

## Demonstration of Cytochemical Reaction of Alkaline Phosphatase, Myeloperoxidase and Glycogen in the Milk Polymorphonuclear Cells of Mastitic and Healthy Sheep and Goats

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### ABSTRACT

*Small ruminant mastitis is an important disease that affects the productivity of the livestock sector and affects both the quality and quantity of milk. The disease is not always life-threatening but it can cause a remarkable economic loss to the country as lambs and kids remain underfed due to the mastitis in their dams. Leucocytes are recruited from the peripheral blood into the milk, which is enhanced during mastitis and can be used as a diagnostic tool. The enzymatic activities associated with leucocytes in milk (somatic cells, polymorphonuclear cells-PMN cells) also alter during the disease. Therefore, the present study was conducted on the milk samples of mastitic and healthy sheep and goats for demonstration of cytochemical reaction of alkaline phosphatase, myeloperoxidase and glycogen in somatic cells of milk, which is not well documented in small ruminants. A total of fifty samples, each of mastitic and healthy milk of sheep and goats were collected and subjected to cytoenzymatic and cytochemical tests. Alkaline phosphatase was demonstrated as blue colour within the cells, and myeloperoxidase activity as blue coloured granules within the cells. A comparison of cytochemical reaction of myeloperoxidase activity of neutrophils of milk and blood was done and showed that the intensity and the number of neutrophils showing myeloperoxidase activity were less in milk than in blood. Glycogen within the somatic cells was demonstrated as magenta colour and the number of somatic cells was more in the mastitic milk sample of both the species. It was concluded that there is alteration of enzymes in context with increase in somatic cells in the mastitic milk in comparison with normal milk in small ruminants.*

**Key words:** Mastitis, Somatic cells, Cytochemistry, Alkaline phosphatase, Myeloperoxidase, Glycogen.

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## INTRODUCTION

Mastitis is the inflammation of mammary gland, which is characterized by a range of physical and chemical changes of the milk and pathological changes in the udder tissues<sup>12</sup>. The importance of mastitis in small ruminants is not an exception where clinical as well as subclinical mastitis directly affect the functionality of mammary gland<sup>6</sup>. The mastitis in small ruminants is important from three perspectives: economic (mortality of animals, treatment costs, reduced quantity and quality of milk); hygienic (the risk of infection or poisoning of consumers by consuming infected milk), and legal (definitions of bacteriological milk quality).

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<sup>3</sup>, 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<sup>4</sup> by chemo-taxis and diapedesis. Series of enzymes are associated with the somatic cells present in the milk whose alteration during mastitis is seen. To combat the infection, these cells phagocytose foreign particles or bacteria<sup>8</sup>, discharges their contents<sup>2</sup> and generates reactive oxygen metabolites<sup>7</sup>. The enzymes stored within the granules of the PMN cells are synthesized at different stages of myelopoietic differentiation<sup>11</sup>. PMN granules contain both antibacterial peptides and enzymes (elastase, collagenase, cathepsins, phosphatases, lysozymes, etc.) that are involved in the elimination of bacteria and are also able to disrupt the extra cellular matrix (ECM). During mastitis, PMN products are also able to modify milk proteins, thus altering the composition of milk<sup>14</sup>. Little is known about the enzymatic capacities of PMN recruited in the mammary gland during mastitis especially in small ruminants, hence, needs further consideration.

## MATERIALS AND METHODS

Fifty milk samples of healthy and mastitic sheep and goats were collected. Detection of mastitis was done by using the California mastitis test. Positive milk sample showed the presence of gel formation. Milk smears of the corresponding samples were made for cytochemical demonstration of alkaline phosphatase, myeloperoxidase activity and glycogen within the leucocytes of the milk.

### **Alkaline phosphatase by the Dorfman-Epstein's method<sup>1</sup>**

The milk smears were fixated with 80% alcohol, for 24 h. After the fixation period, the milk smears were rinsed for once 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 period, the milk smears were successively rinsed in the following solutions: distilled water - 3 min, calcium nitrate solution (2%) - 2 min, ferrous sulphate (1%) - 5 min. The smears were then washed with distilled water - 3 times. The coloring step was done by using the second solution (4 g potassium ferrous 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.

### **Myeloperoxidase activity assay<sup>9</sup>**

Fresh smears of milk were made and fixed at room temperature in 10% formal-ethanol (10 ml of 37% formaldehyde and 90 ml of absolute ethyl alcohol) for 1-2 min. The smears were then gently washed with running water for 15-30 s. Excess water was removed. The wet slides were then placed in incubation mixture (30% ethyl alcohol - 100 ml, benzidine- 0.3g, 0.132 M ZnSO<sub>4</sub>.7H<sub>2</sub>O- 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 s in running tap water, dried and examined under microscope under oil immersion. Same procedure was also applied on the blood smears of the same animal.

### Glycogen in neutrophils by the periodic acid Schiff's staining<sup>1</sup>

The milk smears were kept in a periodic acid bath for 20 min, in dark condition and washed in distilled water. After that, the smears were rinsed in 80% alcohol for 3 min. Staining was done 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 successively rinsed in the following bath solutions: Sulphurous water (three baths for 3 min each), distilled water (two baths) and coloured with hematoxylin for 8 min. Finally, the milk smears were mounted for microscopy under oil immersion.

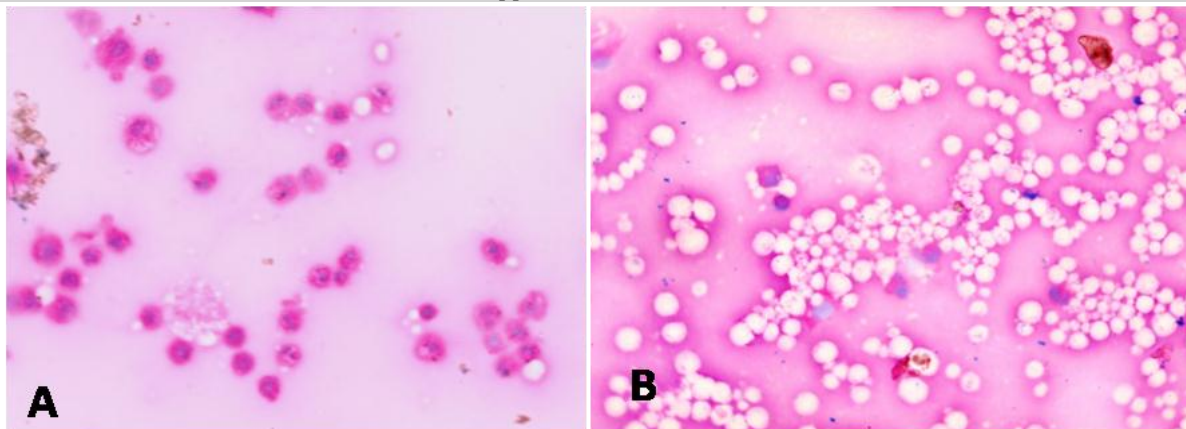
### RESULTS AND DISCUSSION

Alkaline phosphatase within the leucocytes of the milk of both mastitic and healthy sheep and goat as demonstrated by the Dorfman-Epstein method applied on the milk smears showed the presence of blue colour within the cells which indicated the occurrence of enzymatic activity in the somatic cells (Fig. 1). Since in mastitis the number of milk somatic cells increases, therefore, the quantity of alkaline phosphatase also increases. The alkaline phosphatase is an organic catalyser, which regularly appears in all fresh foods, including the raw milk. Alkaline phosphatase is present in the azurophilic granules of the neutrophils and is bounded at the cells' membrane. The majority of enzymes is inactive or presents a diminished activity, when the milk is pasteurized. The quantity and enzymatic activity of the alkaline phosphatase increases in the milk of mastitic cows too, hence, suggesting that its measurement can constitute an indicator for identify an infectious process in mammary gland<sup>1</sup>.

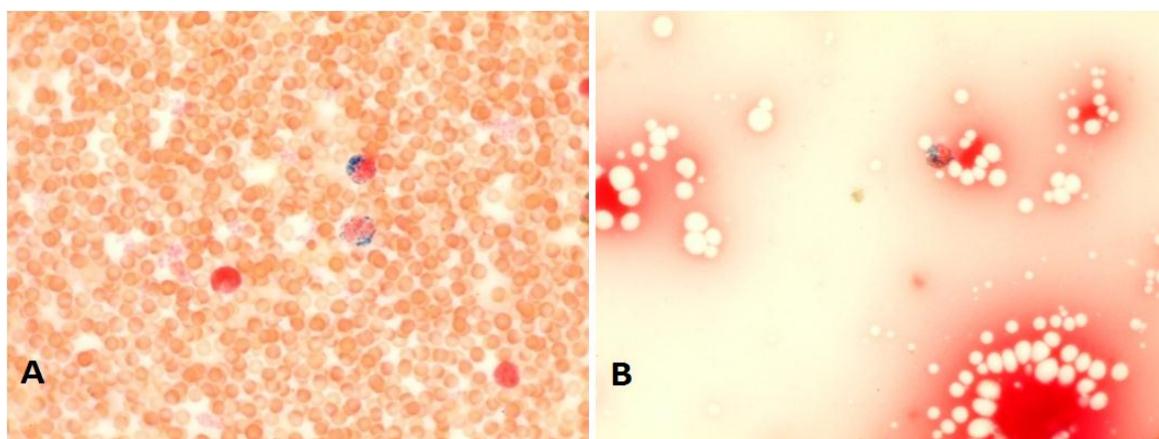
Cytochemical reaction of myeloperoxidase of neutrophils was seen as blue coloured granules within the cells (Fig. 2B). Neutrophils of milk showing the positive cytochemical reaction for myeloperoxidase was compared with blood. It was found that the intensity and the number of neutrophils

showing myeloperoxidase activity was less in milk than in blood (Fig. 2). The release of myeloperoxidase is seen when phagosome containing bacteria fuses with the primary or azurophil granules of neutrophils. This enzyme catalyzes the process of formation of hypochlorite, an oxidizing agent from chlorine and oxygenated water thus playing an important role in host defense against bacterial pathogens and protects against sepsis *in vivo* by producing halogenating species. PMNs that reach the mammary gland thus appear to have used up part of their enzymatic content during their migration in order to cross the endothelium, extra cellular matrix (ECM) and epithelium. Activation may then lead them to go on releasing enzymes locally in milk. The myeloperoxidase content decreases by 39% after diapedesis across the blood-milk barrier *in vivo*<sup>5</sup>. PMN enzymes can be released or expressed on the cells' surface to degrade ECM components (11). A study further demonstrated that diapedesis across the mammary epithelium reduces the phagocytic activity and oxidative burst of PMNs<sup>13</sup>.

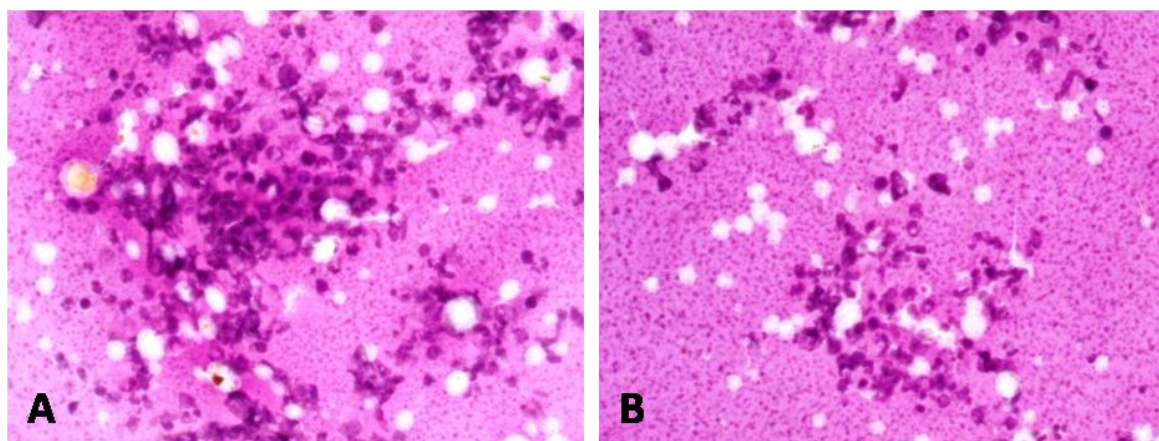
Glycogen granules were demonstrated as magenta colour in the somatic cells by Periodic acid-Schiff's method (Fig. 3). However, the number of the cells was more in mastitic milk than in normal milk. The cytoplasmic glycogen granules of the neutrophils represent the main source of energy for the phagocytosis. The initiation and development of the phagocytosis depend on glycogen reserves. Once complement components and immunoglobulins bind to receptors on the neutrophils' surface, the cells become activated and respiratory burst is initiated generating Hydroxyl radical and Singlet oxygen that kills bacteria. However, some researchers have also found decreased glycogen reserve in the cells of milk in comparison with blood. The poor phagocytic and oxidative burst activity of milk PMNs, compared to that of blood PMNs, was due partially to low energy stores within these cells<sup>10</sup>. There is also a loss of 38% of the initial glycogen content of PMNs after diapedesis into the mammary gland<sup>10</sup>.



**Fig. 1:** Dorfman-Epstein method on the milk smears, the presence and the place of alkaline phosphatase activity in the somatic cells is demonstrated by the occurrence of blue colour within the cell. A- Mastitic milk; B- Normal milk. Original magnification-1000X



**Fig. 2:** Myeloperoxidase activity of neutrophils in the azurophilic granules which are blue in colour, blood (A) and milk(B). Original magnification-1000X



**Fig. 3:** P.A.S. method application on the milk smears, glycogen granules as magenta colour in neutrophils. A. Mastitic milk; B. Normal milk. Original magnification- 1000X

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