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MILK LACTOFERRIN CONCENTRATIONS IN ANATOLIAN BUFFALOES WITH AND WITHOUT SUBCLINICAL MASTITIS

Erhan Özenç[1,*], Esra Şeker[2], Duygu Baki Acar[1], Halit Buğra Koca[3], Ebubekir Yazıcı[1], Hacı Ahmet Çelik[1], Nurhan Doğan[4], Gülcan Avcı[5], Oktay Yılmaz[1], Mustafa Küçükkebapçı[6], Mehmet Uçar[1] and Ayhan Baştan[7]

ABSTRACT

This study aimed to determine the lactoferrin concentration from healthy and subclinical mastitic buffaloes. Lactoferrin levels were determined in a total of 475 mammary quarter milk samples, 391 of which were healthy and 84 suffering from subclinical mastitis. The mean milk lactoferrin level in logarithmic form for healthy and subclinical mastitic milk samples was 1.34±0.27 and 1.85±0.47, respectively. A significant difference ($P<0.001$) was determined between the two groups in terms of the lactoferrin levels. The milk lactoferrin levels in healthy buffaloes were significantly higher in the middle and late periods of lactation than the peak period ($P<0.05$). The mean milk lactoferrin levels in subclinical mastitic buffaloes infected with *S. aureus* or *S. agalactiae* were significantly higher than those from in buffaloes infected with Coagulase-Negative Staphylococci (CNS) or *Candida* spp. ($P<0.05$). An increase in milk lactoferrin levels was observed in parallel with the increase in somatic cell scores. In conclusion, milk lactoferrin levels in buffaloes were found to increase with the lactation stage, the presence of bacterial infection and the somatic cell count. This is the first study to determine the relationship between lactoferrin concentrations and bacterial infection in milk obtained from mammary quarters with subclinical mastitis in Anatolian buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, Anatolian buffaloes, buffalo milk, lactoferrin, subclinical mastitis

[1]Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey, *E-mail: erhanankara@hotmail.com

[2]Department of Microbiology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

[3]Department of Biochemistry, Faculty of Medicine, Afyonkarahisar Health Sciences University, Afyonkarahisar, Turkey

[4]Department of Biostatistics and Medical Informatics, Afyonkarahisar Health Sciences University, Afyonkarahisar, Turkey

[5]Department of Biochemistry, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

[6]Marmara Livestock Research Institute, Balıkesir, Turkey

[7]Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

INTRODUCTION

Mastitis is defined as inflammatory change in the mammary gland characterized by pathologic processes in the mammary tissues and an increase in the milk somatic cell count (SCC) (IDF, 1981). Cases of subclinical mastitis show no changes in the mammary gland or the milk. There is an increase in SCC but it cannot always be diagnosed. Therefore, subclinical mastitis, which can be detected by direct and indirect laboratory tests, is mainly diagnosed by bacteriological examination and the milk SCC (Fagiolo and Lai, 2007; Singh *et al*., 2017; Yigit *et al*., 2018). Even though studies in cows had suggest an SCC value over 100,000 cells/ml as an indicator of mastitis (Berglund *et al*., 2007), reports from buffaloes indicate that a threshold value is 130,000 to 200,000 cells/ml (Dhakal, 2006; Moroni *et al*., 2006; Ozenc *et al*., 2008).

Lactoferrin, which has iron-binding and antibacterial properties, is found in significant quantities on mucosal surfaces and in the milk (Reiter and Oram, 1967; Gaunt *et al*., 1980; Pecorini *et al*., 2010). Milk lactoferrin levels are reported to vary according to the animal's age, lactation stage and parity, SCC and the presence of pathogenic organisms (Harmon *et al*., 1975; Gaunt *et al*., 1980; Hagiwara *et al*., 2003; Giacinti *et al*., 2013). Breed differences are also reported as affecting the lactoferrin levels in the cows (Krol *et al*., 2010). Lactoferrin concentrations in quarter milk samples obtained from cows with subclinical mastitis have been shown to be significantly higher than normal milk (Kawai *et al*., 1999; Hagiwara *et al*., 2003). Lactoferrin has been shown, especially in cow milk, to protect the mammary tissue significantly from *Escherichia coli* and in a certain measure also from *Staphylococcus aureus* and *Streptococcus agalactiae* (Hagiwara *et al*., 2003; Kawai *et al*., 2003).

Systematic breeding studies related to Anatolian buffaloes have just been started in Turkey. Although many studies report the lactoferrin concentration in milk obtained from cows before the year 2000 (Shimazaki *et al*., 2017), there is limited information on the determination of lactoferrin levels in buffalo milk (Elagamy, 2000; Campanella *et al*., 2009; Giacinti *et al*., 2013). However, there is no comparative analysis of lactoferrin levels between bacterial agents and milk lactoferrin levels in buffaloes with subclinical mastitis. Therefore, our objectives were: (i) to determine the normal milk lactoferrin levels and the factors (the animals' age, lactation stage, SCC score) affecting milk lactoferrin, (ii) to determine the relationship between bacterial agents causing subclinical mastitis and milk lactoferrin levels, (iii) to detect the threshold limit value of lactoferrin for diagnosis of subclinical mastitis in Anatolian buffaloes in Turkey.

MATERIALS AND METHODS

Animal selection

Milk samples from 501 mammary quarters were collected from a total of 128 Anatolian water buffaloes aged 3 to 12, in different stages of lactation, on 15 different private farms in western Anatolia in Turkey. Eleven of the sampled mammary quarters were blind. Twenty-six mammary quarters with clinical mastitis were not included in this study. The buffaloes were housed in stalls during the harsh winter months, and moved freely within open-air paddocks in the spring and summer. The animals on the private farms were raised in adequate feeding and barn conditions. In the summer months they were fed from pastures, with the addition of a mixture of bran, barley flakes and hay. Silage was added to this during the winter months. Access to water was *ad libitum.* Animals in all locations were subjected to hand milking twice daily.

The collection of milk sample, determination of SCC and bacteriological examination

After the first streams of foremilk were discarded, 15 ml of milk were collected aseptically from buffaloes in the morning milking. Samples were immediately transported to the laboratory in a cool box on ice. Firstly, the each milk sample was homogenized. Ten µL of each milk sample was inoculated onto Columbia blood agar (Oxoid, Hampshire, England), containing 7% of sheep blood, MacConkey agar (Oxoid, Hampshire, England) and Sabouraud dextrose agar (Oxoid, Hampshire, England). While the blood agar and MacConkey agar plates were aerobically incubated for 24 to 48 h at 37^{o}C, the Sabouraud dextrose agar plates were incubated under aerobic conditions for 5 to 7 days at 24^{o}C. After the incubation, each different colony was examined macroscopically and microscopically. The identification of isolates was achieved by using standard biochemical tests (Holt *et al*., 1994; Quinn *et al*., 2013).

The SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley IBC-M; Bentley Instruments Inc., Chaska, MN, USA).

Milk in which no bacteria were isolated and with no SCC higher than 142×10^3 cells/ml in any sample was considered healthy (Ozenc *et al*., 2008). Milk in which at least one colony of (*S. aureus* or S. agalactiae (≥100 cfu/ml) and 1 to 3 colonies of other microorganism types (≥500 cfu/ml) were isolated in the absence of clinical signs of mastitis (hardness, redness, pain, swelling in the mammary quarters and clots in milk) were evaluated as having subclinical mastitis (Moroni *et al*., 2006).

A total of 475 mammary quarter milk samples (i.e. 84 samples from quarters with subclinical mastitis and 391 healthy samples) were evaluated for lactoferrin levels.

The lactation stage, based on the date of last calving, was characterized as "peak period" (in the 30 to 100 days postpartum), "middle period" (101 to 200 days postpartum) and "late period" (201 days or longer after delivery).

Milk lactoferrin level determination

The milk lactoferrin levels were determined by using a buffalo lactoferrin ELISA kit specially produced for this study (Buffalo Lactoferrin ELISA Kit, BIO K 271, Bio-X Diagnostics S.P.R.L., Jemelle-Belgium).

Statistical analyses

The lactoferrin concentration data (µg/ml) were transformed into logarithmic form. A linear scoring method was used for SCC (Ceron-Munoz and Ark, 2002; Hagiwara and Ark, 2003). The normal distribution of logarithmic values was checked using the Shapiro-Wilks test. Variance analysis was used to compare normal distribution values. The Kruskal-Wallis test was used to compare values with non normal distribution. Tukey test was used to compare different groups. Continuous variables expressed as mean ± standard deviation (SD). Nonparametric ROC curve analysis was used to detect the optimal cut-off points having the highest sensitivity and specificity for determination of subclinical mastitis. All statistical analyses were performed using the MedCalc for Windows (Version 7.1.0.1). $P<0.05$ was considered statistically significant.

RESULTS

The milk lactoferrin levels of the 391 healthy milk samples and the 84 subclinical mastitic milk samples ranged from 2 to 505 µg/ml (mean lactoferrin level: 27.86±1.65 µg/ml) and 6 to 693 µg/ml (mean lactoferrin level 137.70±19.41 µg/ml), respectively. The logarithmic values of mean milk lactoferrin concentrations in healthy milk samples (1.34±0.28) showed a highly significant difference than subclinical mastitic milk samples (1.85±0.47) (P<0.001; Table 1 and 2). The mean lactoferrin level (log) in healthy milk samples in the peak lactation period was significantly lower than that of those in the middle and late periods (P<0.05) (Table 1).

As for mammary quarters with sublinical mastitis, the mean lactoferrin levels (log) in quarter milk samples infected with *S. aureus or S. agalactiae* were significantly higher than those in quarter milk samples infected with Coagulase Negative Staphylococci (CNS) or *Candida* species (P<0.05).

A trend to increase in parallel with SCC was also noted when examining the lactoferrin levels of subclinical mastitic milk. Samples in which more than one infectious agent was isolated could not be subjected to statistical evaluation given their low number (Table 2).

For different cut-off points of lactoferrin levels, the rates of sensitivity, specificity, false positive rate and false negative rate were shown in Table 3. The threshold limit for lactoferrin levels was 32.9 µg/ml (Table 3).

Table 1. The distribution of lactoferrin level (log) with regard to age, stage of lactation and SCC score in quarter milk samples from healthy buffaloes.

Healthy milk		**Number of samples**	**Mean milk lactoferrin level ± standard deviation (logarithmic form)**
Total		391	1.34 ± 0.28
Age	3-4	86	1.39 ± 0.28[a]
	5-6	94	1.36 ± 0.27[a]
	7-8	99	1.34 ± 0.26[a]
	>8	112	1.29 ± 0.28[a]
Stage of lactation	Peak period	126	1.28 ± 0.27[a]
	Middle period	148	1.36 ± 0.27[b]
	Late period	117	1.39 ± 0.26[b]
SCC score	SCC 0	194	1.22 ± 0.21[a]
	SCC 1	77	1.46 ± 0.30[b]
	SCC 2	63	1.43 ± 0.26[b]
	SCC 3	57	1.51 ± 0.25[b]

Significant difference between values symbolized with different letters (a, b) within the column (P<0.05).
Somatic cell count score: 0 ≤ SCC 0 < 18,000; 18,000 ≤ SCC 1 < 36,000; 36,000 ≤ SCC 2 < 71,000; 71,000 ≤ SCC 3 < 142,000

Table 2. The distribution of lactoferrin level (log) with regard to infectious agents and SCC score in quarter milk samples from buffaloes with subclinical mastitis.

Subclinical mastitis	Samples	Number of samples	Mean milk lactoferrin level ± standard deviation (logarithmic form)
Total		84	1.85 ± 0.47
Bacteria	*S. aureus*	13	2.22 ± 0.57[a]
	CNS	23	1.74 ± 0.40[b]
	Candida spp.	26	1.69 ± 0.38[b]
	S. agalactiae	6	2.25 ± 0.48[a]
	Bacillus spp.	5	1.77 ± 0.27[ab]
	E. coli	4	1.64 ± 0.48[ab]
	S. aureus + *Candida* spp.	3	2.08 ± 0.60
	S. aureus + CNS	2	2.20 ± 0.08
	E. coli + CNS	2	1.57 ± 0.18
SCC score	SCC 0	7	1.51 ± 0.33[a]
	SCC 1	7	1.67 ± 0.17[a]
	SCC 2	9	1.73 ± 0.38[a]
	SCC 3	12	1.68 ± 0.36[a]
	SCC 4	17	1.82 ± 0.43[ab]
	SCC 5	11	1.91 ± 0.53[ab]
	SCC 6	9	2.01 ± 0.55[ab]
	SCC 7	7	2.14 ± 0.57[ab]
	SCC 8	5	2.48 ± 0.40[b]

Significant difference between values symbolized with different letters (a, b) within the column ($P<0.05$).
Somatic cell count score: $0 \leq$ SCC 0 < 18,000; 18,000 $\leq$ SCC 1 < 36,000;
36,000 $\leq$ SCC 2 < 71,000; 71,000 $\leq$ SCC 3 < 142,000; 142,000 $\leq$ SCC 4 < 283,000;
283,000 $\leq$ SCC 5 < 566,000; 566,000 $\leq$ SCC 6 < 1,132,000; 1,132,000 $\leq$ SCC 7 < 2,263,000;
2,263,000 $\leq$ SCC 8 < 4,536,000

Table 3. The rates of maximum sensitivity (%) and specificity (%), false positive rate, false negative rate for different cut-off points of lactoferrin level (µg/ml) in receiver-operating characteristic (ROC) curve for any infection compared with no growth.

Cut-off point	Sensitivity	Specificity	False positive rate	False negative rate
15.01	97.62	25.83	74.17	2.38
25.4	91.67	61.89	38.11	8.33
32.9*	82.14	78.77	21.23	17.86
50.3	47.62	92.07	7.93	52.38
101.7	29.76	97.70	2.3	70.24

*The optimal cut-off points having the highest sensitivity and specificity.

DISCUSSION

This report is the first to characterize the correlations between the infectious agents isolated in milk samples with subclinical mastitis and the milk lactoferrin concentrations in buffaloes.

There were different findings about the relationship between age and milk lactoferrin levels in several studies. Hagiwara *et al*. (2003); Al-Majali *et al*. (2007) reported a negative correlation between age and lactoferrin concentration in their study on cows and camels, respectively. On the other hand, Gaunt *et al*. (1980) found a positive correlation between milk lactoferrin levels and age increase in cows. Harmon *et al*. (1975) determined that there is no significant difference between the milk lactoferrin level and age. This study could not establish a significant correlation between age group and lactoferrin levels in water buffaloes.

Hagiwara *et al*. (2003) reported that the mean milk lactoferrin level in cows was higher in the middle and latter period of lactation but not statistically significant. Giacinti *et al*. (2013) determined that the milk lactoferrin level significantly increased with the progress of lactation period. Similarly, Gaunt *et al*. (1980) determined a positive correlation between the advancing lactation and the lactoferrin level. Consistently with the reports by Giacinti *et al*. (2013); Gaunt *et al*. (1980), this study also showed that the milk lactoferrin concentration was lowest at the start of lactation, increasing with the progressing lactation period.

In milk samples obtained from five healthy buffaloes in Italy, milk lactoferrin levels ranged from 5.6 µg/ml to 800 µg/ml while the mean concentration was 232 µg/ml (Campanella *et al*., 2009). In another report, Giacinti *et al*. (2013) showed that the milk lactoferrin level ranged from 30 mg/kg to 813 mg/kg in buffaloes, with a mean value of 332 mg/kg. The mean lactoferrin levels in tank milk from cows and buffaloes were indicated as being, respectively, 76.7 µg/ml and 31.7 µg/ml (Elagamy, 2000). In this study, the mean milk lactoferrin level in the healthy mammary quarters (27.86±1.65 µg/ml) was similar to that of Elagamy (2000), but lower than in the other reports (Campanella *et al*., 2009; Giacinti *et al*., 2013). We suppose that this difference in our results may be a result of the different buffalo breed and the differences in the sampling methods (i.e. sampling at the mammary quarter level or from tank milk).

An increase in milk lactoferrin in cows and camels for the presence of mastitis was already reported (Gaunt *et al*., 1980; Hagiwara *et al*., 2003; Al-Majali *et al*., 2007). Hagiwara *et al*. (2003) indicated that the mean milk lactoferrin levels (log) in healthy and subclinical mastitic cows were 2.23±0.39 and 2.70±0.39, respectively. The milk lactoferrin levels (log) in healthy and subclinical mastitic camels were detected as 2.65±0.88 and 3.8±0.67 (Al-Majali *et al*., 2007). Similarly to previous reports, in our study, the mean lactoferrin level (log) in the milk with subclinical mastitis (1.85±0.47) was significantly higher than in healthy buffalo milk (1.34±0.28). Unlike other reports, in the present study, the mean milk lactoferrin level in healthy lactating buffaloes and subclinical mastitic buffaloes was lower than in cows (Hagiwara *et al*., 2003) and camels (Al-Majali *et al*., 2007). It was reported that the milk lactoferrin level may vary depending on the pathogenicity of bacterial species and milk SCC (Hagiwara *et al*., 2003). Similarly to this report, in the present study, the milk lactoferrin levels in *S. aureus* and *S. agalactiae* infections were found to be significantly higher than other infections. In the present study, the lactoferrin levels increased as SCC increased. This increase corresponds with the reports of the aforementioned researchers.

Bacteriological findings were considered as the gold standard for determining presence or absence of subclinical mastitis in buffaloes (Fagiolo and Lai, 2007; de Oliveira Moura *et al.*, 2017). In this study, the cut-off point of lactoferrin level of milk from Anatolian buffalo infected with any pathogen was 32.9 µg/ml. This lactoferrin threshold limit may be a detection criterion for the diagnosis of subclinical mastitis in Anatolian buffaloes.

CONCLUSION

Milk lactoferrin levels in buffaloes were found to increase with the progressing lactational stage, the presence of bacterial infection and the increase in SCC. Also, the threshold limit of lactoferrin level for diagnosis of subclinical mastitis was 32.9 µg/ml in Anatolian buffaloes. This threshold limit value was only available for the Anatolian buffaloes. Further studies are needed to determine the threshold values of milk lactoferrin in other buffalo breeds.

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REFERENCES

Al-Majali, A.M., Z.B. Ismail, Y. Al-Hami and A.Y. Nour. 2007. Lactoferrin concentration in milk from camels (*Camelus dromedarius*) with and without subclinical mastitis. *Int. J. Appl. Res. Vet. M.*, **5**: 120-124.

Berglund, I., G. Pettersson, K. Ostensson and K. Svennersten-Sjaunja. 2007. Quarter milking for improved detection of increased SCC. *Reprod. Domest. Anim.*, **42**: 427-432.

Campanella, L., E. Martini, M. Pintore and M. Tomassetti. 2009. Determination of lactoferrin and immunoglobulin G in animal milks by new immunosensors. *Sensors*, **9**: 2202-2221.

Ceron-Munoz, M., H. Tonhati, J. Duarte, J. Oliveira, M. Munoz-Berrocal and H. Jurado-Gamez. 2002. Factors affecting somatic cell counts and their relations with milk and milk constituent yield in buffaloes. *J. Dairy Sci.*, **85**: 2885-2889.

de Oliveira Moura, E., A.H. do Nascimento Rangel, M.C.N. de Melo, L.H.F. Borba, D.M. de Lima Júnior, L.P. Novaes, S.A. Urbano and J.C. de Andrade Neto. 2017. Evaluation of microbiological, cellular and risk factors associated with subclinical mastitis in female buffaloes. *Asian Austral. J. Anim.*, **30**: 1340-1349.

Dhakal, I.P. 2006. Normal somatic cell count and subclinical mastitis in Murrah buffaloes. *J. Vet. Med. B.*, **53**: 81-86.

Elagamy, E.I. 2000. Effect of heat treatment on camel milk proteins with respect to antimicrobial factors: A comparison with cows and buffalo milk proteins. *Food Chem.*, **68**: 227-232.

Fagiolo, A. and O. Lai. 2007. Mastitis in buffalo.

Ital. J. Anim. Sci., **6**: 200-206.

Gaunt, S.N., N. Raffio, E.T. Kingsbury, R.A. Damon, W.H. Johnson and B.A. Mitchell. 1980. Variation of lactoferrin and mastitis and their heritabilities. *J. Dairy Sci.*, **63**: 1874-1880.

Giacinti, G., L. Basirico, B. Ronchi and U. Bernabucci. 2013. Lactoferrin concentration in buffalo milk. *Ital. J. Anim. Sci.*, **12**: 139-143.

Hagiwara, S., K. Kawai, A. Anri and H. Nagahata. 2003. Lactoferrin concentrations in milk from normal and subclinical mastitic cows. *J. Vet. Med. Sci.*, **65**: 319-323.

Harmon, R.J., F.L. Schanbacher, L.C. Ferguson and K.L. Smith. 1975. Concentration of lactoferrin in milk of normal lactating cows and changes occurring during mastitis. *Am. J. Vet. Res.*, **36**: 1001-1007.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley and S.T. Williams. 1994. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Lippincott Williams and Wilkins, Philadelphia, USA. 754p.

IDF. 1981. Laboratory methods for use in mastitis work. *Document no: 132*. International Dairy Federation, Brussels, Belgium.

Kawai, K., S. Hagiwara, A. Anri and H. Nagahata. 1999. Lactoferrin concentration in milk of bovine clinical mastitis. *Vet. Res. Commun.*, **23**: 391-398.

Kawai, K., H. Nagahata, N.Y. Lee, A. Anri and K. Shimazaki. 2003. Effect on infusing lactoferrin hydrolysate into bovine mammary glands with subclinical mastitis. *Vet. Res. Commun.*, **27**: 539-548.

Krol, J., Z. Litwinezuk, A. Brodziak and J. Barlowska. 2010. Lactoferrin, lysozyme and immunoglobulin G content in milk of four breeds of cows managed under intensive production system. *Pol. J. Vet. Sci.*, **13**: 357-361.

Moroni, P., C.S. Rossi, G. Pisoni, V. Bronzo, B. Castiglioni and P.J. Boettcher. 2006. Relationships between somatic cell count and intramammary infection in buffaloes. *J. Dairy Sci.*, **89**: 998-1003.

Ozenc, E., M.R. Vural, E. Seker and M. Ucar. 2008. An evaluation of subclinical mastitis during lactation in Anatolian buffaloes. *Turk. J. Vet. Anim. Sci.*, **32**: 359-368.

Pecorini, C., D. Sassera, R. Rebucci, F. Saccone, C. Bandi and A. Baldi. 2010. Evaluation of the protective effect of bovine lactoferrin against lipopolysaccharides in a bovine mammary epithelial cell line. *Vet. Res. Commun.*, **34**: 267-276.

Quinn, P.J., M.E. Carter, B. Markey and G.R. Carter. 2013. *Clinical Veterinary Microbiology*, 2nd ed. Harcourt Publishers Limited, London, England. 720p.

Reiter, B. and J.D. Oram. 1967. Bacterial inhibitors in milk and other biological fluids. *Nature*, **216**: 328-330.

Shimazaki, K.I. and K. Kawai. 2017. Advances in lactoferrin research concerning bovine mastitis. *Biochem. Cell Biol.*, **95**: 69-75.

Singh, K.V., D. Sharma, S.K. Singh, M. Srivastava, S.K. Garg and B.K. Yadav. 2017. Assessment of alteration in metabolic profile and milk composition of buffaloes with subclinical mastitis. *Buffalo Bull.*, **36**: 349-356.

Yigit, A.A., M. Cinar, H.C. Macun, E. Ozenc, R. Kabakci, E. Yazici, H. Kalender, A. Bastan and S. Salar. 2018. Total oxidant and antioxidant activities in milk with various somatic cell count intervals during discrete cow and buffalo lactation periods. *Indian J. Dairy Sci.*, **71**: 72-77.