Lactoferrin Concentration in Milk From Camels (*Camelus dromedarius*) With and Without Subclinical Mastitis

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ABSTRACT

The purposes of this study were to investigate the levels of lactoferrin in 180 normal and 132 mastitic camel milk samples and to elucidate the effect of age, lactation stage, presence of pathogens, and somatic cell counts (SCC) on the concentration of lactoferrin in camel milk using radial immunodiffusion test. The mean log concentration of lactoferrin from mastitic camels (3.8 ± 0.67) was significantly higher than that in normal camels (2.65 ± 0.88). The mean log concentrations of lactoferrin in 3- and 4-year-old lactating camels were significantly higher than that in older camels. A correlation was observed between the levels of lactoferrin in normal and mastitic camel milk and the SCC score. The log lactoferrin concentrations in subclinical mastitic camel milk infected with *Staphylococcus aureus* and coagulase-negative staphylococci isolates were significantly higher than those for other bacterial isolates. No differences in the concentration of lactoferrin were observed in reference to the stage of lactation. These data could help in understanding the mechanisms of udder resistance to infections. In addition, levels of lactoferrin in milk could be used as a diagnostic tool in cases of subclinical mastitis.

INTRODUCTION

Lactoferrin is an iron-binding glycoprotein of the transferrin family that posses antimicrobial activity.[1] Lactoferrin is present in most biological fluids including milk, blood, and mucous secretions. The antibacterial power of lactoferrin against bacteria, some yeast, fungi, viruses, and parasites has been investigated.[2] In addition, the modulatory effect of lactoferrin on inflammatory response and activation of the immune system have been reported previously.[2,3] Lactoferrin works as an antimicrobial compound through chelating the iron ion, making this essential ion unavailable to the invading pathogens.[1]

The content of lactoferrin in milk varies depending on the species. The concentration of lactoferrin in cow's milk is lower than in

human's milk.[4] A factory scale production of bovine lactoferrin at high purity was established long ago.[5]

There have been many reports on the antibacterial effects of lactoferrin of different origins.[3,6,7] In addition, the role of lactoferrin in mastitis has been investigated in cattle.[4] In bovine, lactoferrin concentration was found to be correlated to the milk somatic cell counts (SCCs) and stage of lactation.[4] The mean concentrations of lactoferrin in normal and mastitic cow milk have been reported previously and found to be 2.23 and 2.70, respectively (concentrations are expressed in the logarithmic form).[2,4]

The objectives of this study were to determine the concentrations of lactoferrin in milk of normal and subclinical mastitic camels and to investigate in vitro the antibacterial effect of camel lactoferrin on different bacteria species isolated from camels with subclinical mastitis.

MATERIAL AND METHODS

Animals

A total of 312 quarter milk samples were collected from 78 clinically normal lactating camels (*Camelus dromedarius*) from 10 herds located in the central and the northern parts of Jordan. This study is part of a national epidemiological investigation directed by the principal author to investigate clinical and subclinical mastitis in camels in Jordan. A total of 5-10 mL of milk from each quarter was collected aseptically while the animal was standing. Age and stage of lactation were recorded. Milk samples were classified on the basis of SCCs and bacterial isolation. Milk samples were considered normal if the SSCs were less than 100,000 cell/mL and no bacteria was isolated. Milk samples from which bacteria were isolated were considered subclinical mastitic milk. Out of the 312 analyzed quarter milk samples, 180 were considered normal milk under the above mentioned criteria. Isolated bacteria were identified according to standard procedures.[8] The following bacterial isolates were obtained from the analyzed subclinical mastitic milk: *Staphylococcus aureus* 80 isolates, coagulase negative staphylococci (CoNS) 28 isolates, Arcanobacterium pyogens 15 isolates, *Streptococcus agalactiae* 8 isolates, other streptococci 30 isolates, and *Escherichia coli* 44 isolates.

Determination of SCC

Somatic cell counts in camel milk were determined by spreading 10 µL of thoroughly mixed milk from each sample over a 1-cm^2 area on a glass slide. Slides were stained with Newman-Lampert stain after drying following previously reported procedures.[9] Somatic cell counts were converted into scores as shown in Table 1.

Table 1. Lactoferrin Concentration (log) in Subclinical Mastitic Milk as Classified by Bacterial Isolates and SCC Score.

Criteria	Classification	No. of Samples	Lactoferrin Concentration (log) (mean ± SD)*
Bacterial isolates	*S aureus*	80	4.11 ± 0.12^a
	CoNS	28	4.62 ± 0.32^a
	A pyogens	15	3.12 ± 0.30^b
	S agalactiae	8	3.33 ± 0.25^b
	Other streptococci	30	3.42 ± 0.72^b
	E coli	44	3.02 ± 0.55^c
SCC score	SCC 0	15	3.12 ± 0.41^a
	SCC 1	33	3.31 ± 0.33^b
	SCC 2	42	3.45 ± 0.52^{b,c}
	SCC 3	23	3.52 ± 0.29^{b,c}
	SCC 4	19	4.01 ± 0.38^d

*Values (means) with different letter superscripts statistically different ($P < 0.05$).
SCCs were classified by numbers into the following scores: 0 ≤ SCC 0 < 30,000 ≤ SCC 1 < 50,000 ≤ SCC 2 < 100,000 ≤ SCC 3 < 200,000 ≤ SCC 4.

Lactoferrin Determination

Milk lactoferrin concentrations were measured using a commercially available test kit (BSL, Miyagi, Japan).

Concentrations of lactoferrin were transformed into logarithmic form (log).

Lactoferrin Isolation and Purification From Camel Milk

Lactoferrin from camel milk was isolated and purified using previously reported methods.[10]

Minimal Inhibitory Concentration of Lactoferrin

Minimal inhibitory concentration (MIC) values of lactoferrin isolated and purified from camel milk were determined for the isolated bacterial strains following previously published procedures described for bovine lactoferrin.[11] Briefly, bacterial isolates were cultured in heart infusion broth and incubated at 37ºC for 24 hours. Colony counts were determined using standard plate technique and cultures were adjusted to have 106 cfu/µL. Sixty microliters of each adjusted culture broth were added to a 96-well microplate. After incubation at 37ºC for 1 hour, 5 µL of filter and sterilized lactoferrin at a concentration ranging from 1 µg/mL to 50 mg/mL were added. The plates were mixed thoroughly and incubated at 37ºC for 24 hours. Plates were then mixed and optical density was measured at 600 nm in a microplate reader. Bacterial isolates that were not inhibited at 50 mg/mL lactoferrin concentration were classified as lactoferrin resistant.[11]

Statistical Analysis

The differences in the milk lactoferrin concentrations (log) among the different groups (normal and mastitic milk) were tested by Kruskal-Wallis test. The correlation coefficients among the different factors (age, stage of lactation, and SCC score) were analyzed by Spearman's correlation. A $P \leq 0.05$ was considered significant. All analyses were performed using SPSS® software version 14 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Lactoferrin Concentration

The concentrations of lactoferrin in normal and mastitic camel milk ranged from 20-2100 µg/mL and 44-3100 µg/mL, respectively. The average (± standard deviation) logarithmic concentrations of lactoferrin in normal and mastitic camel milk were 2.65 ± 0.88 and 3.8 ± 0.67, respectively. The mean log concentration of lactoferrin in mastitic milk was significantly higher than that in normal milk. When classified according to bacterial isolates, the log lactoferrin concentrations in subclinical mastitic camel milk infected with *S aureus* and CoNS isolates were significantly higher than those for the other isolates ($P < 0.01$). Mastitic camel milk infected with *E coli* showed the lowest concentration of lactoferrin when compared to mastitic milk infected with other bacterial isolates ($P < 0.01$) (Table 1).

A correlation was observed between the levels of lactoferrin in normal and mastitic camel milk and the SCC score (Table 1), where higher concentration was observed with higher SCC scores (Spearman's coefficient = -0.44, $P < 0.05$).

Table 2. Lactoferrin Concentration (log) in Normal Camel Milk as Classified by Animal Age, Stage of Lactation, and SCC Score.

Criteria	Classification	No. of Samples	Lactoferrin Concentration (log) (mean ± SD)*
Age	2-3 years	45	2.91 ± 0.22[a]
	4-6 years	57	3.01 ± 0.31[a]
	6-8 years	60	2.32 ± 0.28[b]
	>8 years	18	2.23 ± 0.26[b]
Stage of lactation	Beginning	97	2.60 ± 0.31[a]
	Middle	54	2.58 ± 0.14[a]
	Late	29	2.62 ± 0.22[a]
SCC score	SCC 0	101	2.64 ± 0.40[a]
	SCC 1	46	2.99 ± 0.09[b]
	SCC 2	33	3.10 ± 0.35[b]

*Values (means) with different letter superscripts statistically different ($P < 0.05$).

SCCs were classified by numbers into the following scores: 0 ≤ SCC 0 < 30,000 ≤ SCC 1 < 50,000 ≤ SCC 2 < 100,000.

The mean (log) concentrations of lactoferrin in 3- and 4-year-old lactating camels were significantly higher (Kruskal-Wallis test) than those in older camels (Table 2). The concentration of lactoferrin in camel milk was significantly correlated (Spearman's coefficient = -0.66, $P \leq 0.01$) to the age of the lactating camel. No differences in the concentration of lactoferrin were observed in reference to the stage of lactation ($P > 0.05$).

Antibacterial Effects of Camel Lactoferrin Against Selected Isolated Bacteria From Subclinical Mastitic Milk

All tested bacterial isolates were resistant to the camel lactoferrin except 20 *S aureus* isolates, 2 *S agalactiae*, and 12 streptococci other than *S agalactiae* (growth was not inhibited at 50 mg/mL lactoferrin concentration) (Table 3). Lactoferrin failed to inhibit any of the CoNS, *A pyogens*, and *E coli* isolates. The most sensitive isolate to lactoferrin was one of the *S aureus* isolate with an MIC value of 0.006 mg/mL.

DISCUSSION

This is the first investigation on the levels of lactoferrin in camel milk in reference to age, lactation stage, SCC score and bacterial findings. Our study shows strong association between levels of lactoferrin in milk and age in camels, where older camels tend to have lower lactoferrin concentrations. Similar findings were reported in cattle.[4] It has been suggested there is no association between age and lactoferrin concentration in the milk of cattle.[2,4] Unlike previous reports in cattle, stage of lactation in camels has no significant impact on the levels of lactoferrin in the milk.[4,12]

Table 3. The Minimal Inhibitory Concentrations (MIC) of Lactoferrin to Bacterial Isolates From Subclinical Mastitic Camel Milk.

Bacterial Isolate	MIC Value (mg/mL)	Number of Isolates
S aureus	>50	60
	4.1	19
	0.006	1
CoNS	>50	28
S agalactiae	>50	6
	5.1	1
	0.1	1
A pyogens	>50	15
Other streptococci	>50	18
	22	8
	1.8	2
	0.22	2
E coli	>50	44

As expected, the concentrations of lactoferrin in camel milk were enhanced in subclinical mastitic quarters. Similar findings were reported in cattle and human.[4,13] Slight elevation of lactoferrin in cattle with subclinical mastitis has been reported while it was significantly elevated in clinically affected ones.[14] The higher levels of lactoferrin in milk of animals with mastitis may be associated with severity of inflammation.[12,14]

In this investigation, a significant association was observed between lactoferrin concentration and SCC score. Our findings were in agreement with those reported previously in cows.[4] A strong association between udder inflammation and low SCC was reported, rendering low SCC as risk factor for clinical and subclinical mastitis.[15] It is possible that low lactoferrin concentration in camel milk has the same implications as low SCC score. This means that low lactoferrin concentration may be considered a risk factor for udder infection in camels.

The concentration of lactoferrin in camel milk might be associated with the pathogenicity of the bacterial species that present in the mammary gland. In this study, the lowest concentration of lactoferrin was found in quarter milk samples infected with *E coli* and the highest concentration was found in udder infected with CoNS. In cattle, the high levels of lactoferrin were observed in milk infected with *S aureus*.[14]

A low lactoferrin concentration in milk with *E coli* may lead to rapid growth of the bacteria and exaggeration of the clinical disease.[12,16] On the contrary, lactoferrin was significantly increased in cows experimentally infected with *E coli*.[17] Antimicrobial activity

of lactoferrin may be different in Gram-negative and Gram-positive bacteria due to the differences in the cell membrane structure. However, previous studies in cattle and humans showed bacterial isolates (both Gram-positive and Gram-negative) inhibited by in vitro addition of lactoferrin.[2,7,12] Literature has shown that lactoferrin can act as either a bacteriostatic and/or bactericidal agent.[2,4] This difference in the activity may, in part, explain the wide range of MIC values for lactoferrin. Lactoferrin isolated from camels was able to inhibit growth of only 20% of the *S aureus* isolates. None of the CoNS, *E coli*, or *A pyrogens* isolates was inhibited by lactoferrin. Presence of the previously described lactoferrin-binding proteins or lactoferrin receptors on the surface of these microorganisms may partially explain the resistance of these isolates to lactoferrin.[16] More investigations are needed to elucidate mechanisms of resistance of some bacteria species to camels' lactoferrin.

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