Protease-activated receptor 2 expression in the mammary gland tissues in correlation with mastitis severity in goats

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Abstract: Mastitis is a common disease in small ruminant industry. The present study aimed to determine the presence of protease activated receptor-2 (PAR2) mRNA expression in the mammary gland of mastitis challenged goats. 30 clinically healthy mix breed lactating goats were divided into three groups, consisting of Staphylococcus aureus (Group 1), methicillin-resistant S. aureus (Group 2) and sterile phosphate-buffered saline (Control) groups. The data regarding physical condition of udder and clinical parameters of goats were recorded while milk samples and mammary gland tissues were collected at 24 and 48 hours post infection. Somatic cell count (SCC) was measured by direct microscopic method. The presence of PAR2 mRNA in the mammary gland tissue samples was detected by real-time PCR. Goats from group 1 developed mild to moderate clinical signs while Group 2 exhibited moderate to severe clinical signs. SCC was higher in both challenged groups than control group. PAR2 mRNA expression was detected in all mammary gland samples from Group 1 and Group 2. The gene expression was significantly highly in mammary gland tissue with severe clinical signs. The finding of PAR2 expression in caprine mammary gland is novel and important, suggesting serine proteases involved the development of mastitis in goat.

Keywords: PAR2, mastitis, mammary gland, goats, MRSA, qPCR

1. Introduction
Mastitis is an inflammation of mammary glands resulting from imbalance interaction between the agent, the susceptible animal, and the environment [1]. Clinically, it is characterized by fever, warm, painful, and swollen mammary gland [2]. Changes in the physical appearance of milk such as presence of flakes, clot, pus and blood are also common. Staphylococcus aureus (S. aureus) is one of the most common causes of mastitis in goats with a prevalence of 20% to 60% [3,4,5]. In addition, MRSA has also been isolated from bulk tank milk of goats which may impose a significant threat to public health [6, 7, 8].

Protease activated receptor-2 (PAR2), a cell surface G protein-coupled receptor, plays an important role in the induction and amplification of the inflammatory response through proteolytic cleavage by serine proteases [9]. The involvement of PAR2 in inflammation is supported by several studies. Early
studies reported that PAR2 expression was up-regulated by inflammatory mediators such as tumour necrosis factor α, interleukin 1α and lipopolysaccharide [10]. Furthermore, deletion of PAR2 also diminishes and delays the onset of inflammation [11]. PAR2 is widely distributed and expressed by variety of cells including the epithelial cells [12]. In the human mammary gland, activation of PAR2 has been linked to tumour growth and progression [13]. In addition, PAR2 activation also involved in disorders of the cardiovascular, musculoskeletal, gastrointestinal, respiratory and central nervous system [14]. Taking together, these suggest that PAR2 may have an important role in various pathological conditions and have become attractive targets for the development of novel therapeutics.

In ruminant, increased protease activity has been observed in milk from mastitis animals compared to normal [15]. Protease in mastitic milk is originated from milk polymorphonuclear cells (PMN) mainly represented by neutrophils [16]. The proteases contribute to the extracellular killing of microorganisms, tissue degradation and elicit cellular response, at least in part, via the activation of protease activated receptor, which is known to mediate inflammation [17]. However, to date, the roles of PAR2 in the development of mastitis have not yet been defined. Additionally, it has not been determined whether the severity of mastitis is associated with increased PAR2 expression. We hypothesized that PAR2 expression is higher in the mammary tissue of goats with severe mastitis. Thus, the aim of this study was to quantify PAR2 mRNA expression in the mammary gland tissue of goats with and without mastitis and to relate with its severity.

2. Materials and methods

2.1. Experimental animals

Thirty clinically healthy lactating does age 2-4 years were used in this experimental study. The general health status of the does including the condition of the mammary glands was determined by physical examinations, California Mastitis Test (CMT) and bacteriological examination of milk prior to the commencement of the experiment. All goats were free of intramammary infection and had CMT scores of trace or zero with no bacterial growth from milk. The goats were randomly divided into three groups; group 1 (n =10) serve as control and inoculated with 1 ml of sterile pyrogen-free phosphate buffer solution (PBS) intramammarily on both udders; group 2 (n =10) were inoculated with 1 ml of 1.5 × 10^8 cfu/ml of S. aureus intramammarily on both udders; Group 3 (n=10) were inoculated with 1.5 × 10^8 cfu/ml of MRSA intramammarily on both udders. Experimental and control goats were under the same herd management throughout the study and housed at the Pasir Akar Farm, Universiti Sultan Zainal Abidin (UniSZA), Terengganu, Malaysia. The animals were fed ad libitum and water source were made available in the pens. The animals were challenged for 48 hours and were then treated with oxytetracyclin (20 mg/kg) and Mastikel until the animals were fully healed. The experiment was approved by the Universiti Sultan Zainal Abidin (UniSZA) Animal and Plant Ethic Committee (UAPREC) Reff: UAPREC/04/18/006.

2.2. Bacterial growth condition

Staphylococcus aureus (ATCC 25923) and MRSA strains (ATCC 700699) were used as the challenge strain. The S. aureus and MRSA strains was cultured onto nutrient agar and grown overnight (24 hours) at 30˚C. The challenge culture was standardized to 1.5 × 10^8 cfu/ml by comparing the turbidity of the culture to the 0.5 McFarland standard.

2.3. Clinical examinations of experimental goats

The severity of clinical mastitis was scored according to changes in the body temperature, milk appearance, mammary gland and systemic signs. The scoring scheme used was based on that described by [18]. The body temperature was scored on a scale from 0 to 2 (normal score,0; score 1, >39.7- 40˚C; score 2, 41-42˚C).
Table 1. Scoring scheme for body temperature.

| Body temperature (°C) | Score |
|-----------------------|-------|
| 38.5–39.7             | 0     |
| > 39.7                | 1     |
| 41 - 42               | 2     |

The milk appearance was assessed and scored from 0 to 3 (normal score, 0; score 1, small clots; score 2, large fibrin clots; score 3, serous or bloody).

Table 2. Scoring scheme for milk appearances.

| Milk appearance                  | Score |
|----------------------------------|-------|
| Normal                           | 0     |
| Small clots                      | 1     |
| Large fibrin clots               | 2     |
| Serous or bloody                 | 3     |

The changes in the mammary gland were scored on a scale from 0 to 4 (normal score, 0; score 1, swollen; score 2, sore/hardened, score 3, discoloured; score 4, bluish).

Table 3. Scoring scheme for mammary gland appearances.

| Mammary gland appearance         | Score |
|----------------------------------|-------|
| Normal                           | 0     |
| Swollen                          | 1     |
| Sore or hardened                 | 2     |
| Discoloured                      | 3     |
| Bluish                           | 4     |

The systemic signs were scored on a scale from 0 to 4 (normal score, 0; score 1, feverish; score 2, slightly depressed; score 3, deeply depressed; score 4, recumbent).

Table 4. Scoring scheme for systemic signs.

| Systemic signs                  | Score |
|---------------------------------|-------|
| Normal                          | 0     |
| Feverish                        | 1     |
| Slightly depressed              | 2     |
| Deeply depressed                | 3     |
| Recumbent                       | 4     |

The total severity score was determined by summing the individual scores. A global score was determined based on overall assessment of the severity of the clinical examinations (normal score, 0; mild (1), total severity score 1-4; moderate (2), total severity score 5-9; severe (3), total severity score 10-13).

Table 5. Scoring scheme for overall assessment of clinical examinations.

| Overall assessment | Total severity score |
|--------------------|----------------------|
| Normal             | 0                    |
| Mild               | 1-4                  |
| Moderate           | 5-9                  |
| Severe             | 10-13                |
2.4. Milk sampling

Milk samples for clinical examinations, somatic cell count (SCC) and bacteriological analysis were collected from both udders at pre-inoculation (0-h) and at 24 hours and 48 hours post inoculation (hpi). The first few streams of milk were used to score for clinical mastitis as described above. A total of 10 ml and 5 ml of milk samples was collected aseptically for bacteriological examinations and SCC determination, respectively. Milk samples were transported to the laboratory in an ice box and stored at 4°C.

2.5. Mammary gland biopsy

Mammary gland biopsies were performed in the medial region of the udder in all goats. For each animal, one fragment of mammary tissue was collected from the left udder at 24 hpi and the right udder at 48 hpi. The goats were restrained and sedated with 0.5 ml of xylazine administered intramuscularly (0.1 mg/kg, Ilium Xylazil-20). Skin and mammary tissue were locally anesthetized with 1 ml of 2% lidocaine hydrochloride intradermally. The reusable core biopsy instrument comprising a 14-gauge needle was used to collect the mammary parenchymal tissue (Bard® Magnum® Reusable Core Biopsy Instrument) as described by [19]. Tissue samples were placed into RNAlater (Ambion) and stored at 4°C until further analysis.

2.6. Somatic cell count

The SCC of the milk samples was determined within 6 hours post-collection. The milk samples were warmed to 40°C before transferred to glass slides. The samples were shaken thoroughly by rapid inverting movements about 25 times. The samples were allowed to stand for 2 to 5 mins to allow the foam to disperse. 10 µL of milk was transferred onto the center of 1 cm² square area of the slide. The milk was uniformly spread over the entire area and dried at 40 to 45°C within 5 mins using a hot plate or incubator. The slides were stained with Pyronin Y-Methyl Green Stain (New York Modification) and observed under microscope. Single strip factor were determined and the cells were then identified with nucleus stains blue or blue-green and counted within a single strip. The SCC were determined by multiplying the number of somatic cells with the single strip factor. [26].

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from total RNA by using the iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer’s recommendations. cDNA synthesis was carried out on 1 ng RNA, at 46°C for 20 mins. Absence of contamination of RNA with genomic DNA was verified with minus-reverse transcriptase (minus-RT) controls.

2.8. Real-time PCR

Real-time PCR was performed using iTaq Universal Supermix with SYBR Green (Bio-Rad) following the manufacturer’s instructions. Each reaction was conducted in a total volume of 20 µl consisting of 10µl 2× iTaq Universal Supermix with SYBR green (Bio-Rad), 2 µl sample cDNA, 1.2 µl forward and reverse primers and 6.8 µl RNase free water. Each sample was measured in duplicate. The primer was designed using the online primer design tool at the National Centre for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and commercially synthesized by Apical Scientific, Malaysia. *Capra hircus* mRNA sequences of PAR2 was used. Primer details are shown in (Table 6). The reaction was done as follows: initial denaturation at 95°C for 10 mins followed by 40 cycles of denaturation at 95°C for 5 s and data collection at 60°C for 30 s according to the brand’s suggestion. A negative control without a DNA sample was included for every assay. The melting curve was analysed and relative expression of each gene was normalised with the predetermined reference genes, eukaryotic translation initiation factor 3K (EIF3K), peptidylprolyl isomerase A (cyclophilin A) (PPIA) and ubiquitously expressed transcript (UXT2) and estimated as values of 2-ΔΔCt.
Table 6. List of primer used for the detection of PAR2 F2RL1 in the sample.

| No. | Primer | Primer Sequence (5’-3’)                  | Accession Number | Annealing Temperature (˚C) |
|-----|--------|------------------------------------------|------------------|----------------------------|
| 1   | F2RL1  | F - CTGCCAGTGACCACAGGTGA  
R - CAGCATCCCTGC  
GGAAACAC | NC_030817 | 60                          |

2.9. Statistical analysis

A two-way ANOVA and Tukey’s HSD were used to analyse the mammary gland severity score between mastitis and control groups. The normalised values that reflect the PAR2 mRNA levels were analysed using a two-sample t-test to determine whether there was any difference in PAR2 mRNA expression levels between the mastitis and control groups with a significance level (α) set at 0.05. Correlation analysis was determined using Spearman’s Rank Correlation. Correlation coefficient (r_s) results were interpreted according to a modified categorisation of that used by [20]. Correlation was significant at the 0.05 level. All analyses were performed using GraphPad Prism for Windows, version 8.

3. Results

3.1. Clinical parameters

Statistical analysis showed significant differences (P<0.05) in the mastitis severity scores for both mastitis goats (S. aureus and MRSA) and the control goats. At 24 hpi, all goats infected with S. aureus (Group 1) showed mild clinical signs and were given a Global score of 1, whereas all goats infected with MRSA (Group 2) showed moderate clinical signs and were given a Global score of 2. At 48 hpi, the clinical signs in the S. aureus infected group (Group 1) remained mild in four goats, while the other six being moderately affected (Global score 2). However, three of the ten MRSA goats (Group 2) developed into severe mastitis (Global score 3) (Figures 1a-b) and showed systemic signs at 48 hpi. The mastitis severity scores were significantly higher in the MRSA mastitis goats (group 2) than the S. aureus mastitis goats (group 1) at 24 hpi and 48 hpi respectively (P<0.05). No changes were found in the control group.

![Figure 1](image_url). Examples of clinical changes of the mammary gland and milk of goats with mastitis (Global score 3, MRSA group 48 hpi). (a) The right mammary gland and the teat are swelling. The red discoloration is also observed on the skin of the infected gland. (b) The milk from the infected mammary gland is pink in colour, thick in consistency and contains blood and milk clots.
Table 7. Severity scores for control, *S. aureus*, and MRSA groups.

| Group | 24 hpi | 48 hpi |
|-------|--------|--------|
| Control | 0 | 0 |
| 1 | 1 | 2 |
| 2 | 2 | 3 |

3.2. Comparative assessment of PAR2 mRNA levels in mammary gland with and without mastitis

PAR2 mRNA expression was detected in all mammary tissues (10 *S. aureus* mastitis, 10 MRSA mastitis and 10 control). At 24 hpi, the PAR2 mRNA expression levels in the *S. aureus* infected mammary tissue (Group 1) were 0.5 times lower than in control (P>0.05) (Figure 2a). Meanwhile, the PAR2 mRNA expression levels in the MRSA infected mammary tissue (Group 2) were increased by two-fold (P<0.05) than the control (Figures 2a and 2c). At 48 hpi, a significantly higher expression of PAR2 mRNA (P<0.0001) was observed in the *S. aureus* in Group 1 (ten times higher) and MRSA (eleven times higher) infected mammary tissue (Group 2), respectively, than in control (Figures 2b and 2d).

![Figure 2](image-url)

**Figure 2.** Protease-activated 2 (PAR2) mRNA expression in mammary gland tissue. (a) Bar graph shows the fold-change in PAR2 mRNA expression in *S. aureus* mastitis (n=10) and MRSA mastitis (n=10) vs control (n=10) at 24 hpi. The PAR2 mRNA expression levels in the *S. aureus* infected mammary tissue were 0.5 times lower than in control. Meanwhile, the PAR2 mRNA expression levels in the MRSA infected mammary tissue were two times higher than the control group. (b) The PAR2 mRNA expression levels in the *S. aureus* and MRSA infected mammary tissues were ten and eleven times higher than the control group. (c) The PAR2 mRNA expression of the MRSA infected mammary tissues was significantly higher than the control group at 24 hpi. (d) The PAR2 mRNA expressions of the *S. aureus* and MRSA infected mammary tissues was significantly higher than the control group at 48 hpi, respectively. (c,d) Data are presented as mean ± SEM (One-way ANOVA, ****P <0.0001). ns = not significant.
3.3. Somatic Cell Count and its association with PAR2 mRNA levels

At the time point of pre intramammary challenge (0 h), goats across all groups showed mean low SCC (0.15-0.16 x 10^6 cfu/ml). The SCC were profoundly increased (P<0.05) in the infected mammary gland of all 20 goats from the *S. aureus* (Group 1) and MRSA (Group 2) groups 24 and 48 hpi, respectively. Mean values of SCC in the MRSA groups showed significantly higher (P<0.05) than the *S. aureus* groups in both time point.

There was a significant positive correlation between the mean SCC values and the PAR2 mRNA expression in the *S. aureus* (Group 1) (24 hpi, r_s = 0.57, P<0.05; 48 hpi, r_s = 0.51, P<0.05) and MRSA (Group 2) (24 hpi, r_s = 0.68, P<0.05; 48 hpi, r_s = 0.64, P<0.05) infected mammary tissues.

4. Discussion

The present study examined the expression of PAR2 in mammary tissue of goats infected with *S. aureus* and MRSA and compared these patterns with the clinical mastitis severity and SCC at different time point. This study shows for the first time that PAR2 is expressed in caprine mammary tissues of normal and mastitis goats. Quantitative real-time PCR showed that PAR2 expression levels were significantly increased in mastitis compared with normal caprine mammary tissue.

Specific mechanisms that trigger activation of PAR2 in mastitis are not completely understood. PAR2 is expressed in a various cell types, including mammary epithelial cells [12]. PAR2 is activated through proteolytic cleavage by serine proteases found in the microenvironment. Activation of PAR2 initiating an irreversible intracellular signal, which has association with cytokine and chemokine secretions [9]. During intramammary infections, many proteolytic enzymes are released [21,22]. It is likely that the host- and bacterial-derived proteases are involved and trigger the activation of PAR2 in mastitis. It has been reported that indigenous serine proteases, such as elastase, cathepsin and plasmin are associated with leukocytes which constitute the majority of somatic cells [16, 21]. These serine proteases mediate phagocytosis and killing of invading bacteria. The released proteases may at some point interact with PAR2 on the mammary epithelium and trigger the activation of PAR2 which activate distinct signal transduction pathways involved in inflammation, pain and immunity [23]. This explanation is supported by the significant positive correlation between mean SCC values and PAR2 mRNA expression in both *S. aureus* and MRSA infected goats. In addition, expression of PAR2 gene showed a trend to be higher in goats with severe form of mastitis. Future studies should aim to elucidate the role of somatic cell serine proteases in PAR activation during mastitis.
The number of somatic cells reflects the intensity of the inflammatory response to mammary tissue. During inflammation, the rapid increase in SCC is because of the recruitment of 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. In the present study we found that the MRSA infected goats develop a severe form of mastitis than the S. aureus mastitis goats. Additionally, the mean SCC values in the milk of MRSA infected goats increased much more rapidly and consistently higher than the S. aureus mastitis goats with increasing time. These results reflect on the pathogenicity and the virulence factors produced by bacterial strains, which are modulated by a complex transcriptional regulator network [24].

In this study, it was found that the goats challenged with both S. aureus and MRSA showed high SCC values and PAR2 mRNA expressions when comparing with the control group. The number of challenged animals was limited due to several circumstances. Sampling of animals from uncontrolled environment could reflect better understanding of the relationship between PAR2 mRNA gene expression in the mammary tissue and the severity of mastitis.

5. Conclusion
In conclusion, the present study demonstrated that PAR2 mRNA gene expression in the mammary tissue were able to reflects the severity of mastitis. The data reported in this study provide a basis for future studies on the possible relevance of PAR2 as a prognostic marker in mastitis, and for a better understanding of disease development, thus in turn, would enhance newer therapeutic strategies to control and treatment of mastitis in ruminant.

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