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Somatic cell alteration in healthy and mastitic milk of sheep and goats

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Abstract

The present study was conducted from December 2014 to April 2016 in three districts of Kashmir valley including Srinagar, Ganderbal and Budgam. 50 milk samples were collected from healthy and mastitic sheep and goats. California mastitis test was used to confirm the presence of mastitis in the animals. Direct microscopy was done to find the somatic cell count in both the species along with the differential leukocyte count. It was found that neutrophils increases in both sheep (74.08 ± 4.02) and goats (79.10 ± 3.75) during mastitis while as macrophages are predominant in healthy milk of sheep (54.60 ± 4.40) and neutrophils in healthy goats (64.10 ± 5.91) showing the species difference. Gram's positive bacteria were demonstrated in the milk smears. Cytochemical examination showed altered demonstrable activity of glycogen, alkaline phosphatase and myeloperoxidase. It was concluded mastitis in small ruminants increases the somatic cell count and alters the differential cell count affecting both quality and quantity of milk. Cytochemical demonstration of glycogen, alkaline phosphatase and myeloperoxidase shows the high activity of somatic cells during mastitis.

Keywords: Mastitis, California mastitis test, somatic cell count, differential cell count, cytochemistry

1. Introduction

The milk of all mammals contains different types of cells whose origin is in the body itself. In the decade of the 1960s, Paape first coined the concept of "somatic cells" (SC) to refer to these cells [6], which could be divided into two groups according to their origin: blood-borne SC and epithelial SC. Somatic cells are normally present in healthy milk, but in mastitis there is an increased influx of blood leucocytes [12] by chemo-taxis and diapedesis. Blood-borne SC includes macrophages, lymphocytes and particularly, polymorph nuclear (PMN) neutrophils [43]. Increased SCC in milk is considered as an indicator of inflammation of the udder [4]. In dairy goats, PMN neutrophils are the predominant cell type in uninfected glands (45-75%). The cell types present in the milk of healthy ewes are similar to those observed in milk from cows. In both cows and ewes, macrophages are the predominant cell type (45-88%) in healthy udders. There milk secretion in goats is apocrine, compared to merocrine secretion in cows; therefore, SCC for goats is naturally higher than SCC for cows. Epithelial cells in milk result from desquamation of the epithelium of alveoli and ducts of the mammary gland [25]. Besides the presence of SC, there are also extracellular membranous material, nuclear debris and cell fragments in the milk that correspond to large portions of cytoplasm originated from the distal alveolar mammary secretory cells [12]. High somatic cell counts affect the milk quality and milk production as well as milk composition. Due to high SCC, the quality of milk is altered as it causes deterioration in the flavour quality and the shelf life of the milk. These effects result from the breakdown of milk protein and fat and an increase in acid degree value [17].

The apocrine secretion is characterized by the detachment of the apical part of the epithelial cells from their base at the end of the secretory phase and their release into the alveolar lumen [29] resulting in the formation of Cytoplasmic particles. The goat milk shows very high Cytoplasmic particles when compared with other species like ewes and size-wise they are similar to milk Somatic cells [26, 44]. By contrast, the secretion of milk in the cow is of merocrine type without loss of epithelial cytoplasm [23]. These particles have spherical morphology with a size between 5 and 30 μm , and most (99%) lack nucleus [25], and are countered as Somatic cells when specific DNA methods are not used [18]. The objective of study was to see the quantitative and qualitative change in milk of sheep and goats.

2. Materials and methods

Milk samples from ewes and goats, 50 each, affected with mastitis were collected for qualitative and quantitative examination. Besides, milk samples from normal 50 each sheep and goats were collected for comparison of milk parameters under consideration.

2.1 California mastitis test.

California mastitis test (CMT) was conducted on milk samples. The skin of the udder was disinfected before collection of milk from Sheep and Goat. Milk samples after collection were tested according to the Schalm and Noorlander method [35]. Briefly, the CMT was performed by taking equal volumes of milk and the testing reagent, mixing it properly on a test plate. Formation of viscous gel was evaluated by rotating the plate gently. According to the changes of colour and intensity of gel, the results were interpreted as negative and positive (1+, 2+, and 3+) [36].

2.2 Milk somatic cell counts

Milk total and differential somatic cell count was carried out on the milk samples of 50 sheep and goats, each, which showed positive results for mastitis through California mastitis test. Total somatic count was calculated by direct microscopy method. The milk smears were stained by Newman Lampert's stain [36] and milk leukocytes were counted through microscope under oil emersion.

The smears were also stained with Giemsa stain solution for 30-40 minutes and different types of somatic cells were identified using oil immersion lens for both the species [16, 36].

2.3. Demonstration of bacteria and fungi in milk smear

Heat fixed milk smears were also stained for demonstration of Bacteria by Gram's staining Kit [47].

2.4. Demonstration of Cytoenzymes and cytochemistry in milk smear

Milk samples from affected mammary glands were subjected to following enzymatic tests:

2.4.1. Glycogen in neutrophils

Glycogen in neutrophils was demonstrated by the periodic acid Schiff's method [2], (modified). The milk smears were kept in a periodic acid solution for 20 min, in dark condition and washed in distilled water. After that, the smears were rinsed in 80% alcohol for 3 min. Slides were stained with Subici solution (0.5g of alkaline fuxine + 80ml, 96% alcohol + 20 ml of hydrochloric acid + 100 ml of water) and the smears were kept in this solution for 30 - 35 min. The milk smears were then rinsed in the following solutions: Sulphurous water (three times for 3 min each), distilled water (two times) and coloured with hematoxilin for 8 min. Finally, the milk smears were mounted for microscopy under oil immersion.

2.4.2. Alkaline phosphatase

Alkaline phosphatase activity was demonstrated by the Dorfman-Epstein's method [2] (modified). The milk smears were fixed for 24 hours with 80% alcohol. After fixation, the milk smears were rinsed with distilled water and incubated in a first solution (25 ml of sodium barbitone (2%) + 25ml of sodium beta-glycerophosphate (2%), 5 ml of calcium nitrate (1%), 2 ml of magnesium sulphate (1%) and 50 ml of distilled water) for 15 min. After the incubation, the milk smears were rinsed in the following solutions: distilled water - 3 min,

calcium nitrate solution (2%) - 2 min, iron sulphate (1%) - 5 min. The smears were then rinsed in distilled water thrice. The coloring of milk smears was done by using the second solution (4 g potassium iron cyanide + 80 ml of distilled water and 0.4 ml of hydrochloric acid) for 1 min and eosin (1%) for 5 min. Then the milk smears were mounted for microscopy for examination under oil emersion.

2.4.3. Myeloperoxidase

Myeloperoxidase within the leucocytes was demonstrated by Kaplow method [13]. Fresh smears of milk were made and fixed at room temperature in 10% formal-ethanol for 1-2 min. The smears were then gently washed with running water for 15-30 sec. The wet slides were then placed in incubation mixture (30% ethyl alcohol - 100 ml, benzidine- 0.3g, 0.132 M ZnSO₄.7H₂O- 1ml, sodium acetate- 1g, 1.0 N sodium hydroxide- 1.5ml and safranin O- 0.2 g) for 1-2 min. The smears were washed for 30 sec in running tap water, dried and examined under microscope under oil immersion.

Statistical analysis

The statistical analysis of the data was performed by using test statistics. Independent sample t-test for difference of two means and chi-square for nominal data. These tests were two sided and were referred for p-value for their significance. Any p-value less than 0.05 (i.e. $p < 0.05$) was taken as statistically significant, otherwise non-significant. The analysis of the data sets was done by using comprehensive statistical package for social sciences (SPSS ver 20.00, Chicago U.S.A for windows).

3. Results

Qualitative examination of milk in live mastitic animals

Fresh milk samples from the sheep and goat whether normal or affected with mastitis were brought to the laboratory (Fig.1). The mastitic milk of the live animals selected for comparison with the normal animals ranged from +1 to +3 score with California Mastitis Test (Fig. 2).

50 negative and 50 positive milk samples each from both the species (sheep and goat) after conducting California mastitis test were further subjected to other examinations.

3.1 Milk total and differential somatic cell count

The counting of somatic cells (Fig. 3) was done in both healthy and infected animals. The means±SD of total milk somatic cells and differential (neutrophil, macrophages and lymphocytes) (Fig. 4) counts for sheep and goat are presented in Tables 1, 2 and 3.

The total milk somatic cell count in either species (sheep and goat) was significantly ($p < 0.0001$) higher in mastitic than in healthy animals. In both the species, neutrophil population was significantly ($p < 0.0001$) higher in infected animals, while the macrophages were significantly ($p < 0.0001$) lower in mastitic animals. The values of lymphocytes were found to be significant in case of sheep and mastitic goats.

Table 1: Milk total somatic cell (Mean±SD, n=50) of infected and healthy sheep and goat

Species	Healthy animal	Infected animal	t-value	p-value
Sheep	2.41±0.52 × 10 ⁵	5.69±1.35 × 10 ⁵	7.136	0.001
Goat	6.08±0.87 × 10 ⁵	11.18±2.12 × 10 ⁵	7.03	0.001

Table 2: Differential score (Mean±SD, n=50) of infected and healthy sheep

Parameter	Healthy sheep	Infected sheep	t-value	p-value
Neutrophil%	33.90±5.24	74.08±4.02	19.58	0.001
Macrophage%	54.60±4.40	16.90±3.70	20.74	0.001
Lymphocyte%	11.50±1.78	8.30±1.16	4.76	0.001

Table 3: Differential score (Mean±SD, n=50) of infected and healthy goat

Parameter	Healthy goat	Infected goat	t-value	p-value
Neutrophil%	64.10±5.91	79.10±3.75	6.77	0.001
Macrophage%	24.30±4.78	12.90±2.56	6.64	0.001
Lymphocyte%	11.60±2.63	8.00±1.76	3.59	0.002

3.2 Demonstration of bacteria in the milk smear

Gram’s staining was done on the milk smears which revealed Gram positive bacteria in mastitic milk. Bacteria were found in pairs, chains and tetrads (Fig.5).

3.3. Cytochemistry

3.3.1. PAS for demonstration of glycogen

Glycogen granules were demonstrated as magenta colour in the milk cells (Fig. 6).

3.3.2. Alkaline phosphatase

Dorfman-Epstein method was applied on the milk smears, the presence and the place of enzymatic activity in the somatic cells were demonstrated by the blue colour occurrence within

the cell (Fig. 7). The enzyme was well demonstrable in all somatic cells of mastitic milk.

3.3.3. Myeloperoxidase

Milk smears stained for demonstration of the Myeloperoxidase activity of neutrophils revealed as blue colour (Fig. 8).

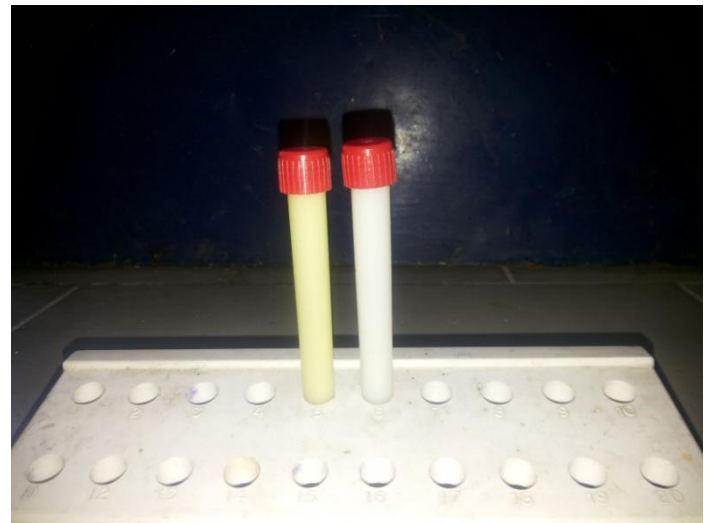


Fig 1: Milk sample from two halves of the mammary gland one affected with mastitis while the other half was normal.

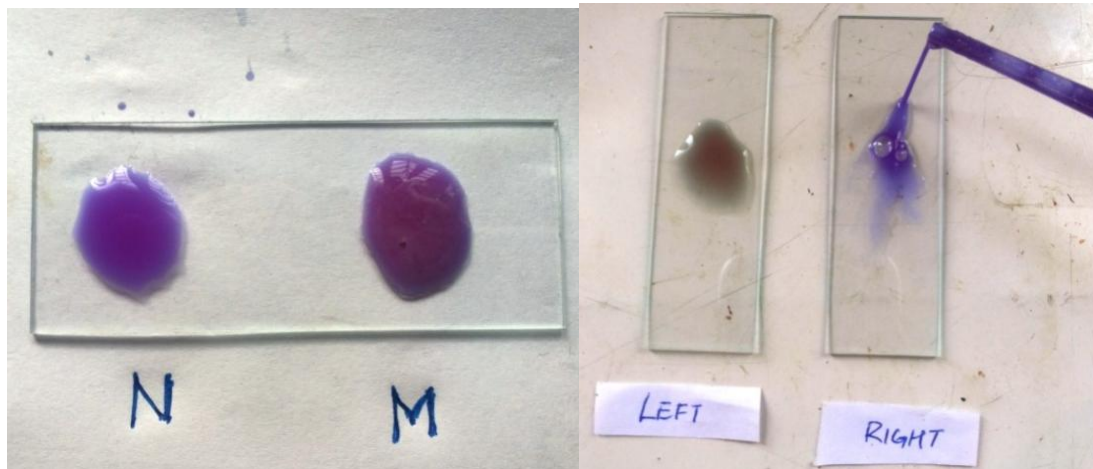


Fig 2: California mastitis test. Positive +++.

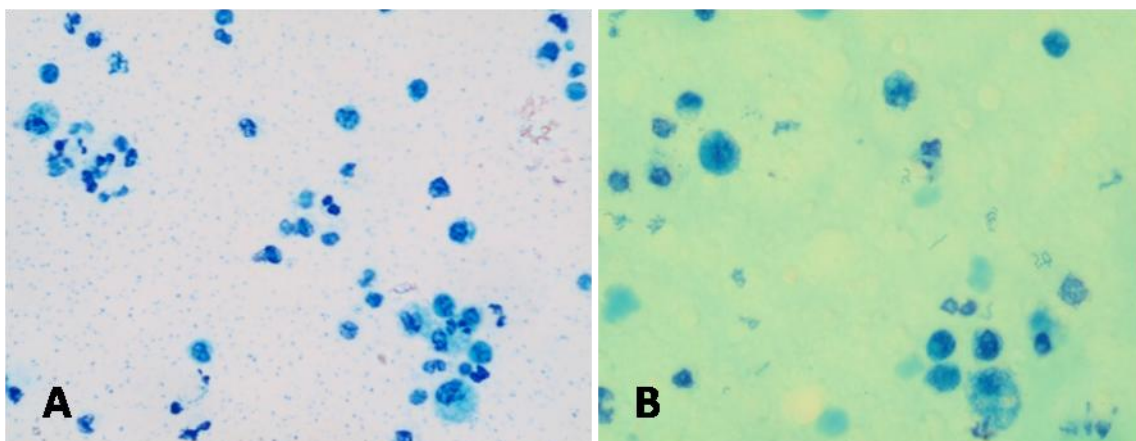


Fig 3: Direct microscopic method for counting of somatic cells - Newman Lampert’s stain. Original magnification- 1000X.

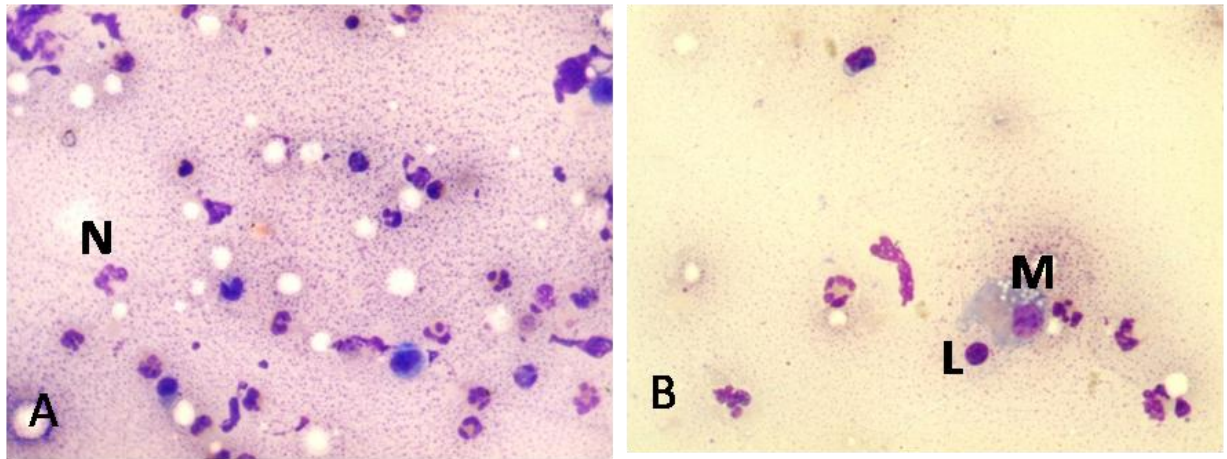


Fig 4: Differential cell count of milk showing neutrophils (N), macrophages (M) and lymphocytes (L). Original magnification-1000X.

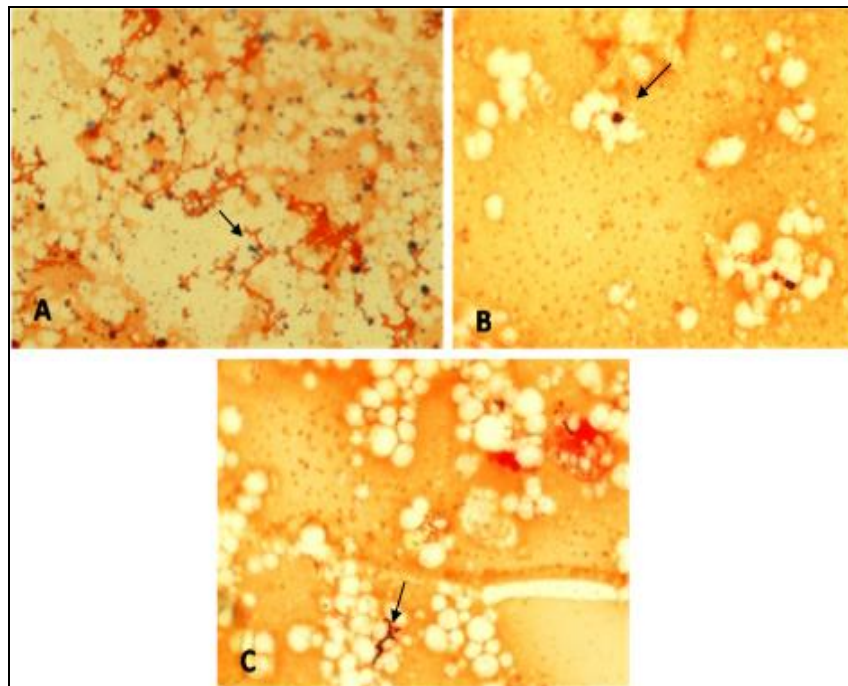


Fig 5: Gram's staining on the milk smear showing Gram positive bacteria in pairs (A), in tetrads (B) and chains (C). Original magnification-1000X.

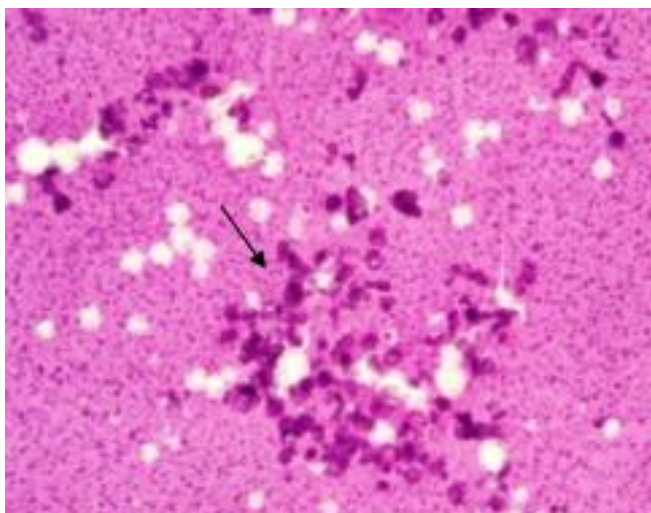


Fig 6: Milk smears showing glycogen granules as magenta colour in neutrophils. PAS stain. Original magnification- 1000X.

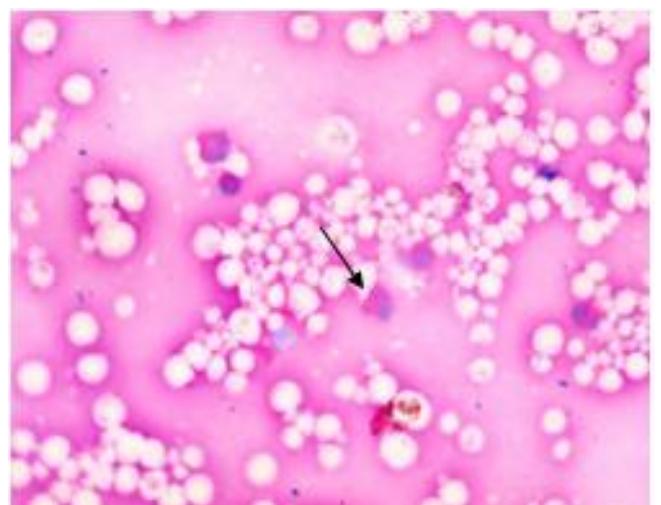


Fig 7: Neutrophils showing alkaline phosphatase activity in the azurophilic granules which is blue in colour. Original magnification-1000X.

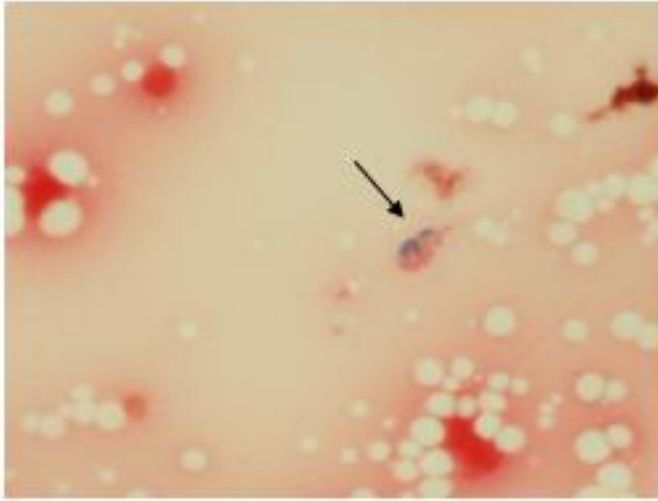


Fig 8: Neutrophils showing Myeloperoxidase activity in the azurophilic granules which is blue in colour. Original magnification-1000X.

4. Discussion

Milk somatic cell counts play vital role to measure the inflammatory response present in mammary glands. Milk somatic cell counts are usually used as diagnostic tool. In the present study mastitis was detected in field cases using California mastitis test being simple, cheap, quicker and reliable [5], however, it was also reported that CMT has low sensitivity as diagnostic method of mastitis in ewes [33]. The mean somatic cell count in healthy ewes was $2.41 \pm 0.52 \times 10^5$, while in does was $6.08 \pm 0.87 \times 10^5$, while in mastitic ewes and does these values were 5.69 ± 1.35 and $11.18 \pm 2.12 \times 10^5$, respectively. A studied was carried out on 274 milk samples from both udder halves of 137 local breed does and found SCC/ml in the range of 1.2×10^6 and 2.15×10^6 cell/ml [1]. Non-specific stains have detected the mean SCC in goats varying from 680 000 to 880 000 [22, 28, 30]. A report suggested that the SCC/ml in healthy goat should be less than 1000, in infection with weak pathogen it is 500,000 - 2,000 while over 1,500,000 in severe infection [39]. SCC of milking ewes can be used to define subclinical mastitis and a threshold of 200,000 to 400,000 cells/ml and above will accurately identify most infected ewes [5, 33, 34] showed that the SCC levels vary greatly, reaching a count up to 1.5×10^6 ml⁻¹, however, a count limit above 250,000 cells ml⁻¹ or below 500,000 cell.ml⁻¹ indicated mastitis in ewes.

There appears to be higher values of somatic cells in goats than sheep. In present study significant difference was observed in total milk somatic cell and differential count in sheep and goat. There are three characteristics that distinguish goat milk from sheep or cow: higher values of SCC, CP and PMN [26, 27]. Due to the apocrine secretion in goats, large numbers of cytoplasmic particles occur in normal goat's milk, hence, increases SCC in goats while as in case of sheep there is merocrine milk secretion, containing a relatively low number of epithelial cells, therefore, SCC is comparatively lower. Cell content in goat milk increases with the progression of lactation and this elevation is larger with infected mammary glands. The response to udder infections and advancing lactation with a rise in SCC is greater in goat milk than in cow milk [30]. Thus when predicting whether a goat is infected with mastitis by evaluating the number of somatic cells, physiological factors such as stage of lactation, parity, breed and state/area, have to be considered as important as the infectious status [19, 27, 45]. In the present study, the neutrophil percentage of healthy sheep and goat

was found as $33.90 \pm 5.24\%$ and $64.10 \pm 5.91\%$ while as in mastitic sheep and goat the percentage was $74.08 \pm 4.02\%$ and $79.10 \pm 3.75\%$, respectively. The percentage of macrophage and lymphocytes in healthy sheep was $54.60 \pm 4.40\%$ and $11.50 \pm 1.78\%$ while as in mastitic sheep it was found $16.90 \pm 3.70\%$ and $8.30 \pm 1.16\%$. Similarly, in healthy goats percentage of macrophage and lymphocytes was $24.30 \pm 4.78\%$ and 11.60 ± 2.63 while as in mastitic goats the percentage was $12.90 \pm 2.56\%$ and $8.00 \pm 1.76\%$, respectively. The results were found significant statistically. In healthy does, neutrophil population was higher (64.10 ± 5.91), while in healthy ewes macrophages (54.60 ± 4.40) were higher in number. Similar findings in normal milk of ewe and goat have been previously been reported [15, 24, 32]. The total somatic cell and neutrophil count in present study was significantly increased in infected sheep and goats indicating mammary gland infection. In goat milk secretion PMN are the main cellular component in both healthy and in infected glands representing 70% of the SC while as in case of healthy sheep and cow macrophages are highest in the milk [8, 41]. As inflammatory response of udder is directly related to neutrophil influx, it is, therefore, understandable that whenever the inflammatory process starts, the neutrophil number increases. Similar findings have been previously reported by many workers [7, 9, 15, 16, 20, 21, 32, 39, 46, 48]. A study reported that determination of neutrophil population in milk is useful indicator in the evaluation of mammary gland infection [48]. However, it was observed that lymphocyte, monocyte and macrophage population significantly decreased in milk of infected animals. Similar findings regarding differential cell count had been reported earlier [11, 14, 20, 38]. In infected does and ewes the predominant cells were neutrophil followed by macrophages and lymphocytes. Similar reports had been made earlier in mastitic animals [3, 38, 41]. As the neutrophil act as first line of defense in the body and are considered most active phagocyte, therefore, during udder infections these have been observed in higher number at the initial stages of infection.

Gram positive bacteria were seen in pairs, tetrad and chains in the milk smears from mastitic sheep and goats indicating that Gram positive bacteria were mainly responsible for causing mastitis in the animals which is also reported by other workers [10, 40].

Glycogen content in the milk neutrophils was found as magenta red stain. Glycogen is present in the cytoplasm of neutrophils and in the nucleus of all somatic milk cells. Cytoplasmic glycogen granules of the neutrophils represent the main source of energy for the phagocytosis. The optimal initiation and development of the phagocytosis depend on glycogen reserves. The demonstration of both was in context with the findings of Argherie [2].

Alkaline phosphatase activity was found increased in the neutrophils of mastitic milk samples and was visible as blue coloured material in the cells. Alkaline phosphatase is present in the azurophilic granules of the neutrophils and is bounded at the cell membrane. Since in mastitis the numbers of neutrophils in the milk increases, hence, the activity of alkaline phosphatase also increase. The enzymes majority is inactive or presents a diminished activity, when the milk is pasteurized. This present observation was in accordance with a study which reported that the quantity and enzymatic activity of the alkaline phosphatase was increased in the mastitic milk, therefore suggested that its measurement could constitute an indicator for identify an infectious process in mammary gland [2].

Myeloperoxidase was detected as blue coloured granules in the neutrophils. The release of myeloperoxidase is seen when phagosome containing bacteria fuses with the primary or azurophil granules of neutrophils ^[13].

5. Conclusion

It was concluded from the present study that Mastitic milk from both sheep and goat revealed increased numbers of somatic cells with preponderance of neutrophils. In normal milk of sheep macrophages are predominant while in goats neutrophils are predominant. The somatic cells of mastitic milk showed well demonstrable activity of alkaline phosphatase, myeloperoxidase and glycogen indicating highly active somatic cells. Control of mastitis in small ruminants will go in the long run to improve productivity and profitability from these animals.

6. Acknowledgement

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