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Milk DLC in early lactating Sahiwal cows for identification of udder health

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Abstract
Mastitis, an inflammation of the udder predominantly caused by intramammary infection, poses significant economic and welfare challenges to the dairy industry. While somatic cell count (SCC) serves as a traditional indicator of udder health, its variability and inability to distinguish cell types limit its diagnostic utility. This study focuses on assessing the efficacy of milk differential leukocyte counts (DLC) in early lactating Sahiwal cows for identifying udder health status. Thirty-six Sahiwal cows in early lactation were categorized into healthy, subclinical mastitis, and clinical mastitis groups based on SCC and California mastitis test. Milk samples were analyzed for SCC and DLC, including neutrophils, macrophages, and lymphocytes. Results revealed a significant increase in neutrophil percentage and a decrease in macrophage percentage as udder health deteriorated. Conversely, lymphocyte percentage decreased, albeit not significantly, from healthy to subclinical mastitis. These findings underscore the importance of DLC in complementing SCC for a comprehensive assessment of udder health in early lactating Sahiwal cows. Implementation of DLC could enhance diagnostic accuracy and facilitate targeted interventions for mastitis management in dairy herds.

Keywords: Sub clinical mastitis, somatic cell count (SCC), differential leucoyte count (DLC), polymorphonuclear neutrophils (PMN), macrophage, lymphocyte

Introduction
Inflammation of the udder, known as mastitis, mostly caused by intramammary infection (IMI), is one of the expensive disease in milk production and related industry worldwide and approximately 70-80% of the financial losses are caused by its subclinical form [1-2]. Besides its substantial financial losses to the dairy industry, mastitis adversely affects the dairy cow welfare and is one of the prominent reason for the usage of antimicrobials in the adult cattle (e.g., approximately 70% of all antimicrobials are used for mastitis treatment; van Werven, 2014) [3] on dairy farms. Somatic cell count (SCC) which has been used for a long time as an indicator of udder health is considered as a vital parameter in the industry of dairy since it affects the price of milk and milk products. However, because SCC parameter is ephemerally and individually variable during lactation, it is not every time a clear indicator of a prospective infection. It has been previously demonstrated that the immune cells and especially the neutrophils, play vital role in the immunity of the mammary gland, for the effective defense against attacking pathogens and the resolution of infectious disease [4-5]. A commonly used cut-off margin to differentiate between cows likely to be infected and normal is at 200, 000 cells/mL of milk [6]. The somatic cell count depicts the immune cells in the milk, which are chiefly lymphocytes, polymorphonuclear neutrophils (PMN), and macrophages [7]. Furthermore, macrophages recognize infringing pathogens and initiate an immune reaction, by the rapid recruitment of PMNs [7-9]. The motive of PMN is to neutralize the invading pathogen and guard the mammary glands at the start of an acute inflammatory reaction [7, 9]. As the immune cells have various functions, their distributions in normal healthy and mastitic milk differ [10]. In milk samples collected from healthy udders, macrophages and lymphocytes dominate the total cells, while PMNs command the cell count in milk that is obtained from infected udder or milk with high SCC. The proportion of these leucocytes in the milk changes following infection.

Once the mammary glands are infected, an influx of PMN can be observed [11] triggered by the inhabitant cells (lymphocytes, macrophages, and epithelial cells) [12].

There is a consensus on the association between an increase in milk SCC with the change in the proportion of the inflammatory cells in the cell population, and it has been put forward that the amount of polymorphonuclear neutrophils (PMN) may be a more useful index in the evaluation of udder health than SCC [13-17]. In the healthy udder, the resident cell types are macrophages followed by lymphocytes, polymorphonuclear cells (PMNs), and the epithelial cells. It was proposed that the type of leucocytes, as well as their numbers and functions, differ among invading pathogens, stages of the infection and in individual cows. Therefore, differences in leucocyte populations and the change of recruitment and functioning are instrumental in the recovery of the udder from mastitic events [18]. The prominent reason why early lactating cows were selected is that, in early lactation, there is relatively more stress on the production of milk in animal, and thus possibility of infection of the mammary gland increase and thus mastitis. This study made an attempt to compare the different leucocyte populations in milk from mammary gland of healthy cow, sub clinically affected cow, and clinical mastitic cow in early lactating Sahiwal breed.

Materials and Methods

The present study was carried out at the Livestock Research Centre (LRC), of ICAR National Dairy Research Institute (NDRI), Karnal, Haryana-132001. This location comes under a semi-arid region where the climatic conditions are hot during summer (45 °C) and cold (4 °C) during the winter season. A total of 36 indigenous (Sahiwal) cows that were in early stage of lactation, i.e., up to 90 days of lactation were selected and subdivided into three groups *viz.*; healthy (n=12), subclinical (n=12), and clinical mastitis (n=12) according to their somatic cell count (SCC) and California mastitis test. The colostrum of these animals was not taken into account and only normal milk SCC and DLC was done. All the selected animals were in parity numbers of 1 to 6. All the cows were maintained under a loose housing management system. The housing space for the cows was specified as per "BIS" standards. Standard feeding practices were being followed. The Sahiwal cows were machine-milked and hand-milked twice daily i.e., in the morning (5:00 to 6:00 am) and evening (5:00 to 6:00 pm). For machine milking, the pulsators were adjusted to give a pulsation rate of around 50 pulsations per minute with a uniform vacuum level of 400 mm Hg. After washing the cows, each cow was stimulated for a let-down for one minute before connecting to the milking machine.

Chemicals and equipment used in this objective were CMT reagent, xylene and methylene blue dye, Olympus Simple microscope CH20i for DLC and for estimating milk composition- 1 x PBS, Lactoscan SCC kit x 4 (manufactured by Milkotronic Ltd.) consisting of Lactochip, Sofia green lyophilized dye and Lactoscan and Milk analyser type MCC WS.

SCC of milk samples collected were measured by two methods. Firstly, milk somatic cells were estimated by the machine Lactoscan milk SCC counter (Milkotronic Ltd. StaraZagora, Bulgaria). The Lactoscan milk SCC analyzer is based on the fluorescent microscope technique principle of counting cells. About 100 µl of fresh milk which was collected from the animal was diluted 1:2 with distilled water and mixed with Sofia Green lyophilized dye in a micro tube. Then 8 µl was pipetted onto the single lactochip. After this, the chip was loaded into the machine. The analysis of the sample was done between 10 seconds and 2 minutes and the duration was depending on the number of filmed fields. The camera of lactoscan SCC focuses automatically on the chip loaded and the dyed cells. The algorithm analysis of digital images captured determines the number of fluorescent cells and recognizes their concentration and size. The result obtained was displayed on the monitor. Secondly, milk smears were prepared on fresh grease free slides to double check the findings of SCC obtained from the Lactoscan milk SCC counter machine and also measure the milk differential leukocyte counts (DLC) by employing an inverted microscope as described by Dang *et al*. (2007) [19].

Differential cell counting procedure was performed to determine the existence of different cell types such as neutrophils, macrophages, and lymphocyte in the collected milk sample. For performing DLC, around 10 µl of fresh milk was spread on a 1 cm² (1 x1 cm) area of a degreased microscopic slide and was dried in a horizontal position. The films were air-dried and then duplicate smears were fixed with 96% ethyl alcohol (min) air-dried, defatted with xylene (12 min), and rinsed smoothly with 60% ethyl alcohol, air-dried. Subsequently dyed with Methylene blue solution for 2 min and then rinsed with water and air-dried. SCC of milk samples were also determined microscopically at 40 X zoom and differential cell counts of milk were carried out at 100 X zoom to know the presence of different cell types like lymphocytes, neutrophils, and macrophages in milk. Milk neutrophils are identified by their multi lobed nuclei with bridges, milk lymphocytes were seen as having a deeply stained nucleus, which may be seen in center and having a relatively lesser amount of cytoplasm. Largest leucocyte seen in the milk sample was macrophage. The differential leukocyte count is expressed in percentage of total cell counts.

$$\text{Count of that particular cell type (\%)} = \frac{\text{Count of that particular cell}}{\text{Total no. of cells in the sample}} \times 100$$

The differential leukocyte counts of healthy, Sub clinical mastitic and clinical mastitic animals were analysed and tabulated as mean with standard error. For the determination of significant differences in DLC of healthy, Sub clinical mastitic and clinical mastitic animals, one-way ANOVA test was done using IBM SPSS statistics 26 software.

Results and Discussion

This study finds that early lactating healthy Sahiwal cows having CMT scores 1-2 with no visible symptoms have a somatic cell count of up to 2x 10^5 cells/ml of milk. Cows with subclinical mastitis with a CMT score of 3 and with no visible symptoms had a SCC in a range of 2 x 10^5 to 5 x 10^5 cells/ml of milk. Clinical mastitis cows with a CMT score of more than 3 with symptoms like swelling had a somatic cell count of more than 5x 10^5 cells/ml of milk which is in accordance with IDF, 2013 and Damm *et al*., 2017 [6-7].

The mean milk neutrophil percentage in early lactating healthy Sahiwal cows was 17.25±0.99 percent. These values increased significantly ($p<0.05$) as the animal went from healthy to subclinical mastitis to clinical mastitis form. It was observed the mean neutrophil percentage in subclinical mastitis and clinical mastitis in early lactating Sahiwal cows were 32.67±1.52 and 79.08±0.59. In the case of milk

macrophages, their number decreased significantly ($p<0.05$) as udder condition deteriorated from healthy to subclinical to clinical mastitis in early lactating Sahiwal cows. In healthy, subclinical, and mastitis, the mean macrophage percentage was 64.58±1.19, 53.17±1.89, and 17.00±0.68 respectively.
Mean milk lymphocyte percentage in healthy, subclinical, and clinical mastitis cases in early lactating Sahiwal cows were 18.17±1.89, 14.17±1.27, and 3.91±0.65 respectively. Although the values decreased from healthy to subclinical mastitis to clinical mastitis but significant ($p<0.05$) decrease was observed only from healthy to subclinical mastitis.

Table 1: Mean DLC of healthy, Sub clinical mastitic and clinical mastitic animals

	Healthy	SCM	CM
Neutrophil	17.25±0.99^a	32.67±1.52^b	79.08±0.59^c
Macrophage	64.58±1.19^a	53.17±1.89^b	17.00±0.68^c
Lymphocyte	18.17±1.89^a	14.17±1.27^b	3.91±0.65^b

Differential leucocyte counts (DLC) were observed in the milk of Healthy, Subclinical and Clinical mastitis in early lactating Sahiwal cows. Values within a row with different superscript letters (a, b, c) differ significantly ($p<0.05$) between groups within the breed.
These changes in milk DLC were because of the inflammatory response, reflects the immunological response of the mammary glands against invading pathogens and other antigenic factors. The resident cell population of the healthy gland initiates the immune response following pathogen penetration through the teat canal. These cells initiate the inflammatory response and together with the newly recruited leucocytes are necessary to eliminate the invading bacteria.

Conclusion

In early lactating Sahiwal cows, it was found that as milk SCC increased, the mean milk neutrophil percentage also increased i.e., there was significant increase in neutrophil count in both subclinical and mastitis milk. Mean milk macrophage percentage decreased as milk SCC increased i.e., there is significant decrease in macrophage count in both subclinical and mastitis milk. The mean lymphocyte percentage decreased from the healthy to subclinical mastitis group of cows significantly and decreased further in clinical mastitis cases but not significantly. From this study, it was concluded that though milk SCC gives a picture of udder health in early lactating Sahiwal cows but it does not talk about the type of cells present in it. For that milk DLC can be done which gives a clear image of types of cells in healthy, subclinical, and clinical mastitis in early lactating Sahiwal cow.

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