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Characterization of Bacterial Isolates, Antibiogram Profile and Pro-Inflammatory Cytokines in Subclinical Mastitis in Cross-Bred Dairy Cows

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ABSTRACT

Subclinical mastitis (SCM) is a major problem in the dairy industry creating a great loss both in quantity and quality of milk. The aim of this study was to characterize bacteria, antibiogram profile, evaluation of immune cells and proinflammatory cytokines (IL-2, IL-6, TNF-α) associated with the inflammatory response in SCM. Out of total twenty six (26) milking cows, microbial and biochemical tests revealed that fifteen (15) cows (57.68%) having a total of 18 bacterial isolates of which the prevalence of coagulase negative *Staphylococci spp.*, *Staphylococcus aureus*, *Streptococci spp.*, *E. coli*, and *Corynebacterium spp. were revealed as* 38.89%, 16.67%, 22.22%, 16.67%, *and* 5.56%, respectively. On antibiogram profile, it was found effective against isolated organisms in ranking order as chlortetracycline, bacitracin, chloramphenicol, amoxicillin, nalidixic acid, penicillin, ampicillin, etc. although resistant isolates to penicillin, bacitracin, ampicillin, and chloramphenicol were detected. Blood and milk samples taken from those SCM+ cows further underwent for characterizing the pro-inflammatory cytokines IL-2, IL-6, and TNF-α, where both IL-2 and IL-6 revealed significant differences($P < 0.05$); and then justified with the SCC, differential leucocytes differentiations. SCM+ milk and blood samples revealed a significantly higher ($P < 0.05$) level of total leukocytes and a marked neutrophilia prevailed in SCM+ cows than those of healthy ones. However, there were no significant differences found in aspect of macrophages, eosinophils, and lymphocytes population. Blood and milk albumin, but not globulin, showed significant differences ($P < 0.05$) in SCM cow blood and milk compared to control cows. Therefore, our findings suggest that microbes related with SCM cases result in increase pattern of neutrophilic inflammation and subsequent induction of pro-inflammatory cytokines.

1. INTRODUCTION

Bovine subclinical mastitis is still a very common multi-factorial disease resulting in reduction of milk yield, quality along with imposing serious economic losses to the farmers, animal welfare, and the dairy industry (Halasa et al., 2007, Mekonnen et al., 2017). Due to silent nature, it is very difficult to diagnose subclinical mastitis in field level, and thus widening the provision for public health risk through the possible transmission of zoonotic bacteria, toxins, and also some antibiotic-resistant genes (Abebe et al., 2016, Ruegg, 2017). Nowadays, it is a big challenge to combat subclinical mastitis, and concerns stressing on its etiological microbes, basically gram negative and/ or positive bacteria, and their virulent toxin-producing genes, and resistant to different antibiotics (Botrel et al., 2010, Oliver and Murinda, 2012, Rozanska et al., 2019).

Due to animal and human wellbeing concerns, the microbial consequences, udder inflammations and immune responses to subclinical mastitis in dairy industries skewing logical concerns (Molenaar et al., 2009, Tanamati et al., 2019). Subclinical mastitis results with the induction of mammary gland inflammation and the primary defensive roles start with the trafficking of leukocytes in inflamed udders (Berg et al., 2011).Then the immediate innate immune response starts with the phagocytosis process directed by infiltrated neutrophils and macrophages and thus eliminate cellular aggregates (Paape et al., 2003). As a result of bacterial hyper populations in inflamed udders, the innate and adaptive cells become activated and produce different cytokines streams to orchestrate the subsequent inflammatory process and overwhelm the pathogenesis (Trigo et al., 2009). The milk protein levels, along with pro-inflammatory cytokines (IL-2, IL-6, TNF-α etc.) activation and induction (Bochniarz et al., 2017a, Berg et al., 2011) may results in the overwhelming development of acute-phase proteins (APP) those are key inducers for the development of acute form of mastitis, and subsequent economic loss (Nielsen et al., 2004, Eckersall et al., 2006, Sadek et al., 2017). Along with herd health management and economic impacts, there is a big chance to have zoonotic potential also, and so proper screening, treatments are the prerequisites for effective management of subclinical mastitis (Rollin et al., 2015, Abebe et al., 2016). The utmost problem in the treatment and combating subclinical mastitis is the advent of acquired antimicrobial resistance by pathogenic bacteria, and those should be monitored clearly for public health issues, recommended by OIE (Oliver and Murinda, 2012). Moreover, indiscriminate and haphazard use of antibiotics along with various epidemiological risk factors may overwhelm the changing patterns of resistance (Srinivasan et al., 2007, Ruegg, 2017, Botrel et al., 2010).The patterns of drug resistance continue to change in a particular area depending upon various epidemiological factors and indiscriminate use of antibiotics (Nielsen et al., 2004, Das et al., 2017, Su et al., 2016).

Given the above perspectives, we have characterized the bacterial pathogens harboring in the subclinical mastitic udder, and antibiogram status of those bacteria against available antibiotics used in veterinary practices. Besides, we also itemized the immune cellular trafficking to induce pro-inflammatory cytokines, and proteins on the way to the inflammatory mechanism and progression of subclinical mastitis in cross-bred cows.

2. MATERIALS AND METHODS

A total number of twenty six (26) subclinical mastitis (Eeckhaut et al.) suspected cows of Sylhet Government Dairy Farm (SGDF) having same management, nutrition, and external health conditions with 3rd-4th parity, underwent for sampling through very rapid field test (VRFTscm) following techniques documented elsewhere, with a simple modification by using 3% detergent available in market (Muhammad et al., 2010). During sampling the somatic cell count (SCC>2x10^6 cells/mL), and history of systemic antibiotics used last 30 days taken in account accordingly. The VRFTscm positive 15 milking SM+ cows were further verified with SCC, milk pH.

A representative *Staphylococcus aureus*, *Streptococcus spp.*, *Escherichia coli,* and *Corynebacterium spp*. colony were characterized morphologically based on cultural and biochemical traits (Al Azad et al., 2019, Hossain et al., 2012). The antibacterial susceptibility of *Staphylococci*, *streptococci*, *E. coli*, and *Corynebacterium spp*. isolates to different antibacterial agents were performed by disc diffusion method to determine the drug sensitivity pattern (Srinivasan et al., 2007, Hossain et al., 2011). The inhibition of the growth was demonstrated by a clear zone of growth inhibition around the dices due to the result of two processes viz (a) diffusion of the antibiotics and (b) growth of the bacteria. Sensitivity was expressed as 3+, 2+, 1+ and were expressing 'high', 'moderate', 'sensitive' and 'resistant' level of susceptibility, respectively (Oliver and Murinda, 2012).

Both blood and milk samples were collected aseptically as duplicate in Eppendorf tubes and were undergone for hematological studies, and separated plasma were used to detect total protein, albumin and globulin using Haematology –Sysmex XN2000, in Novo Diagnostic Centre, Bangladesh. Proinflammatory cytokines (IL-2, IL-6, TNF-α) were also measured from blood plasma following commercial ELISA Kit recommendations(eBioscience) following the method described earlier (Dervishi et al., 2017, Trigo et al., 2009). It is noted that all samples were compared with healthy (mock) samples taken from eleven (11) cows.

The statistical significance of ELISA was evaluated by the Mann–Whitney test or unpaired two-tailed Student's *t*-test. All data were analyzed using GraphPadPrism4 software (GraphPad Software, Inc., San Diego, CA, USA). The other data are expressed as the means ± SEs and were analyzed using Microsoft Excel. All data are expressed as Means ± SD for groups. **P*<0.05, ***P*<0.01 and ****P*<0.001.

3. RESULTS

A total of twenty six (26) milking cows having gradual decreasing milk yield history underwent for subclinical mastitis (SCM) test, and resulted with fifteen (15) cows as positive (57.68%) for subclinical mastitis upon milk VRFTscm, SCC (Fig. 1), and pH test.

3.1. Microbial characterization and antibiogram profile:

Milk SCC showed significantly increased pattern (*P < 0.001) in SCM +ve cows (7.5±2.14) than those of healthy control (1. 8± 1.17) (Fig. 1), and there was also a similar and intense relation of the bacterial population revealed on culturing. Upon various colony morphology and biochemical tests, we have recovered a total of 18 isolates, and out of them the predominant bacterial flora was characterized as seven (07) isolates of coagulase negative *Staphylococcus spp.*(38.39%), three (03) isolates of *Staphylococcus aureus* (16.67%), four (04) isolates of *Streptococcus spp.* (22.22%); three (03) isolates of *E. coli* (16.67%), and then one (01) isolate as *Corynebacterium spp.* (5.56%). (Fig.2). The antibiotic sensitivity test of *Staphylococcus aureus* found to be highly sensitive (+++) to chlortetracycline (80.0%) and nearly completely resistant to ampicillin (80%). On the contrary, *Streptococci spp* demonstrates higher sensitivity to chlortetracycline (66.66%) and highly resistant to bacitracin and chloramphenicol (both were 33.33%). Majority of the *E. coli* and *Corynebacterium spp*. isolates were highly sensitive (+++) to amoxicillin and chlortetracycline (50.0%) with the same value of resistant pattern.

3.2. Blood and milk leukocytes population:

In this study, SCM blood samples revealed a significantly higher (****p*<0.001) level of total leukocytes (Fig. 3a), and neutrophil population, compared to those of healthy ones. There were no differences in aspect of macrophage, eosinophils, and lymphocytes population (Fig. 3b,c). A similar pattern was also observed in aspect of neutrophil in SCM milk samples (****p*<0.001), though other eosinophils, and lymphocytes showing an increased shape in SCM cows in an insignificant manner (Fig. 4 a,b). However, healthy cow's milk samples exhibited more macrophages population, compared to SCM positive samples, in an insignificant manner.

3.3 Blood and milk cytokine stream:

Our results suggest that upon ELISA, both blood plasma (Fig. 5a-c) and milk samples (Fig. 6a-c) of SCM cows exposed with a higher level of pro-inflammatory cytokines (IL-2, IL-6, TNF-α) than those of control ones in a significant manner.

3.4. Serum protein levels:

In our study, we observed a significantly increased pattern of serum total proteins (***p*<0.005), than those of healthy control (Fig. 7a). However, though the albumin and globulin levels are higher in SCM serum, compared to control; but there were no significant differences found in aspect of albumin and globulin level (Fig. 7b,c).

4. DISCUSSIONS

Subclinical mastitis (SCM) is the utmost prevalent and the single leading costly disease that quivers worldwide dairy industry by reducing milk production, milk quality, and reproductive performance. However, subclinical infections do not trigger any visible alteration of udder but increase the Somatic Cell Count (SCC) in milk. Thus, SCC is a practical interpreter to monitor the level of existence of SCM in herds. The prevalence of SCM is higher in crossed breed dairy cattle compared to local zebu cattle in Bangladesh (Quaderi et al., 2013). However, the prevalence of SCM has been decreasing over recent decades (Hiitiö et al., 2017) due to the improvement of udder health management. In this study it was found that SCM prevalence in cross-breed dairy cows was 57.68% which was lower than the previous findings of (Quaderi et al., 2013) who reported 68% positive cases, but our findings are slightly higher than the findings of (Kabir et al., 2017b) who reported around 51% SCM cases at Sirajganj and Pabna districts in Bangladesh. Previously, in Bangladesh, prevalence of SCM in dairy herds was recorded around 70 to 60% (Badiuzzaman et al., 2015). Despite the condition that has been refining slowly, a wide range of bacterial involvement and the possibility of antibacterial resistance is a great concern.

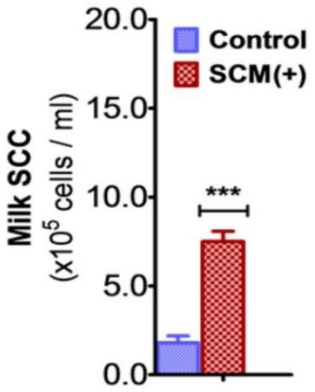

Fig. 1: Somatic cell count (SCC) is the most widely accepted indicator of subclinical mastitis in dairy cows (Fig. 1). Due to subclinical mastitis, the total milk SCC becomes increased as four times more, compared to healthy cows. Data represent the average ± SEM of the levels derived from the SCM +ve (n=15) and healthy dairy cows (n=9). ****p*<0.001 compared between the indicated groups.

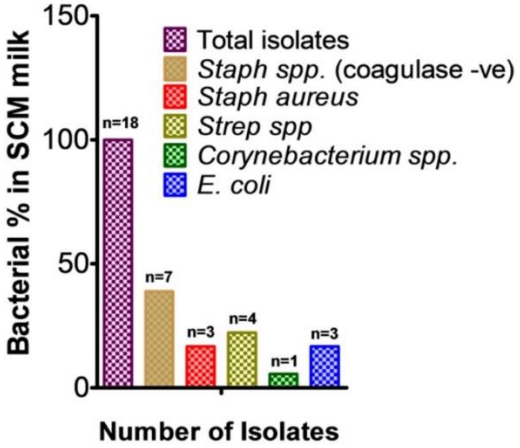

Fig. 2: Monitoring of bacterial burdens in SCM milk. The presence of different bacterial isolates was characterized on the basis of cultural and biochemical traits. The bacterial population was threshold in SCM +ve milk, and a total of 18 isolates were recovered (Fig. 2). The prevalence of isolates was characterized as coagulase negative *Staphylococcus spp.* (38.39%), *Streptococcus spp.* (22.22%), *Staphylococcus aureus* (16.67%), *E. coli* (16.67%), and as *Corynebacterium spp.* (5.56%).

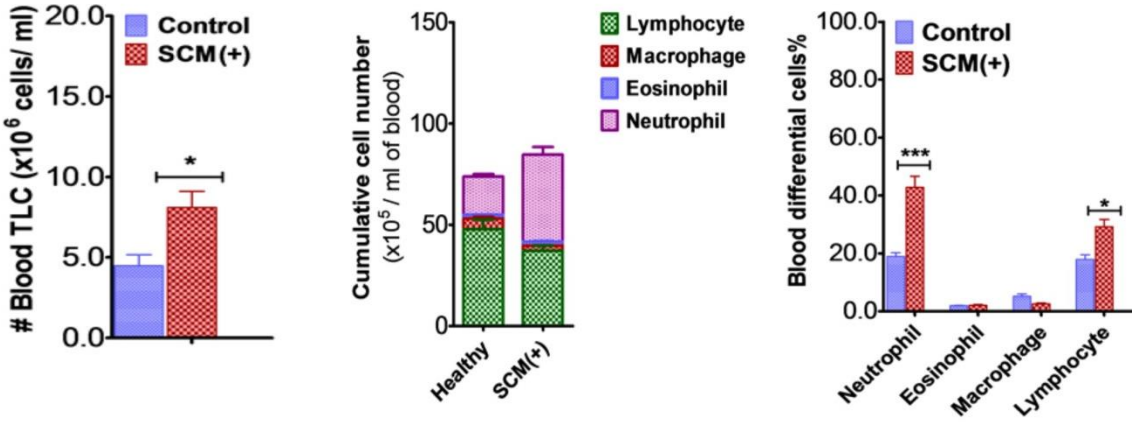

Fig.3: Presence of different leukocytes in blood: The total and differential leukocytes population was measured in blood collected from both healthy and SCM +ve cows (Fig. 3a-c). The cumulative cell number also found to be increased in SCM +ve blood samples (Fig. 3b). The SCM +ve blood revealed with a significant influx of neutrophils and lymphocytes, than those blood samples of healthy control (Fig. 3c). Data show the average ± SEM of the levels derived from SCM +ve (n=15) and healthy dairy cows (n=9). **p*<0.05, ***p*<0.01 and ****p*<0.001 compared between the indicated groups.

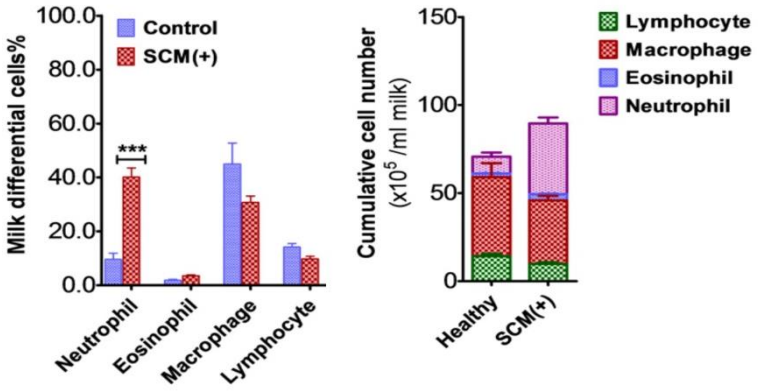

Fig. 4: Infiltration of different leukocytes in milk of SCM +ve cows. The different leukocytes population in SCM +ve milk samples revealed with significant infiltration of neutrophils (40.20±13.06)%, but other cells like eosinophils, macrophages and lymphocytes showing no significant differences (Fig. 4a). The cumulative population also reflecting the increased neutrophil and macrophage in SCM +ve milk samples (Fig. 4b). Data show the average ± SEM of the levels derived from SCM +ve (n=15) and healthy dairy cows (n=9). **p*<0.05compared between the indicated groups.

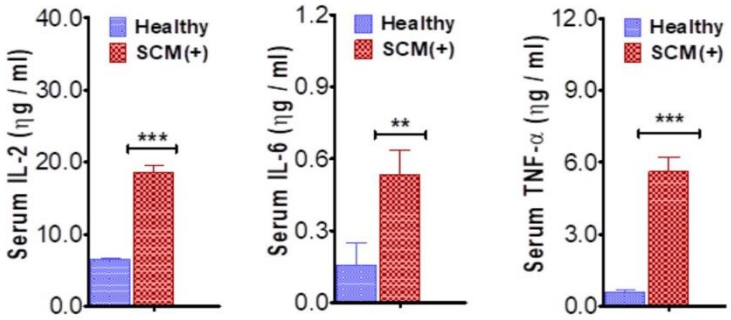

Fig. 5: Induction of pro-inflammatory cytokines in serum. Upon Sandwich ELISA, the blood serum samples taken from SCM +ve cows showed significant induction of IL-2 (Fig. 5a), IL-6 (Fig. 5b) and TNF-α (Fig. 5c), compared to samples from healthy cows. Data show the average ± SEM of the levels derived from SCM +ve (n=15) and healthy dairy cows (n=9). **p*<0.05, ***p*<0.01 and ****p*<0.001 compared between the indicated groups.

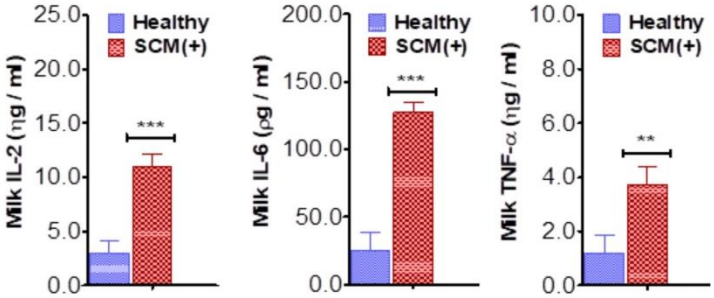

Fig. 6:SCM results with higher induction of pro-inflammatory cytokines in milk .Likewise the blood samples, the milk samples also revealed with significant production of IL-2 (Fig. 6a), IL-6 (Fig. 6b) and TNF- α (Fig.6c) in SCM +ve cows, than those of healthy control. Data show the average ± SEM of the levels derived from SCM +ve (n=15) and healthy dairy cows (n=11). **p*<0.05, ***p*<0.01 and ****p*<0.001 compared between the indicated groups.

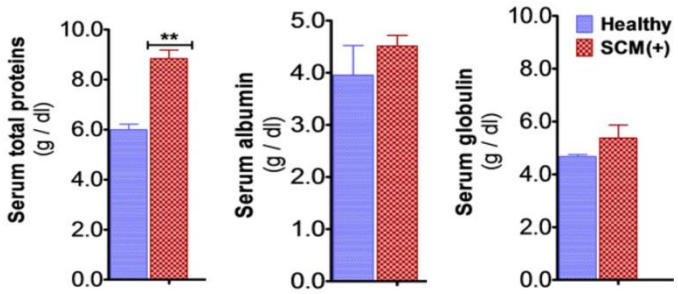

Fig.7: There is an increased (**p<0.01) level of total serum proteins in SCM +ve cows, compared to healthy ones (Fig. 7a). But, there was no differences found in aspect of albumin (Fig. 7b) and globulin (Fig. 7c) concentration in serum. Data show the average ± SEM of the levels derived from SCM +ve (n=15) and healthy dairy cows (n=11).

Staphylococci followed by *Streptococci* and *Escherichia* coli have been reported as the major etiological agents associated with SCM in dairy cattle (Padhy et al., 2015, Singh and Baxi, 1982, Hegde et al., 2013). In the present study, most of the mastitiscases (38.89%) were caused by *Staphylococcus* (coagulase negative), *Streptococci spp*. (22.22%), then both *Staphylococcus aureus* and*E. coli* (20%), and *Corynebacterium spp*. (5.56%). These findings are also more or less similar toothers(Shrestha and Bindari, 2012), who reported the highest prevalence of *Staphylococcus* followed by *E. coli*, *Streptococci* and *Corynebacterium*. Similar study in Bangladesh revealed that the cows were mostly infected with mixed infection by *Staphylococcus spp*., *Streptococcus spp*., *Escherichia coli, and Salmonella spp(Kabir et al., 2017a).*A recent study in Bangladesh reported 45.68% Staphylococcus sp., 14.81% *Streptococcus uberis* and 9.88% *Escherichia coli in SCM cases by using PCR which was nearly similar to our present findings (Kabir et al., 2017b).* Comparatively, low prevalence was observed in Rwanda where the prevalence of *Staphylococcus aureus was* 20.6%, *and Streptococcus* species was 10.3% in SCM affected cows (Mpatswenumugabo et al., 2017). In the current study, the high predominance of staphylococcal mastitis can be explained by lack of proper milking hygienic practices in the farms, augmented by nonuse of teat dips and inadequate routine mastitis screening tests; as a result environmental opportunistic pathogens get easy entrance to invade the udder from the skin and develop into an intramammary infection. Simultaneously, staphylococcal mastitis is highly contagious as the organisms can spread from infected to clean cows through hands or equipment from one udder to another (Bagley, 1997). *Several factors like* climatic condition, animal species, and disease management practices *may be influenced in the variation of* etiological agents of SCM from place to place and case to case.

The antibiotic sensitivity test was performed against penicillin, ampicillin, amoxicillin, chlortetracycline, chloramphenicol, bacitracin, and nalidixic acid. All of the isolated organisms showed variable sensitivity to chlortetracycline, bacitracin, chloramphenicol, amoxicillin, nalidixic acid, penicillin, and ampicillin in ranking order where, they exposed resistance to penicillin, bacitracin, ampicillin, and chloramphenicol. It was observed that *Staphylococcus aureus* (80.0%) and *Streptococci spp*. (66.66%) were highly sensitive (+++) to chlortetracycline, whereas *E. coli* (50.0%) and *Corynebacterium spp*. (50.0%) were highly sensitive (+++) to both amoxicillin and chlortetracycline. However, variation was observed in case of resistance pattern where *Staphylococcus aureus* was nearly complete resistant to ampicillin (80%), *Streptococci spp* to bacitracin and chloramphenicol (both were 33.33%), *E. coli* and *Corynebacterium spp to* amoxicillin and chlortetracycline (both were 50%). In a similar study, 90% penicillin and 70% ampicillin resistant pathogens were detected from SCM dairy cattle in Mexico (Mpatswenumugabo et al., 2017) which was more than our present finding. This may be due to their prophylactic use of antimicrobial (penicillins) into the udder at the end of period of lactation in dairy cattle for the prevention of future mastitis. This procedure might generate penicillin-resistant microorganisms in this region (Leon-Galvan et al., 2015). In Bangladesh, (Kabir et al., 2017a) reported that microorganisms isolated from SCM cows were sensitive to Amoxicillin, and were resistant to Penicillin. Resistant to antibiotics may result from the excessive use of certain antibiotics for the treatment

and prevention of mastitis by the farmers in Bangladesh and there is also some possibility of getting resistant genes by horizontal gene transfer as there is overuse or misuse of antimicrobial drugs in livestock production that induces selection pressure on bacteria to become resistant against those antibiotics (Hammerum and Heuer, 2009).

For any microbial infections, either in acute or chronic forms, blood leukocytes particularly neutrophil infiltrates in the infection sites along with characteristic immune activities (Trigo et al., 2009, Tanamati et al., 2019). After the onset of pathogen invasion to mammary glands, the resting macrophages, dendritic cells (DCs) and other leukocytes start to direct the immediate inflammatory response (Bochniarz et al., 2017a). Blood leucocytes mainly neutrophils and macrophages are considered one of the important biomarkers of SCM that may found in both blood and milk of affected animals. TLC rises during SCM because of the invasion of any pathogen inside the mammary gland which indicates altered physiology of the gland (Paape et al., 2003b).Besides, a high level of neutrophils in milk migrates from the blood to employ cascades of biochemical reactions to destroy the pathogens (Alhussien et al., 2015). In this study, a significantly higher ($P < 0.05$) level of TLC specifically neutrophils were detected in both blood and milk of SCM affected cows than those of healthy ones. Similarly, an increased level of milk neutrophils was reported by many reporters (Alhussien et al., 2015, Sarvesha et al., 2017, Hussain et al., 2013) in SCM cases. This high level of neutrophils is indicative of the innate immune response elicited by the pathogens in the mastitis affected mammary gland.

Cows having SCM is highly associated with higher SCC in milk, increased total protein and globulin concentrations in serum, and those are interlinked with the immune response of the mammary glands (Bobbo et al., 2017a). In such mammary gland infections, due to innate immune response the serum total proteins, particularly albumen and globulin become increased and involved in udder defense mechanisms (Jadhav et al., 2018). Blood serum proteins (i.e., total protein, albumin, and globulin) are other possible important indicators of SCM as the level of these proteins increase in response to any inflammatory reaction induced by pathogens (Piccinini et al., 2004). The availability of blood serum proteins in milk is due to the altered capillary permeability following the inflammatory condition. Pathogenic microbes when invading inside the teat cistern there is activation of the innate immune system. For initiation of inflammation, a series of pro-inflammatory cytokines such as IL-2, IL-6, TNF-α,etc. are secreted from recruited leucocytes in the affected area following synthesis of acute-phase proteins like α-globulins serum amyloid A and haptoglobin (Viguier et al., 2009). It has been reported that cows with high SCC in milk had greater total protein and globulin levels in blood serum suggesting the involvement of α- and γ-globulins in the immune response of the mammary gland (Bobbo et al., 2017b). However, in this present study total proteins and albumen in blood and milk were significantly differed (**p<0.01) in SCM compared to the healthy one; whereas there were no significant differences observed in the aspect of globulin level. The opposite findings were observed by others where there was a non-significant change in albumin and a significant rise inglobulin level in mastitis (Pandey et al., 2012, Zilaitis et al., 2006, Bobbo et al., 2017b). Environmental factors (e.g., herd productivity), breed, individual cow factors (stage of lactation and parity) and different etiological agents can induce differential immune responses leading to synthesize different pro-inflammatory cytokine (Oviedo-Boyso et al., 2007, Bobbo et al., 2017b) that may be responsible for the variation of blood and milk protein concentrations in our findings compared to others.

Cytokines are immune-modulatory natural proteins that promote host defense by conferring either specific or non-specific immunity. The potential role of pro-inflammatory cytokines is to stimulate the synthesis of acute-phase protein from the liver. Thus, the high concentration of these cytokines in blood and milk is considered as an early but nonspecific indicator of various inflammatory conditions as well as a possible marker for the detection of SCM (Bochniarz et al., 2017b). Several key pro-inflammatory cytokines and anti-inflammatory cytokines become key indicators in their relation with pathological disorders in cows having SCM (Sadek et al., 2017, Bochniarz et al., 2017a); and thus overwhelm the immunomodulatory signatures. In the current research, upon ELISA, both blood plasma and milk samples of SCM cows exposed significantly higher level of pro-inflammatory cytokines IL-2, and IL-6 ($P < 0.05$) than those of control ones which was similar to the findings of others (Hagiwara et al., 2001, Osman et al., 2010). Another reporter also revealed 20 times and 2.5 times more IL-

6 in milk and serum respectively in SCM cases (Bochniarz et al., 2017b). The high level of TNF-α indicate an influx of neutrophils in the infected mammary gland during SCM, and the level of TNF-α was significantly higher in our findings.

Hence, SCM is associated with the over trafficking of leukocytes, particularly the neutrophil influx, and the induction of pro-inflammatory cytokines (IL-2, IL-6, TNF-α) to mediate the pathogenesis and inflammatory process in the udder. Of note, hitherto both farmers and milk collection centers have completely neglected the testing of milk for subclinical mastitis for generating the quality milk at the production level. For better food safety points of view, farmers should screen the health status of cows as a routine work. Haphazard uses of antibiotics to treat SCM are also creating a big problem for the dairy industry and human health as well. In this regards, farmers should strictly follow the legislative rules to treat the cows using antibiotics. Further studies should be performed to identify the mechanistic pathways and signaling process of innate and adaptive immune balance to widen the window of research in combating SCM.

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