count (DMSCC)

The somatic cell count was determined using the direct microscopic technique (Paape et al., 2001). Smears of milk samples prepared on glass slides (1 cm^2) were fixed with Carnoy's fixative solution and stained with Pyronin-Y Methylene Green (PYMG) stain for six min. The milk films were then dried overnight, followed by washing in butanol and cleared in two changes of xylene. Ten high power fields were examined on a thin section of each milk film under light microscopy using an oil immersion objective lens (x100), to count the number of PYMG stained somatic cells. Somatic cell count >1 x 10^6 cells/mL was classified as subclinical mastitis infections.

Acute Phase Proteins measurements

The quantitative detection of Hp, SAA and AGP in sera and milk was done using a commercial sandwich ELISA test kit (Hp sensitivity: 46.8 ng/mL; SAA: 0.1 µg/mL; AGP: 0.06 ng/mL) supplied by MyBioSource, California, US (catalogue number: MBS765761 (Hp); MBS031629 (SAA); MBS266196 (AGP)). The samples were assayed in triplicate. In brief, 50 µl of the standard solution was added to the designated wells. 40 µl of samples and 10 µl of anti-Hp/SAA/AGP antibody and 100 µl of streptavidin-horse radish peroxidase-conjugated were added to the sample wells. The plate was incubated for 60 min at 37°C followed by a washing step. The chromogen solution A and B were added to each well, followed by a final incubation for 15 min at 37°C. A stop solution was added to each well and the optical density (OD) of the assay was measured immediately using a microplate reader (Sunrise Tecan, Switzerland). The OD obtained were quantitatively analyzed using a Four Parameter Logistic curve fit to calculate the concentrations of APPs in the sera and milk samples (myassays.com).

Statistical Analyses

The data obtained were statistically analyzed using GraphPad Prism for Windows, version 8. Two-way ANOVA with Tukey's HSD post-test analysis was performed, with a significance level (α) set at 0.05. Correlation analysis was determined using Spearman's Rank Correlation. Correlation coefficient (r_s) results with 0.05 significant level were interpreted according to a modified categorization as explained by Mukaka (2012). The correlation was significant at the 0.05 level.

RESULTS

We investigated the responses of APP and SCC during experimental induced subclinical mastitis in goats. The infected goats did not show clinical signs of infection following intramammary inoculation of *S. aureus*. The rectal temperature, respiratory and pulse rates in the infected groups were within the normal range. Before inoculation, the milk SCC readings obtained from the infected and control groups was below the threshold value of 1 x 10^6 cells/mL. The mean SCC was increased significantly ($p<0.05$) above the threshold in infected groups than in the control group. The mean SCC was consistently above the threshold value in infected groups relative to control at 6, 24, 48 and 72 h post-infection (hpi), respectively (Figure 1).

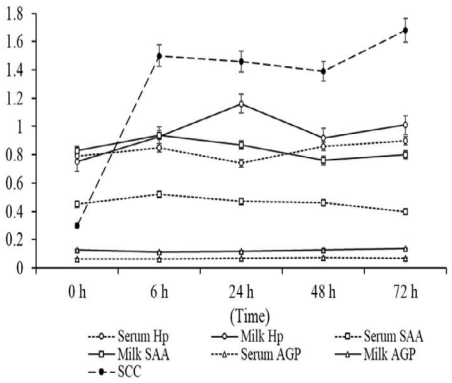

Figure 1: Dynamic between the acute phase protein concentration and somatic cell count (SCC) in the infected group throughout the study period. Acute phase protein is expressed as mean ± SEM (µg/mL) and SCC is expressed in 1 x 10^6 cells/mL. The SCC is consistently above the threshold values of 1 x 10^6 cells/mL at 6, 24, 48 and 72 hpi of *S. aureus* (n=15).

Prior to the experiment (0 h), bacteriological examinations of milk samples from both infected and control groups were conducted and no *S. aureus* was isolated. *Staphylococcus aureus* was reisolated 6, 24, 48 and 72 hpi. Reisolation of *S. aureus* (1.47 x 10^2 cfu/mL) was found as early as 6 hpi, and the bacterial count declined by 24, 48 and 72 hpi. All milk samples from the control group were found to be negative for *S. aureus* throughout the experimental period. Changes in serum and milk Hp, SAA and AGP in a goat model of subclinical mastitis.

The results of the serological assay of APP's revealed a

Table 1: Mean (10^{-3}) ± SEM concentrations (µg/mL) of serum Hp, SAA and AGP in the infected and control group at different time intervals.

Serum Hp					
Hour after administration of inoculum					
Group	0 h	6 h	24 h	48 h	72 h
Infected	0.79 ± 0.01[a]	0.85 ± 0.01[b]	0.74 ± 0.01[a]	0.86 ± 0.01[b]	0.90 ± 0.00[b]
Control	0.80 ± 0.01[a]	0.77 ± 0.01[a]	0.73 ± 0.01[a]	0.76 ± 0.01[a]	0.72 ± 0.00[a]
Serum SAA					
Hour after administration of inoculum					
Group	0 h	6 h	24 h	48 h	72 h
Infected	0.45 ± 0.01[a]	0.52 ± 0.03[b]	0.47 ± 0.02[a]	0.46 ± 0.01[a]	0.40 ± 0.03[a]
Control	0.47± 0.02[a]	0.40 ± 0.02[a]	0.40 ± 0.01[a]	0.44 ± 0.01[a]	0.39 ± 0.02[a]
Serum AGP					
Hour after administration of inoculum					
Group	0 h	6 h	24 h	48 h	72 h
Infected	0.065 ± 0.01[a]	0.066 ± 0.00[a]	0.068 ± 0.00[a]	0.075 ± 0.00[b]	0.071± 0.00[a]
Control	0.062 ± 0.01[a]	0.063 ± 0.00[a]	0.063 ± 0.00[a]	0.066 ± 0.01[a]	0.068± 0.01[a]

Values with different superscript letters are significantly different at $p<0.05$. Comparison is between control and induced groups at different time intervals.

Table 2: Mean (10^{-3}) ± SEM concentrations (µg/mL) of milk Hp, SAA and AGP in the infected and control group at different time intervals.

Milk Hp					
Hour after administration of inoculum					
Group	0 h	6 h	24 h	48 h	72 h
Infected	0.75 ± 0.01[a]	0.93 ± 0.06[a]	1.16 ± 0.08[b]	0.92 ± 0.05[b]	1.01 ± 0.06[b]
Control	0.78 ± 0.01[a]	0.67 ± 0.02[a]	0.70 ± 0.02[a]	0.69 ± 0.01[a]	0.76 ± 0.02[a]
Milk SAA					
Hour after administration of inoculum					
Group	0 h	6 h	24 h	48 h	72 h
Infected	0.83 ± 0.01[a]	0.94 ± 0.02[b]	0.87 ± 0.03[b]	0.76 ± 0.01[a]	0.87 ± 0.03[b]
Control	0.83 ± 0.01[a]	0.83 ± 0.01[a]	0.78 ± 0.01[a]	0.71 ± 0.01[a]	0.74 ± 0.01[a]
Milk AGP					
Hour after administration of inoculum					
Group	0 h	6 h	24 h	48 h	72 h
Infected	0.128 ±0.01[a]	0.111 ±0.01[a]	0.116 ±0.01[b]	0.129 ±0.01[b]	0.138±0.01[b]
Control	0.111 ±0.00[a]	0.097 ±0.01[a]	0.088 ±0.0[a]	0.103± 0.01[a]	0.099±0.01[a]

Values with different superscript letters are significantly different at $p<0.05$. Comparison is between control and induced groups at different time intervals.

time-dependent change of Hp, SAA and AGP in the infected goats compared to the control groups. Overall, APP levels in the milk were significantly higher ($p<0.05$) than those in the serum. The changes of serum and milk APP levels in the infected groups were shown in Table 1, Table 2 and Figure 1 respectively.

The mean serum Hp was increased significantly ($p<0.05$) in the infected group at 6 hpi relative to the control group. However, the serum Hp decreased at 24 hpi and gradually increased and peak at 72 hpi than in the control group (Table 1). Milk Hp levels were higher in the infected group with 2.4 folds increase ($p<0.05$) at 24 hpi than in the control group. However, milk Hp declined at 48 hpi then showed a slight increase at 72 hpi (Table 2).

A significantly ($p<0.05$) higher concentration level of serum SAA was found in the infected group at 6 hpi than in the control group (Table 1). Similarly, the mean milk SAA concentrations increased earlier and reached higher

levels at 6 hpi ($p<0.05$) in the infected groups than in the control. However, the mean milk SAA concentrations fall gradually from 24 to 48 hpi before fluctuating again at 72 hpi ($p<0.05$) (Table 2).

The mean serum AGP in the infected group increased and reached its highest concentration at 48 hpi ($p<0.05$) than in the control (Table 1). The mean milk AGP in the infected group was significantly elevated ($p<0.05$) from 24 to 72 hpi (Table 2).

There was a significant-good positive correlation between milk Hp concentrations with SCC values (r_s=0.73; $p<0.05$). Correlation analysis revealed a slight positive relationship between the milk SAA concentrations and SCC values (r_s=0.19; p=0.37). However, the relationships were not statistically significant. Moreover, there was a negligible, negative relationship between milk AGP concentrations and SCC, which was not statistically significant (r_s=-0.13; p=0.53).

DISCUSSION

This study investigates the changes in APP and SCC levels in goats during experimental induced subclinical mastitis with *S. aureus* obtained from a field strain. Clinical examinations on the infected goats revealed no changes in gross appearances of the udder or the presence of any abnormal clinical signs. This condition reflects a successful subclinical infection in the experimental setting that could be achieved using the inoculation dose of1×10^3 cfu/mL. In the present study, the SCC peaked at 72 hpi and was followed by a decline of *S. aureus* counts. These findings suggest a bacteriological cure for the infected mammary tissue.

Serological assay on APPs revealed the presence of time-dependent change in the serum and milk Hp, SAA and AGP levels in all the infected goats compared to the control groups. There was a significant change in serum and milk Hp concentration during induced *S. aureus* subclinical mastitis infection. Increase serum Hp observed in this study agrees with the results of a previous study that reported an increased concentration of serum Hp during an experimental clinical mastitis model in goats (Fasulkov et al., 2014; Simplicio et al., 2017). Both studies reported early evidence of APP involvements in the pathogenesis of *S. aureus* clinical mastitis in small ruminants. A recent study by Sadiq et al. (2019) reported that significant changes in serum Hp can be detected as early as 24 hours post-infection in lactating goats during another similar experimental condition. The ability of the Hp molecule to bind to iron prevents the use of iron for bacterial cell metabolism. In addition, the Hp binds with free haemoglobin to reduce oxidative stress and prevents the formation of oxygen radicals (Ceciliani et al., 2012).

In the present study, we describe for the first time the profile of milk APPs changes that occur in goats with experimentally induced *S. aureus* subclinical mastitis. In this study, the milk Hp peaked at 24 hpi and was followed by a significant decrease of serum Hp. The findings suggest a possible breakage of the blood-milk barrier integrity thus sufficiently allowing Hp in the serum to leak into the milk in an early event of subclinical mastitis (Hiss et al., 2004). In bovine mastitis, Hp, SAA and c-reactive protein have been reported in milk, suggesting their potential as biomarkers of mastitis (Thomas et al., 2015; Simões et al., 2018). Previous studies have also shown that milk Hp is more sensitive in cattle and buffalo than serum Hp as an early acute phase response during intramammary infection (Eckersall et al., 2001; Pedersen et al., 2003; Singh et al., 2015). Moreover, the Hp is reported to be locally expressed in the mammary gland of cattle with mastitis (Eckersall et al., 2001). However, the expression of Hp protein and mRNA in the caprine mammary tissue has not been reported and it is necessary to investigate its potential as a marker for *S. aureus* based subclinical mastitis in small ruminants.

Sadiq et al. (2019) found the serum SAA concentration levels were significantly increased in goats with experimentally induced staphylococcal mastitis. Contrary to previous findings, we found no significant changes in serum SAA concentration levels in our study. Dalanezi et al. (2020) reported the levels of APP in milk from cattle with clinical mastitis caused by different pathogenic bacteria. They found that bacteria that cause chronic and long-term infection as *S. aureus* induced a slighter immune response and therefore lower concentration of SAA could be expected. SAA is produced and secreted primarily by the hepatocytes to modulate immune reactions during systemic inflammation opsonizing microbial cells and to prevent cholesterol crystal formation at the site of inflammation (Shah et al., 2006; Jain et al., 2011).

In the present study, serum AGP levels in goats with subclinical mastitis were significantly increased only after 48 hpi. In comparison with serum AGP, milk AGP levels increased earlier at 24 hpi and the values were consistently higher than in the control group at 48 and 72 hpi, respectively. A previous study has reported that AGP is produced by the mammary alveolar cells, although the main source of AGP in milk is released by somatic cells (Ceciliani et al., 2012). AGP reduces the local inflammatory response by protecting cells from apoptosis induced by inflammation. Therefore, it is shown that mastitis leads to apoptosis in epithelial cells of the mammary gland, and consequently

trigger the synthesis of AGP (Dalanezi et al., 2020). Moreover, AGP has a long half-life, thus it remains longer in the body fluid and can serve as an indicator of chronic inflammation in small ruminants and other species too (Eckersall and Bell, 2010; Gannon et al., 2019).

We have demonstrated that APP levels were significantly higher in milk than in the serum samples of goats during experimentally induced subclinical mastitis. This is possibly due to the leakage of APP out of the blood vascular system into milk as a result of the blood-milk barrier dysfunction during inflammation, or due to the local production of APP in the mammary gland as a response to mastitis (Cray et al., 2009; Heller and Johns, 2015). Previously, Nielsen et al. (2004) reported that the presence of APP in an organ can be a reliable and specific indicator of local inflammatory responses in that organ. Therefore, the changes of milk APP could be used as a specific indicator of intramammary infection in goats. There is a need for more studies to fully elucidate the local expression of major APPs in the caprine mammary tissue during both healthy and diseased conditions.

The use of APP present in milk can be measured routinely for the early detection of mastitis in goats. In addition, milk samples are feasible to collect and manipulate. The collection of milk samples is relatively inexpensive, non-invasive and it provides a satisfactory amount for use in a mammary health screening (Pyorala et al., 2011).

The number of somatic cells reflects the intensity of the inflammatory response to mammary tissue. During inflammation, the rapid increase in SCC is due to the recruitment of polymorphonuclear cells (PMN) into the milk. The function of PMN is to mediate intracellular killing of the invading pathogen and defend the mammary glands at the beginning of an acute inflammatory reaction. Determination of SCC has been widely used in clinical practice to detect subclinical mastitis in ruminants and previous works have been demonstrated to show a positive correlation with Hp level. Hence, Hp could be established as an indicator for the diagnosis of subclinical mastitis in goats and merits further investigation.

However, some limitations should be noted. The study only reflects acute phase response in experimentally induced subclinical mastitis in goats. The APP levels in goats might be different in the naturally occurring subclinical mastitis conditions caused by either the same or different pathogens (Dalanezi et al., 2020).

CONCLUSION

Altered expression of the serum and milk APPs was seen during *S. aureus*-induced subclinical mastitis infection in goats that unlock the potentials of acute phase protein assessment as recommended and alternative tools for the early detection of subclinical mastitis in goats. In addition, the significant relationship observed between milk Hp concentration and SCC level could be used to reflect the intensity of the inflammatory response that occurs in the mammary tissue. Therefore, determination of APP level in milk during a natural state of infection may be useful to confirm the validity of Hp to be the candidate biomarker for subclinical mastitis in small ruminants.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

NOVELTY STATEMENT

This study is the first to investigates the milk APP level in goat mastitis with experimentally induced S. aureus. We have found that milk Hp are highly potential to be the protein marker candidate for the early diagnosis of caprine mastitis.

AUTHORS CONTRIBUTION

All authors contributed equally according to their tasks and approved the final manuscript.

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