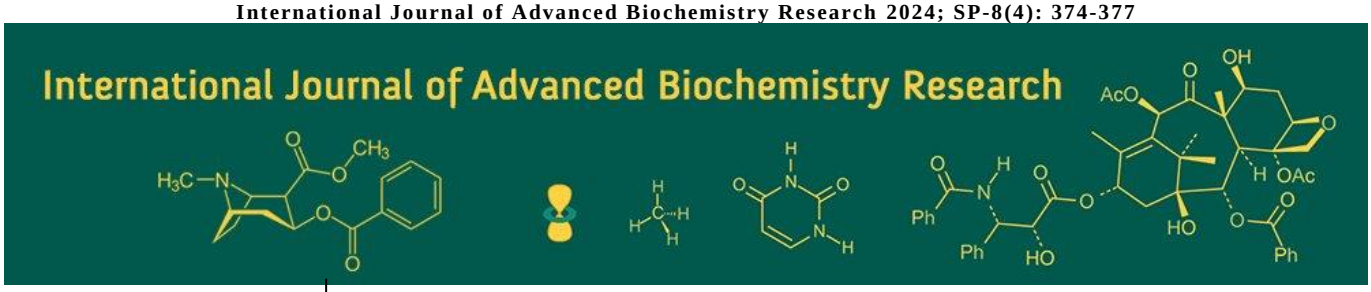

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Milk DLC in early lactating Karan Fries cows for identification of udder health

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Abstract
Monitoring udder health in dairy herds is crucial for maintaining milk quality and animal welfare. Somatic cell count (SCC) serves as a primary indicator of udder inflammation, but its reliability has diminished with decreasing global SCC averages. This study investigates the utility of milk differential leukocyte count (DLC) in early lactating Karan Fries cows for identifying udder health status. A total of 36 cows were categorized into healthy, subclinical mastitis (SCM), and clinical mastitis (CM) groups based on SCC and California Mastitis Test scores. Milk samples were collected and analyzed for SCC and DLC using both automated and microscopic methods. Results revealed a significant increase in neutrophil percentage and a decrease in macrophage percentage as udder health deteriorated from healthy to SCM to CM. Lymphocyte percentage decreased but not significantly across the groups. The study highlights the potential of DLC as a complementary tool to SCC for precise udder health assessment in early lactating Karan Fries cows.

Keywords: Milk differential leukocyte count (DLC), somatic cell count (SCC), Sub clinical mastitis, neutrophils, macrophages, lymphocytes

Introduction
The most straightforward, practical, and sustainable approach currently utilized for monitoring udder health in dairy herds is individual somatic cell count (SCC). Although not as precise as microbiological analysis [1], SCC serves to indicate inflammation rather than the presence of a specific pathogen. Generally a threshold value of 200,000 cells/ml is employed to detect subclinical mastitis under field conditions [2-5]. However, the global decrease in mean SCC across dairy herds has led to a reduced reliance on SCC for identifying diseased cows. There's a consensus regarding the correlation between increased SCC in milk and a shift in the proportion of inflammatory cells within the cell population. Consequently, the quantity of polymorphonuclear neutrophils (PMN) has been proposed as a potentially more informative indicator for evaluating udder health than SCC [6-10]. Indeed, alterations in milk composition have been observed even below the 100,000 cells/mL mark [11-14]. This body of evidence supports ongoing research with an aim of utilizing differential somatic cell count (DSCC) as a tool either in conjunction with SCC or independently to identify mastitis.
In healthy dairy cows, the milk cell population typically consists of over 97% leukocytes and less than 3% of mammary epithelial cells (MECs) [11, 15]. The presence of leukocytes in the milk of healthy cow udders is because they are able to traverse the blood-milk barrier (diapedesis) as a part of the natural immune survilleance to safeguard the mammary gland against infections. MECs are cast-off into the milk as a consequence of milk secretion process and also to carry out continuous renewal of the mammary gland epithelium. The milk secretion by the merocrine process in bovine aids in cell preservation, whereas the apocrine milk secretion process in human breasts is cell-destructive in nature and is responsible for comparative predominance of MECs in human milk [16]. Inflammation of the mammary gland i.e., mastitis can be characterized by the presence of leukocytes - primarily polymorphonuclear leukocytes (PMN) into the udder's lumen [11, 17, 18]. This migration of leukocytes causes the somatic cell count (SCC) to increase. Consequently, SCC is the internationally used predominant parameter for managing udder health in dairy herds. It's assessment can be done on-site and semi-quantitatively by visual inspection using California

Mastitis Test (CMT), or by accurate counting them using a flow cytometer. [19]

Materials and Methods

This particular study was carried out at the Livestock Research Centre (LRC), of ICAR National Dairy Research Centre (NDRI), Karnal-132001, Haryana, India. The location of study comes under semi-arid region. In total 36 early lactating crossbred Karan Fries (Holstein - Friesian x Tharparkar) cows were selected and subdivided into three groups *viz*.; healthy group (n=12), subclinical mastitis group (n=12), and clinical mastitis group (n=12) in accordance with their Somatic Cell count (SCC) and California Mastitis Test (CMT). Milk collection was done under hygienic conditions involving udder cleaning of all cows and screening them for their mammary health using CMT. 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 number of 1 to 6.

Loose housing system was adopted for maintenance of all the animals. The housing space for the cows was as specified under "BIS" standards. This type of housing system facilitates free movement and sufficient physical activity for the animals. Feeding standards were being followed at NDRI were based on Nutrient requirements of cattle and buffalo (ICAR, 2013) published by the Indian Council of Agricultural Research, New Delhi. During the postpartum period, cows were fed ad-lib available fodders and concentrates based on their milk production level.

All the Karan Fries cows were machined milked thrice daily i.e., in the morning batch (5:00 to 6:00 am), in the noon batch (12:00 to 1:00 pm) and evening batch (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 vaccum level of 400 mm Hg. After washing the cows, each cow was stimulated for let-down for one minute before connecting to the milking machine. Chemicals, equipment used in this study were CMT reagent, for DLC-Xylene and methylene blue dye and for estimating milk composition- 1 x PBS, Lactoscan SCC kit x 4 (manufactured by Milkotronic Ltd.) consisting of Lactochip, Sofia green lyophilized dye, Olympus Simple microscope CH20i and Lactoscan Milk analyser type MCC WS.

SCC of milk samples were measured by two methods. Firstly, milk somatic cells were estimated by the Lctoscan milk SCC counter (Milkotronic Ltd. StaraZagora, Bulgaria) . The Lactoscan milk SCC analyzer is based on the fluorescent microscope technique of counting cells. Around 100 µl of fresh milk was diluted with distilled water at the ratio of 1:2 and then mixed with Sofia Green lyophilized dye in a microtube and the 8 µl was pipetted onto the single lactochip. After that, the chip was loaded into the machine. The analysis was done between 10 seconds and 2 minutes and this duration varied based on the number of filmed fields loaded. This Lactoscan SCC system focuses automatically on the chip and the dyed cells are filmed by the sensitive CCD camera. The algorithm analysis of digital images determines the number of fluorescent cells and counts their concenteration and size. The result was automatically displayed on the monitor. Secondly, milk smears were prepared on fresh slides to crosscheck the results of SCC obtained from the Lactoscan milk SCC counter and also to estimate milk differential leukocyte counts by a microscopic method using an inverted microscope as described by Dang *et al*. (2007) [20].

Differential cell counting (DCC) was carried out to determine the presence of different cell types like neutrophils, lymphocytes, and macrophages in milk. For milk slide preparation around 10 µl of fresh milk was spread on a 1cm ^{2} (1 x1 cm) arear 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 rinse with water and air-dried. Milk SCC was corrected for the dilution done for final readings.

SCC of milk samples was also measured by microscope at 40X and DCC of milk were carried out and 100X to determine the presence of different cell types like lymphocytes, neutrophils, and macrophages in milk. Milk neutrophils had multilobed nuclei with bridges, milk lymphocytes were distinguished by having a depply staining nucleus, which may be eccentric in location, and having a relatively small amount of cytoplasm. Milk macrophages were identified as the largest cell type seen in milk. The differential leukocyte count is expressed in percentage of total cell counts.

$$\text{DLC of a particular cell type (\%)} = \frac{\text{no. of that particular cell}}{\text{Total no. of cells}} \times 100$$

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

Results and Discussion

This study finds that early lactating healthy KF 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 somatic cell count in a range of 2x 10^5 to 5x 10^5 cells/ml of milk while Clinical mastitis cows with a CMT score of more than 3 with symptoms like swelling, painful udder etc. had a somatic cell count of more than 5x 10^5 cells/ml of milk.

The mean milk neutrophil percentage in early lactating healthy Sahiwal cows was 21.83±1.00 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 sub-clinical mastitis and clinical mastitis in early lactating Sahiwal cows were 41.58±1.76 and 78.58±1.13.

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 64.83±1.05, 46.25±1.63, and 17.33±0.90 respectively.

Mean milk lymphocyte percentage in healthy, sub-clinical mastitis and clinical mastitis cases in early lactating Sahiwal cows were 13.33±0.67, 12.16±0.98, and 4.08±0.78

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.

Due to inflammatory response due to invading pathogens or other antigenic factors, the immunological responses of the mammary glands were observed which resulted in change in milk SCC and milk DLC. Due to these invading pathogens or antigens, a newly recruited leucocytes are necessary to eliminate the invading pathogens which in case of mastitis enter through the teat canal thus DLC in milk in mastitis and sub clinical mastitis animal varies significantly from healthy animal.

	Healthy	SCM	CM
NEUTROPHIL	21.83±1.00^{a}	41.58±1.76^{b}	78.58±1.13^{c}
MACROPHAGE	64.83±1.05^{a}	46.25±1.63^{b}	17.33±0.90^{c}
LYMPHOCYTE	13.33±0.67^{a}	12.16±0.98^{b}	4.08±0.78^{b}

Conclusion

It is concluded from this study that milk SCC increased in early lactating KF cows as the animals went from healthy to sub clinical mastitis to clinical mastitis. It was also found that 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. It was also concluded from this study that one cannot get a clear image of type of cells from milk SCC in case of early lactating KF cows. For that, milk DLC is done which gives a clear picture of type of cell in different milk samples from healthy, sub-clinical mastitis and clinical mastitis animal milk.

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