

Original Article

Neutrophil surface adhesion molecule and toll like receptor dynamics in crossbred cows suffering from *Staphylococcus aureus* subclinical and clinical mastitis

Dilip Kumar Swain, Mohar Singh Kushwah and Ajay Kumar Dang

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AFFILIATIONS

- Dilip Kumar Swain
- Mohar Singh Kushwah
- Ajay Kumar Dang

Lactation and Immuno Physiology
Laboratory, National Dairy Research
Institute, Karnal 132 001, Haryana,
India.

ABSTRACT

Objective: The present study was an attempt to delineate the roles played by the neutrophil surface adhesion molecules and toll like receptors (TLRs) in crossbred cows suffering from *Staphylococcus aureus* subclinical and clinical mastitis.

Materials and methods: Thirty six Karan Fries (KF) cows were categorized into three groups namely healthy (n=12), subclinical mastitis (SCM; n=12) and clinical mastitis (CM; n=12) after screening 146 cows. The grouping was done based on evaluation of collected milk samples by routine procedures like Californian Mastitis Test (CMT) scoring, microscopic counting of milk cells (SCC), bacterial culture of milk samples and observing gross changes in milk. Culture of milk and blood was done for the identification of *Staphylococcus aureus* subclinical and clinical mastitis.

Results: Healthy cows expressed significantly ($P<0.05$) higher L-selectin (CD62L) in both milk and blood neutrophils as compared to the animals suffering from SCM and CM; however, no significant difference was noticed between milk and blood neutrophils. Significant ($P<0.05$) increase in the expression of beta integrin (CD11b) was observed in the CM group of cows as compared to SCM and healthy cows. Similar trend in the expressions of TLR2 and TLR4 in both blood and milk neutrophils was observed in the CM cows as compared to the healthy and SCM cows. Milk neutrophils revealed a higher expression of TLR as compared to blood neutrophils.

Conclusion: Host elicits stage specific expression of surface adhesion molecules and TLR2 and TLR4 as dynamic host innate immune response against *Staphylococcal* mastitis.

CORRESPONDENCE

Dilip Kumar Swain, PhD

Assistant Professor, Department of
Physiology, DUVASU, Mathura-281001
(Uttarpradesh, India).

E-mail: dilip_swain@yahoo.com

KEYWORDS

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INTRODUCTION

Mastitis is considered as one of the most complex diseases of bovines due to multiple involvements of pathogens, their toxins and immune cells like neutrophils, macrophages and lymphocytes ([Burton and Erskine, 2003](#); [Paape et al., 2003](#)). With the invasion of pathogens in to udder, dynamic cell crosstalk signaling cascades are initiated consequently bone marrow is stimulated to release neutrophils as first line of cellular defense in to blood circulation which migrate into udder and show phagocytosis of pathogens ([Borregard, 2010](#)). Neutrophils get terminally differentiated to bring out immune functions by employing both oxidative and non-oxidative mechanisms of pathogen killing ([Fuchs et al., 2007](#)).

Udder employs number of antimicrobial systems to overcome the attack of pathogens causing infection. Massive influx of neutrophils to the udder via the blood capillaries is one of the most significant processes that occur during the invasion of pathogens in to the udder ([Burton and Erskine, 2003](#)). In the process of recruitment of neutrophils, surface receptors play the central role mediating the events like adherence, rolling and diapedesis along the vascular endothelial cells. The surface receptors include surface adhesion molecules and toll like receptors (TLRs) ([Aitken et al., 2011](#)). The tolls like receptors are considered as the receptors that recognize the foreign pathogens and their associated molecules. The expression of receptors depends on type of pathogen, degree of stimulation of immune cells by pathogens, degree of interaction of pathogen with host immune system, and cytokine-chemokine signaling eliciting cellular host response against the invaded organism ([Bannerman et al., 2004](#); [Baumert et al., 2009](#); [Bannerman, 2009](#)).

Neutrophil recruitment to the mammary lumen during a pathogen challenge is mediated by the tethering of the neutrophils to the endothelium and followed by their transmigration to the mammary lumen. This process of migration is mediated through the surface expression of cell surface adhesion molecules namely L-selectins (CD62L) and $\beta 2$ integrins (CD11b) on neutrophils which interact with their corresponding ligands on the endothelial cell ([Diez-Fraile et al., 2002](#)). Both the receptors are expressed in stage specific manner and mediate differential roles like rolling, squeezing along the endothelium to reach the mammary lumen. L-Selectins (CD62L) mediate loose binding and rolling of polymorphonuclear cells (PMN) with the endothelial lining and $\beta 2$ integrins are associated with tight binding of PMNs with the endothelial cells ([Diez-Fraile et al., 2003](#)).

The expression of adhesion molecules on cell surface is mediated by the signal transduction pathways which are activated by the interaction of the pathogens and their toxins with the toll like receptors (TLRs) of neutrophils ([Futosi et al., 2013](#)). Interaction of pathogens with the TLRs is mediated by the pattern recognition molecules released by the pathogens ([Sabroe et al., 2005](#)). These interactions with the TLRs activates the signal transduction pathways which ultimately lead to prolonged cell survival, cell adhesion, enhanced release of cytokines, chemokines and production of ROS leading to enhanced phagocytosis. Both toll like receptors 2 and 4 are involved in the downstream activation of caspase dependent pathways causing faster onset of apoptosis during a pathogen challenge to the host ([Souza et al., 2012](#)).

The higher incidence of *S. aureus* caused mastitis has been reported in subtropical countries and is also the common most pathogen that causes mastitis in cows ([Sharma et al., 2012](#); [Smith, 2015](#)). Till date, scanty information is available in literature regarding differential pattern of expression of neutrophil cell surface adhesion molecules, and TLRs in crossbred KF cows suffering from subclinical mastitis (SCM) and clinical mastitis (CM) caused by *Staphylococcus aureus*. Therefore, the present study was undertaken to investigate the differential expression of CD62L, CD11b, TLR2, and TLR4 in neutrophils isolated from both blood and milk of crossbred KF cows suffering from *Staphylococcal* SCM and CM.

MATERIALS AND METHODS

The proposed work was accomplished in crossbred (Holstein Friesian \times Tharparker) Karan Fries (KF) cows having an average milk yield of 4500L/lactation. The cows were kept under semi intensive system of farming practices and were given ration as standardized in the farm. A total of 146 KF cows were screened for the study and milk samples were taken for screening. Hand milking was employed to collect milk samples in sterile vials from four quarters. Collected milk samples were analyzed for detection of mastitis by employing standard tests ([Swain et al., 2014, 2015](#)). Californian mastitis test (CMT) was employed by using a commercial CMT solution (Masti Check Reagent, GEA Westfalia Surge, India) and CMT scoring was done as per the specifications mentioned by the company. Grouping of cows to healthy, SCM and CM groups was done as per the method described earlier ([Swain et al., 2015](#)). Bacteriological examination of milk samples were carried out as per method established in the laboratory and has been reported earlier ([Dang et al., 2007](#); [Swain et al., 2014, 2015](#)).

Sampling of milk and blood: Collection of milk and blood samples was done as per the guidelines of the institutional animal ethics committee. Disinfection of the teats was carried out by using 70% ethyl alcohol prior to collection of milk. Milk samples were collected by hand milking method from each quarter. 50 mL of milk was collected from each quarter in sterile falcon tubes and were pooled for screening of cows. Milk samples were kept in icebox and transferred immediately to the laboratory for processing and further analysis. From the same cows, blood samples were collected in heparinised vacutainers (9 mL from each cow) from external jugular vein with least disturbance to the animal.

Isolation of blood and milk neutrophils: All materials and reagents used for the isolation of polymorphonuclear neutrophils (PMN) were sterile and of cell culture grade. Isolation of PMN from the blood samples was performed as per the method described earlier (Mehrzad et al., 2004; Swain et al., 2014, 2015). The purity of the blood PMN was found to be >90%, whereas, purity of milk PMNs was found to be >85%, as evaluated by Field's stain under oil immersion (100X). Different types of blood and milk PMNs were estimated by Field's stain and May Grunwald Giemsa stain and were observed under oil immersion (100X).

Expression of CD62L, CD11b, TLR (2 and 4) in blood and milk neutrophils: Relative quantification of CD62L, CD11b, TLR2 and TLR4 were done by flow cytometry as reported earlier (Swain et al., 2015). Details of the procedure followed during quantification of surface expression of receptors were established in the laboratory (Swain et al., 2015). For the quantification 1×10^5 cells were taken. For studying the expression of CD11b and CD62L, the primary antibody (CD11b-FITC) was used at 1:50 dilutions (BiorByt, Catalog no- orb26347, 100 µg/mL), whereas, CD62L-FITC was used at 1:100 dilutions (BiorByt, Catalog no- orb15311, 0.5mg/mL). For studying the expression of TLR2, the primary antibody (TLR2-FITC) was used at 1:50 dilutions (BiorByt, Catalog no- orb 16416, 0.5 mg/mL), whereas, for TLR4, the primary antibody (TLR4-FITC) was used at 1:100 dilutions (BiorByt, Catalog No. orb16418, 0.5 mg/mL).

Flow cytometry: Flow cytometry was carried out for the relative quantification of receptors by using FAC Scan flow cytometer (Becton Dickinson Immunocytometry Systems, BD Bioscience, USA). After gating of the PMNs, dot plots were recorded based on forward scattering of light and granularity of the cells. Ten thousand events were acquired for the quantification of expressions of receptors on neutrophils of all the three

groups of cows. Details of procedure of flow cytometry and quantification have been reported earlier (Swain et al., 2015).

Statistical analysis: Data obtained from the study was analyzed by using Sigma plot software (7.01 version). Mean and standard error of the mean (SEM) of both blood and milk neutrophils were analyzed by using two way analysis of variance (ANOVA). Data were considered significance at 5% (0.05) for all observations. To compare the groups considering different variables, two-way analysis of variance (ANOVA) was used, and the significance was tested at 0.05 (5%) for all the observations.

RESULTS

CMT score was found negative for healthy cows and single positive score was detected in subclinical group of cows. Clinical mastitis group of cows exhibited double and three positive score of CMT along with formation of thick gel. Clinical mastitis group of cows also exhibited presence of clots and flakes in the milk samples. Culture of milk sample identified the causative agent as *S. aureus* in milk samples of both subclinical and clinical group of cows. Significant ($P < 0.05$) increase in microscopic count of milk somatic cells was found in subclinical and clinical group of cows as compared to healthy group (Figure 1).

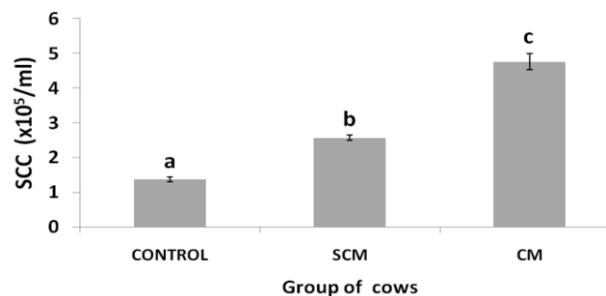


Figure 1. Somatic cell counts ($\times 10^5$)/mL of milk in different groups of KF cows. Superscripts (a, b, c for three groups of cows) indicate significance of the difference among the three groups ($P < 0.05$; $n = 12$). Bars represent the standard error of the mean.

The major part of the milk SCC is formed by the neutrophils. The degree of migration of neutrophils to milk depends on the type of pathogen, degree of pathogen interaction with the host immune system as well as cytokine-chemokine signaling. The degree of release of secondary metabolites is also another factor for the migration of neutrophils (Sordillo and Streicher, 2003). In the present study, significantly ($P < 0.05$) higher influx of neutrophils to udder during clinical mastitis as compared to SCM and control cows.

The results of expression of CD62L in the blood and milk neutrophils have been presented in **Table 1, Figure 2a and 2b**. In control/healthy animals, CD62L expression was significantly higher ($P<0.05$) as compared to the infected group of cows. Blood and milk neutrophils did not show any significant difference with respect to expression of CD62L in infected groups of cows.

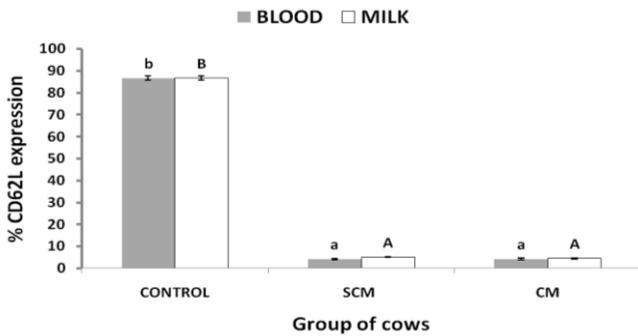


Figure 2a. Percent CD62L expression on neutrophils of different groups of KF cows. Superscripts (a, b, c for blood neutrophils and A, B, C for milk neutrophils) indicate significance of the difference among the three groups of cows ($P<0.05$; $n=12$). Bars represent the standard error of the mean.

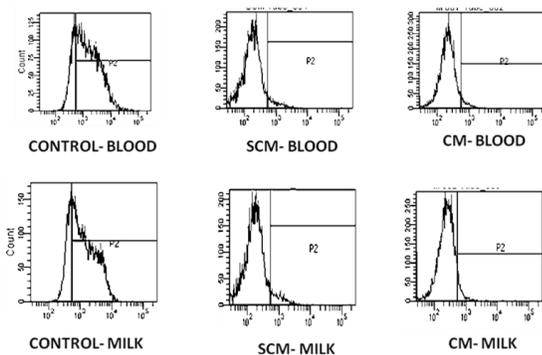


Figure 2b. Histogram showing percent CD62L expression on neutrophils of different groups of KF cows.

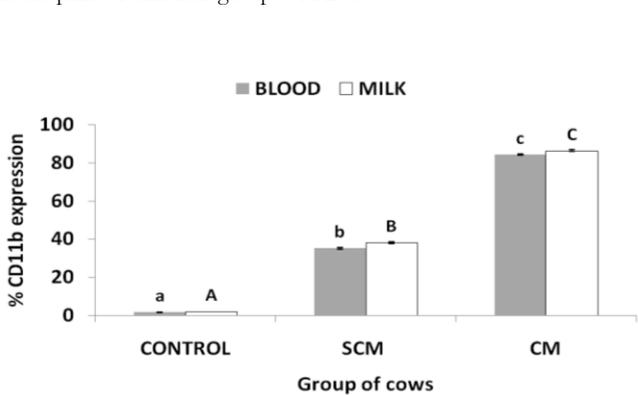


Figure 3a. Percent CD11b expression on neutrophils of different groups of KF Cows. Superscripts (a, b, c for blood neutrophils and A, B, C for milk neutrophils) indicate significance of the difference among the three groups of cows ($P<0.05$; $n=12$). Bars represent the standard error of the mean.

Relative expression of CD11b in different groups of cows has been presented in **Figure 3a and 3b**. Significant ($P<0.05$) up regulation of CD11b positive cells was evident in clinical mastitis group of cows with respect to subclinical and healthy group of cows. Reverse trend of expression of CD62L (L-selectins) and $\beta 2$ integrins (CD11b) was seen in neutrophils of both blood and milk. Along with this, the relative expression of CD11b on both blood and milk neutrophils did not show any significant difference. Relative expressions of toll like receptors (TLR2 and TLR4) in neutrophils isolated from different groups of cows have been presented in **Table 1, Figure 4a and 4b, 5a and 5b**. Significantly ($P<0.05$) higher expression of both TLR2 and TLR4 on blood and milk neutrophils was seen in clinical mastitis group as compared to healthy and subclinical group. In the study, higher expressions of both TLR2 and TLR4 in milk neutrophils but these were not significantly different from blood neutrophils.

The relative expressions of surface adhesion molecules and toll like receptors (TLRs) on both blood and milk neutrophils isolated from KF cows suffering from results of our study stand similar with earlier reported studies in

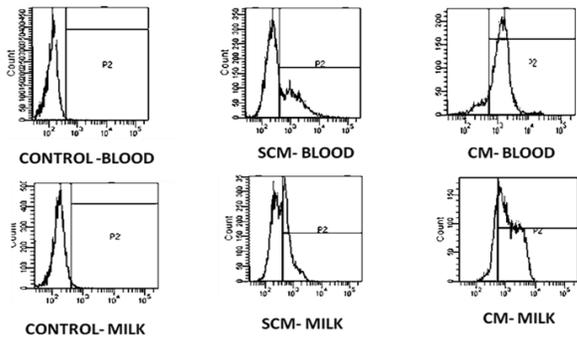


Figure 3b. Histogram showing percent CD11b expression on neutrophils of different groups of KF cows.

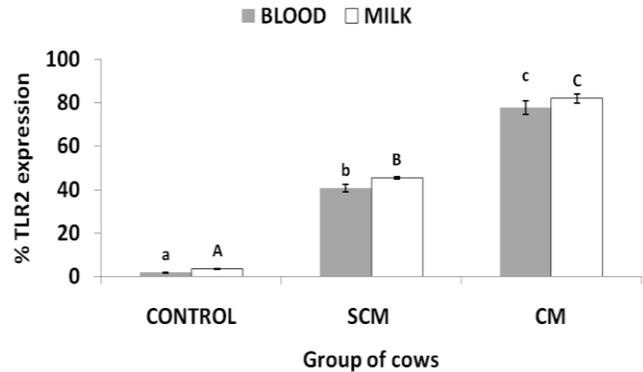


Figure 4a. Percent TLR2 expression on neutrophils of different groups of KF cows. Superscripts (a, b, c for blood neutrophils and A, B, C for milk neutrophils) indicate significance of the difference among the three groups of cows ($P<0.05$; $n=12$). Bars represent the standard error of the mean.

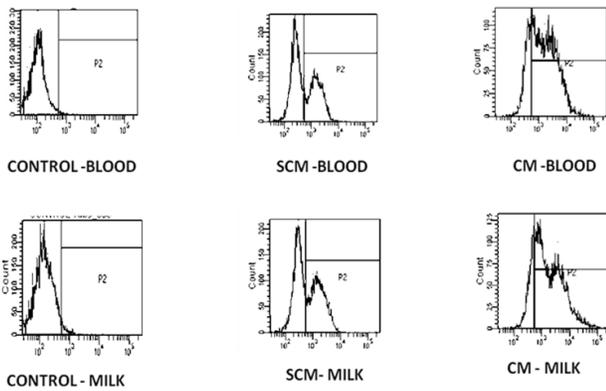


Figure 4b. Histogram showing percent TLR2 expression on neutrophils of different groups of KF cows.

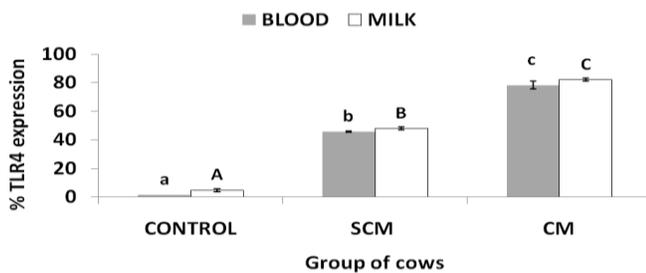


Figure 5a. Percent TLR4 expression on neutrophils of different groups of KF Cows. Superscripts (a, b, c for blood neutrophils and A, B, C for milk neutrophils) indicate significance of the difference among the three groups of cows ($P < 0.05$; $n = 12$). Bars represent the standard error of the mean.

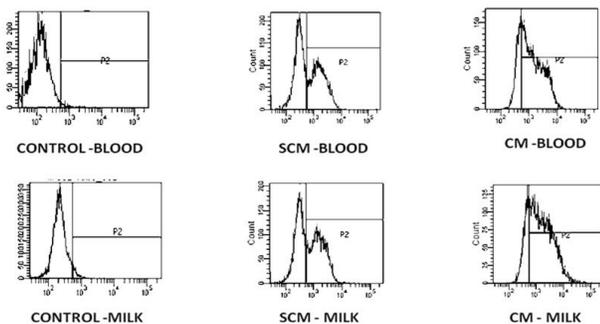


Figure 5b. Histogram showing percent TLR4 expression on neutrophils of different groups of KF cows.

terms of surface expressions of CD11b and CD62L during different forms of Staphylococcal mastitis.

The expression of CD62L increases in early phases of infection, and with progression of course of infection, its expression decreases which is followed by increase in expression of CD11b. During severity of infection, PMNs shed CD62L and express more CD11b so as to have tight contact with endothelial cells. This dynamic interplay between the surface adhesion molecules on the surface of the PMNs regulates the degree of PMN function along with their recruitment (Diez-Fraile et al.,

2004). Similar trend of expression of adhesion receptors on milk PMNs was seen in our study with *Staphylococcus aureus* infection but the level of expression of adhesion receptors was less in milk as compared to blood neutrophils (92% to the blood PMNs).

Subclinical mastitis group of cows revealed significantly ($P < 0.05$) lower expression of surface receptors as compared to clinical mastitis group of cows. Early phases of pathogen entry stimulates higher expression of L-selectins (CD62L) and with progression of pathogenesis, L-selectins get dislodged from the neutrophils. Along with the reduction in the CD62L expression on neutrophils, expression of CD11b increases. This dynamic kinetics of selectins and integrins on the surface of PMNs is the potential regulator of PMN function in terms of recruitment. However, in the present study, we were not able to understand why this kinetics was not seen in SCM group of cows.

Migration of the neutrophils along with the endothelial cells during diapedesis is mediated by β chains of integrins (CD11b) (Hoeben et al., 2000). Shedding of L-selectin (CD62L) provides an intrinsic signal to neutrophils to express more $\beta 2$ integrin (CD11b). This may be a probable reason behind our results where we noted significantly ($P < 0.05$) higher expression of L-selectins (CD62L) in control neutrophils as compared to both subclinical and clinical group of cows.

In control animals, the higher expression of L-selectins is an indication of slower and lower rolling of neutrophils along with their recruitment but with the progression of infection, the expression of $\beta 2$ -integrins increased, that provides an indication of strong affinity based binding of neutrophils with vascular endothelium (tethering) leading to higher transmigration of neutrophils towards the site of pathogen entry. Decreased expression of L-selectin causes diminished tethering as well as diapedesis of neutrophils to the site of inflammation. Consequence to this is a resultant reduction in the chemotaxis of the PMNs (Kolaczowska and Cubs, 2003). This may be one of the reasons behind higher expression of CD11b in PMNs isolated from blood and milk of cows suffering from SCM and CM. Another possibility which can't be undermined that, host exhibits lower degree of neutrophil stimulation during subclinical infection as compared to clinical infection. Pathogen mediated stimulation of host immune system is poor during subclinical mastitis as compared to clinical mastitis. On the other hand, the expression of CD62L was more in the control healthy group as compared to the infections groups. It is the dissociation of the CD62L that allows the interaction of CD11b with the endothelial surface (Hoeben et al., 2000).

Significant ($P<0.05$) increase in blood and milk neutrophil toll like receptor 2 and 4 was observed in CM and SCM groups of KF cows as compared to healthy control group (Figure 4a, 4b, 5a, and 5b). Higher TLR expression on neutrophils is an indication of higher recognition of the pathogens as well as immune response against the pathogens (Fuchs et al., 2007). Interaction of pathogens with the pattern recognition receptors is mediated by the pattern recognition molecules (TLR2 and TLR4) which are released by the pathogens (Petzl et al., 2005). These interactions with the TLRs activates the signal transduction pathways which ultimately lead to a prolonged cell survival, cell adhesion, enhance release of cytokines, chemokines and production of ROS leading to enhanced phagocytosis. These events enhance the microbicidal activities of the neutrophils. Both toll like receptor 2 and 4 are also involved in the downstream activation of caspase dependent pathways causing the faster onset of apoptosis (Akira and Takeda, 2004). Results of the present study are in corroboration with our earlier reports in indigenous Sahiwal cows suffering from Staphylococcal subclinical and clinical mastitis (Swain et al., 2014).

In SCM group, the expressions of TLR2 and TLR4 were 50% lower to that of the CM group of cows. During CM, released toxins and LPS by the pathogens is more as compared to SCM which leads to the activation of both TLR2 and TLR4 signaling pathways (Baumert et al., 2009). Significant ($P<0.05$) increase in percent of both blood and milk PMNs was shown by CM group of cows as compared to SCM and healthy group (data not shown). This may be due to higher TLR4 signaling as observed during TLR4 quantification by flow cytometry. In Indian dairy herds, 70 % of SCM and CM are caused by *S. aureus* (Dang et al., 2007; Sentitula et al., 2012). *S. aureus* employs lower expression of TLRs to escape the mammary defense and carryout the initiation of SCM (Yang et al., 2008). However, these hypotheses need further investigation to justify findings of the present study.

CONCLUSION

Present work was an attempt to decipher the dynamic role played by the first line of cellular defense that is neutrophils in crossbred KF cows during Staphylococcal subclinical and clinical mastitis in terms of expression of cell adhering molecules and toll like receptors 2 and 4. The study revealed that, crossbred cows with clinical mastitis exhibit higher milk SCC levels, along with higher expression of CD11b, TLR2 and TLR4. This study also clearly indicated the differential expression of various receptors which depends on the type as well as severity of

mastitis. More investigations are warranted for the validation of the specific and early cell signaling pathways associated with the onset and progression of *Staphylococcal* mastitis in crossbred cows.

CONFLICT OF INTEREST

No conflict of interest was declared by authors with any other people or organizations in any financial or personal relationship.

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