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# MORPHOMETRIC AND PATHOLOGICAL STUDIES ON MAMMARY GLAND OF SLAUGHTERED NILI-RAVI BUFFALOES

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In the present study morphometric, quantitative and histopathological effects associated with mastitis were recorded in mammary glands of naturally infected buffaloes. The milk samples of 200 buffaloes were subjected to California Mastitis Test to detect mastitis at Faisalabad abattoir. Mastitis was significantly higher in buffaloes having cylindrical and round teats, bowl and round udder shape ($P<0.0004$). Teat diameter was significantly higher while teat and streak canal length was significantly lower in mastitic buffaloes. The total milk leukocytes and neutrophil ($P<0.0001$) was significantly higher, while lymphocytes and macrophages population was significantly ($P<0.0001$) lower in mastitic buffaloes. Alveolar diameter (short and long), number of alveoli and alveolar cell population were significantly ($P<0.0001$) decreased in mastitic buffaloes. Tissue sections from infected animals indicated marked leukocyte infiltration, atrophy of alveoli, cellular exudates, connective tissues proliferation and abscesses formation. Histochemically, tissue sections from infected udder showed low alkaline phosphatase activity and density of protein staining. The results revealed that some udder traits play significant role to prevent mastitis and mammary function is severely decreased due to altered cellular activity in infected glands.
**Keywords:** Buffaloes, mastitis, somatic cell counts, histopathology, histochemistry

## INTRODUCTION

Black gold, the buffalo (*Bubalus bubalis*) is the world's second most important dairy animal in Pakistan. Pakistan is the fourth largest milk producing country in the world with annual milk production of 46.66 million tones (Ali *et al*., 2012). Major share of milk production is contributed by the buffalo, which is predominant dairy specie in Pakistan. The total milk produced in the country is contributed by 28.69 million heads of buffalo (Hussain *et al*., 2012a). There are so many diseases which decrease the production of milk in Pakistan; among them mastitis is one of the major problem hindering the development of dairy sector (Hussain *et al*., 2012b). Mastitis is a complex and multifactor disorder which increases risks of premature culling of animals and requires understanding of exact mechanism of its pathogenesis (Parker *et al.,* 2007; Karahan *et al.,* 2011; Islam *et al.,* 2012). Previously different studies have indicated the potential effects of teat length, teat apex diameter, teat-end size and morphology and pendulous udder on risks of developing clinical mastitis (Seykora and McDaniel, 1985a; Slettbakk *et al.,* 1995).These traits play important role for farmers at the time of selection of animals for milking purpose and lower the incidence of mastitis (Seykora and McDaniel, 1985a).

The different forms of mastitis occur according to the invasion and multiplication of micro-organisms and host response (Costa, 1998). The movement of neutrophil and macrophages during inflammatory process triggered by different pathogens is crucial against intra-mammary. These factors determine the cruelty of the symptoms that can vary from increased milk somatic cells counts with no macroscopic alterations in milk to severe toxaemia to establish fibrosis (Fragkou *et al.,* 2007; Abera *et al.,* 2010). During inflammatory reaction, the inflammatory cells and damaged epithelial cells of udder release various products those include hydrolytic enzymes; non-lysosomal such as lactate dehydrogenase or *β*-galactosidase lysosomal enzyme (Oliszewski *et al.,* 2002). Free radicals which are produced during phagocytosis at the time of mastitis result in damage to mammary epithelial cells and thus decrease milk production (Barbano *et al.,* 2006). Mammary gland is a compound organ, both in configuration and occupation. Milk production and protein synthesis by mammary secretary epithelial cells is a complex process under the influence and controlled of several systemic and multiple local steroid hormones (Silanikove *et al.,* 2006; Elsayed *et al.,* 2009; Hussain *et al.,* 2012a). The histopathological findings in mammary tissues due to indirect action of the inflammatory process or due to multiplication of pathogens and studies on different teats/udder lesions are mainly experimental (Kennedy and Muller, 1993). The aim of the present study was to investigate the changes in secretary mammary cells and in the mammary gland of naturally infected buffaloes.

## MATERIAL AND METHODS

***Udder biometry and milk somatic cells*:** After gross examination (inspection and palpation) milk samples from

200 buffaloes of different ages (age was determined through dentition) were collected and tested using California Mastitis test (Schalm *et al.,* 1971; Nazifi *et al*., 2011). Briefly, the CMT was performed by taking equal volumes of milk and the testing reagent, mixing it properly on a test plate with four quarter wells. Formation of viscous gel was evaluated by rotating the plate gently. According to the changes of color and intensity of gel, the results were interpreted as negative and positive. The udder of all the buffaloes was washed, dried and disinfected with 70% alcohol. The milk samples were collected aseptically in sterilized plastic bottles for milk total somatic cell and differential leukocyte count (Gargouri *et al.,* 2008). The teat-end shape, teat/udder lesions and udder morphology was recorded (Bhutto *et al.,* 2010; Hussain *et al.,* 2012b). Teat diameter, teat and streak canal length of all the quarters of slaughtered animals were carried out with the help of vernier caliper (Shukla *et al.,* 1997; Hussain *et al.,* 2012c).

***Histological and histochemical investigations*:** Fragments from parenchyma of teat and udder approximately 2-3 $cm^3$ were collected from 100 each California Mastitis Test positive and negative animals slaughtered due to low milk production and preserved in 10% neutral buffered formalin for histological and histochemical study (Mayer and Klein, 1961). Briefly, tissue samples were fixed in Bouin's solution for 12 h, then in 70% ethanol and processed for histopathology. About 4-5 µm thick sections were cut with microtome, processed through paraffin embedding technique and stained with hematoxylin and eosin (Bancroft and Gamble, 2008). The alveolar diameter was determined using computer assisted software (Hussain *et al.,* 2012c).

Alkaline phosphatase activity, density of protein staining and frequency of alveolar cell population was determined by the modified techniques (Elsayed *et al.,* 2009). Prior to histochemical investigations for alkaline phosphatase, protein expression and mammary alveolar cell population, 4-5µm thick sections were cut and processed. After deparafinization for 10 min in xylene the sections were cleared in different grades of ethanol (100, 90, 70 and 50%) for 3–4 min in each grade and washed in running tap water for 2 to 3 min (Elsayed *et al.,* 2009). Thereafter, these sections were subjected to detect enzyme activity, protein expression and cellular frequency with some specific steps for each activity as described below.

For activity of alkaline phosphatase after deparaffinization, clearing and washing the tissue sections were fixed in formol methanol for 45 second at 4°C and again washed in tap water for 2-3 min. These sections were dried at room temperature and then incubated at room temperature in a mixture of substrate Tris buffer+Naphthol AS phosphate for 15 minutes. Then the sections were washed for 2 min in running tap water and kept to dry. Finally, the sections were stained with safranin for 50 min, dehydrated and mounted in DPX. The activity of alkaline phosphatase in these sections was observed with the help of light microscope.

For protein expression after above mentioned steps the sections were stained in mercury–bromophenol blue solution for 2 h. The differentiation was done for 5 min in 0.5% acetic acid, transferred to tertiary butyl alcohol for 5 min and then cleared in xylene for 5 min. Finally the sections were mounted with DPX and examined under light microscope.

After deparaffinization, clearing and washing the sections were then immersed in 1-N HCl for 2 min at room temperature, followed by 10 min at 60°C, returned back to 1-N HCl solution for 2 min at room temperature and then these sections were directly transferred to Schiff's reagent for 90 min, followed by thrice time washing in 0.5% sodium metabisulphite and distilled water for 2 min. Finally, sections were dehydrated in each grade of ethyl alcohol mentioned above, cleared in xylene for 2 min and mounted in DPX. Cell population /DNA frequency was examined using light microscope.

***Statistical analysis*:** The data collected in present study was analysed by SAS 9.1 statistical software (SAS, 2004) using Duncan multiple test, Chi- square and general linear model (GLM) procedures.

## RESULTS

The results of bivariate frequency analysis for udder shape, udder configuration, teat/udder lesions and teat shape are presented in Table 1. The frequency analysis revealed that udder configuration and teat/udder lesions were not significantly different in infected (n=100) and healthy (n=100) buffaloes. However, there was a significant association for udder shape ($P<0.0004$) and teat shape ($P<0.0006$). The mastitis was significantly increased in buffaloes having round and bowl shape udder and cylindrical, round and flat teat compared to cup shape udder and pointed teat. The results of analysis of variance for teat diameter, teat length and streak canal length in infected and healthy buffaloes are presented in Table 2. The results revealed that these parameters have a significant association with mastitis. The mastitis was significantly increased in buffaloes having large teat diameter (Teat apex, mid teat and teat base) of various ages which was recorded in aged buffaloes, while small teat ($P<0.001$) and streak canal length ($P<0.001$).

***Histopathology*:** In present study significantly increased total milk leukocyte count and differential leukocyte count in milk samples collected from infected buffaloes were recorded (Table 3). The total milk leukocyte count ($P<0.0001$) and neutrophil number was significantly increased in mastitic buffaloes. However, the percentage of lymphocytes and macrophages was significantly ($P<0.0001$) decreased in mastitic buffaloes.

**Table 1. Bivariate frequency analysis of different parameters in mastitic and healthy buffaloes slaughtered at abattoir**

| Parameters | Positive | | Negative | Mental-Haenszel Chi |
|---|---|---|---|---|
| | n | % | | |
| **Udder shape** | | | | |
| Cup | 30 | 32.61 | 62 | <0.0004 |
| Round | 33 | 63.46 | 19 | |
| Bowl | 37 | 58.70 | 19 | |
| **Udder configuration** | | | | |
| Non pendulous | 48 | 43.24 | 63 | >0.1165 |
| Pendulous | 52 | 58.42 | 37 | |
| **Teat shape** | | | | |
| Pointed | 18 | 25.71 | 52 | <0.0006 |
| Cylindrical | 40 | 64.52 | 22 | |
| Round | 27 | 69.23 | 12 | |
| Flat | 15 | 51.72 | 14 | |
| **Teat/udder lesions** | | | | |
| None | 30 | 38.46 | 48 | >0.2503 |
| Teat apex | 16 | 69.57 | 7 | |
| Skin abrasion | 12 | 60.00 | 8 | |
| Inflammation | 15 | 65.22 | 8 | |
| Cord formation | 6 | 33.33 | 12 | |
| Hemorrhages | 9 | 60.00 | 6 | |
| Necrosis | 9 | 50.00 | 9 | |
| Udder edema | 3 | 60.00 | 2 | |

**Table 2. Measurements (Mean ± SD) of various parts of quarters collected from healthy and mastitic buffaloes**

| Parameter/Quarter | Healthy (n=100) | Mastitic (n=100) | P -Value |
|---|---|---|---|
| **Teat Length (cm)** | | | |
| Right Rear | 6.31±0.42 | 5.70 ±0.47 | <0.001 |
| Left Rear | 6.51± 0.31 | 5.72 ±0.52 | <0.001 |
| Right Front | 5.89± 0.45 | 5.55± 0.34 | < 0.050 |
| Left Front | 5.84±0.21 | 5.74± 0.48 | <0.001 |
| **Teat Apex Diameter (cm)** | | | |
| Right Rear | 0.77± 0.13 | 0.94± 0.15 | <0.001 |
| Left Rear | 0.74±0.11 | 0.91±0.12 | <0.050 |
| Right Front | 0.77 ±0.08 | 0.90± 0.11 | <0.050 |
| Left Front | 0.71±0.09 | 0.96±0.13 | <0.001 |
| **Mid Teat Diameter (cm)** | | | |
| Right Rear | 2.36 ±0.22 | 2.45±0.06 | <0.001 |
| Left Rear | 2.40± 0.18 | 2.40±0.07 | <0.005 |
| Right Front | 2.41±0.18 | 2.51± 0.06 | <0.005 |
| Left Front | 2.41±0.21 | 2.47±0.06 | <0.010 |
| **Teat Base Diameter (cm)** | | | |
| Right Rear | 3.35±0.32 | 3.48±0.09 | <0.001 |
| Left Rear | 3.39±0.22 | 3.53±0.28 | <0.001 |
| Right Front | 3.41±0.27 | 3.45±0.08 | <0.010 |
| Left Front | 3.36±0.27 | 3.47±0.08 | <0.061 |
| **Teat canal length (cm)** | | | |
| Right Rear | 5.07 ±0.68 | 4.77±0.46 | <0.001 |
| Left Rear | 5.21±0.09 | 4.79±0.37 | <0.001 |
| Right Front | 5.11±0.08 | 4.91±0.39 | <0.001 |
| Left Front | 5.01±0.14 | 4.85±0.31 | <0.001 |

**Table 3. Histopathological results of various parameters in slaughtered dairy buffaloes**

| Parameter | Healthy (n=100) | Mastitic (n=100) | P-Value |
|---|---|---|---|
| **Milk leukocyte counts** | | | |
| Total milk somatic cell count ($x10^5$) | 3.95±0.26 | 90.34±7.44 | <0.0001 |
| Neutrophil (%) | 21.62±1.77 | 61.06±1.88 | <0.0001 |
| Macrophages (%) | 25.68±2.43 | 11.70±1.27 | <0.0001 |
| Lymphocyte (%) | 32.28±2.86 | 17.44±2.11 | <0.0001 |
| **Alveolar diameter (µm)** | | | |
| Long diameter | 111.65±9.08 | 77.92±13.64 | <0.0001 |
| Short diameter | 67.32±12.11 | 49.38±6.13 | <0.0001 |
| Minimum long diameter | 71.38±12.14 | 55.23±5.15 | <0.0001 |
| Maximum long diameter | 119.57±13.11 | 89.14±17.02 | <0.0001 |
| Minimum short diameter | 59.22±12.71 | 37.34±6.63 | <0.0001 |
| Maximum short diameter | 84.33±14.72 | 54.66±16.13 | <0.0001 |
| No. of alveoli/plate | 76.071±4.19 | 53.64±4.98 | <0.0001 |
| Alveolar cell number | 38.21±4.99 | 9.92±1.89 | <0.0001 |

Microscopical examination of tissue sections from teat exhibited severe histological changes in cistern and lamina propria. In most of the teat, severe cellular infiltration and presence of lymphoid nodules were observed beneath the epithelium of teat duct. Nodular proliferation of the mucosa was also present at teat cistern. Histologically, sections from mastitic udder revealed chronic inflammation. Marked Infiltration of inflammatory cells mainly neutrophil and macrophage in milk alveoli was observed. In present study cellular exudates, abscesses formation (Fig.1a), acinar atrophy, inter-alveolar fibrosis and degenerated acinar epithelium were the consisting findings (Fig. 1b). The histo-morphometric analysis revealed significantly less number and size of acini (Table 3) and lower alveolar secretary epithelial cell population in infected mammary parenchymal tissues.

***Histochemistry*:** The activity of alkaline phosphatase in tissue sections of healthy buffaloes was apparent on the outer boundary of alveolar secretory cells indicating the high activity of mammary gland (Fig. 2a). However, in tissue sections from mastitic buffaloes few alveoli indicated weak activity of alkaline phosphatase while most of the alveoli showed disappearance of this enzyme (Fig. 2b) reflecting mild activity. Similarly the density of protein staining was observed in thick-walled alveoli of the mammary tissues of healthy buffaloes (Fig. 3a). The tissue sections of the mastitic buffaloes showed weak to no protein staining (Fig.3b).

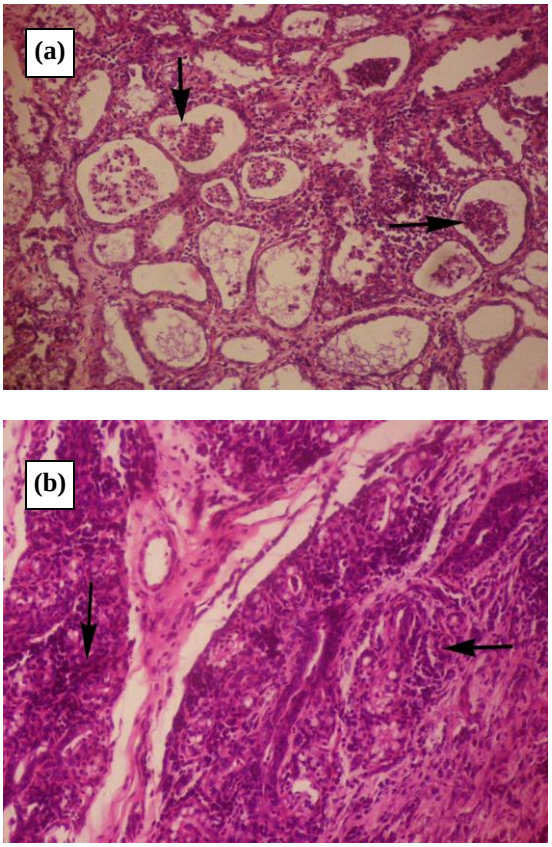


**Figure 1. Sections of mammary tissue from mastitic buffalo (a) showing atrophic alveoli containing cellular exudates and infiltration of mononuclear cells (arrow). Mammary tissue section (b) showing disappearance of alveoli and connective tissue proliferation from mastitic buffalo (arrow). 200X, H & E Stain.**

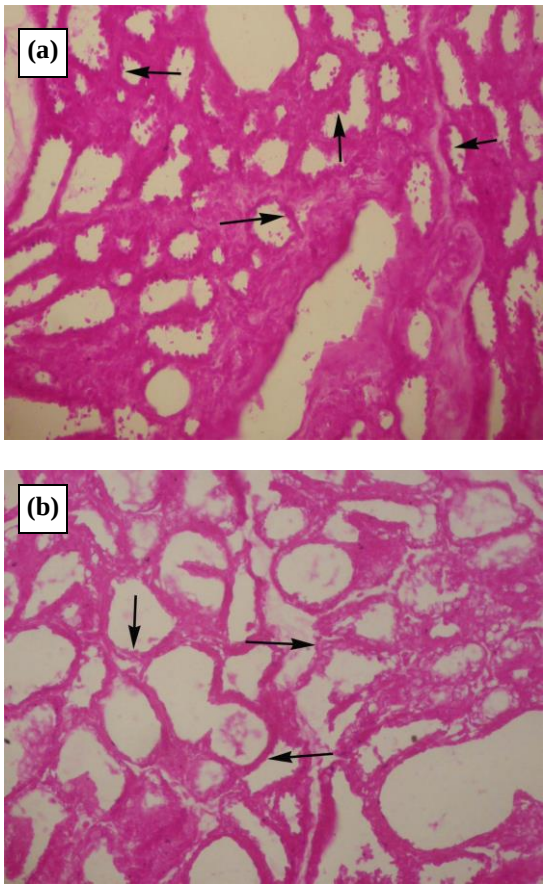


**Figure 2. Section of mammary tissue from healthy buffalo (a) showing high level of alkaline phosphatase activity (arrows). Part b, mammary section from infected buffalo showing no alkaline phosphatase activity (arrows), 400X.**

## DISCUSSION

The control of bovine mastitis is of paramount importance in dairy animals and its incidence can be reduced by identification of exact pathogenesis and removal of different potential risk factors associated with mastitis in individual animal. Selection of dairy animals which are less susceptible to mastitis and develop resistance against udder infections is important. Previously different studies have been conducted to explore the various host factors associated with mastitis in dairy herds (Bannerman *et al.*, 2008a; Bhutto *et al.*, 2010; Hussain *et al.*, 2012b). The results of present study did not showed clear association between different individual risks factors and occurrence of intra-mammary infection. In present study no significant association between udder configuration and teat/udder lesions was recorded. In contrast to these findings different studies have reported significant association of these factors with mastitis (Compton *et al.*, 2007; Bhutto *et al.*, 2010). Results revealed significant association for teat and udder shape (P<0.0004).

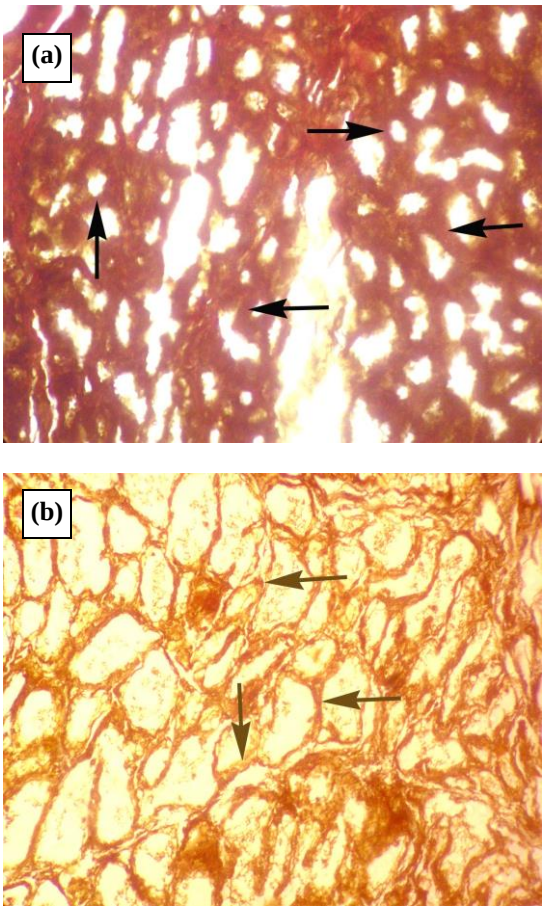


**Figure 3. Section of mammary tissue from healthy buffalo (a) showing high protein expression (arrows). Part b, mammary tissue from mastitic buffalo showing no protein expression (arrows), 200X.**

The mastitis was significantly increased in buffaloes having bowl and round shape udder while cylindrical, round and flat teats as compared to cup shape udder and pointed teats. It could be due to the reason that impaired udder provide easy entry for pathogens. Moreover the milk drops spreads at teat apex in flat and cylindrical teats thus enable the entry and multiplication of different mastitis pathogens in udder. Similar findings have also been reported (Slettbakk *et al.*, 1995; Bhutto *et al.*, 2010; Hussain *et al.*, 2012b). Results of analysis of variance for teat diameter, teat length and streak canal length revealed significant association with mastitis. These findings suggest that in teat having large apex diameter remains open for longer period and the pathogen enter through teat orifice. The short teat and streak canal length also favors the pathogens to travel short distance to establish infection in mammary parenchyma. These results

have supported by different other studies (Shukla *et al.,* 1997; Compton *et al.,*, 2007). In present study total milk leukocyte count and neutrophil population was significantly increased indicating mammary gland infection. The milk leukocyte count is considered the best biomarker for inflammatory reaction in udder infection. The increased number of milk leukocyte in present study could be due to microbial infection in udder as these are the major part of the immune response of the animals (Djabri *et al.,* 2002; Gargouri *et al.,* 2008).

Microscopical examination of tissue sections from teat of infected buffaloes exhibited severe histological changes. In most of the teat, beneath teat duct epithelium severe cellular inflammation, presence of lymphoid nodules and nodular proliferation of the mucosa was present at teat cistern. Different researchers hypothesized that the epithelium of the teat cistern mucosa is more vulnerable to injuries and bacterial toxins (Ngatia *et al.,* 1991; Fragkou *et al.,* 2007). Previously similar pathological changes in teat sections from experimentally induced cases of mastitic animals have been reported (Fragkou *et al.,* 2007; Ahmed *et al.,* 2010). Histologically, the most of examined sections from udder of mastitic animals showed lymphocytic mastitis. Marked Infiltration of inflammatory cells, abscesses formation, discrete intralobular fibrosis, connective tissues proliferation, degenerated acinar epithelium and acinar atrophy observed in present study are also reported by different workers (Geishauser *et al.,* 2005; Mavrogianni *et al.,* 2007; Castro-Alonso *et al.,* 2009).

In present study morphometric procedures showed that the number and size of acini and alveolar secretary epithelial cell population was significantly reduced in infected mammary tissues. The degenerative changes in mammary parenchyma could be due to infectious pathogens which results poor biosynthetic capacity of udder and decreases cellular differentiation. Up to the best of our knowledge in accessible literature such reports are not available. However, the decrease number of alveoli, luminal area and less number of alveolar secretary cells has been determined in advance stage of lactation (Akers *et al.,* 2006). In addition the decrease number of alveolar secretory cell could be due to cell death induced by milk accumulation in alveoli (Singh *et al.,* 2005).

In present study few alveoli in tissue sections from mastitic buffaloes indicated weak activity of alkaline phosphatase (AP) and most of the alveoli showed disappearance of this enzyme reflecting impaired activity. AP, a glycoprotein enzyme is distributed in the body of animal and increases the hydrolysis of phosphates (Goor *et al.,* 1989). This enzyme is mainly located on the outer membrane of alveolar secretary epithelial cells where it helps in active transport processes. In accordance with the results of present study, Silanikove and Shapiro (2007) and Silanikove (2008) have reported that AP is located on the outer membrane of secretary epithelial cells of alveoli. The disappearance of alkaline phosphatase in present study could be due to negative effects of different pathogens or stress factors related to increase milk yield. However, different studies have indicated that the activity of this enzyme is reduced at the late stage of lactation (Hassan, 2004; Bhutto *et al.,* 2010). The expression of weak protein in our study could be due to the impaired and weak activity of mammary parenchymatous cells and endoplasmic reticulum. The weak to low protein expression may also be related to connective tissue proliferation and various degenerative changes induced by microbial agents. These histochemical evidences could not be found in published literature in mammary glands naturally infected animals. Hassan (2004) and Bhutto *et al.* (2010) reported that weak protein expression mainly occurs in mammary glands of animals during last stage of lactation due the replacement of functional parenchyma by connective tissue proliferation.

***Conclusions*:** The results of present study suggest that the chances of mastitis are higher in buffaloes with short, flat, round and cylindrical teat while, round and bowl udder shape. Different aspects of teat and udder morphology are useful to decrease the incidence of mammary gland infection.

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