



Evaluation of Sialic Acid and Acute Phase Proteins (Haptoglobin and Serum Amyloid A) in Clinical and Subclinical Bovine Mastitis

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ABSTRACT

The present study was conducted to evaluate the concentrations of sialic acids (total, lipid bound and protein bound) and their correlation with acute phase proteins (haptoglobin and serum amyloid A) in clinical and subclinical mastitis of cattle. Thirty subclinical mastitic cows with positive California mastitis test (CMT) test and no clinical signs of mastitis, 10 clinical mastitic cows and 10 healthy cows with negative CMT test and normal somatic cell count were selected. Milk and blood samples were collected after confirmation of clinical and subclinical mastitis by somatic cell count and bacterial identification. Serum haptoglobin (Hp), serum amyloid A (SAA), total sialic acid (TSA), lipid bound sialic acid (LBSA) and protein bound sialic acid (PBSA) were measured by validated standard methods. Haptoglobin and SAA increased significantly in both types of mastitis compared with control group ($P < 0.001$). However, the ratio of HP/SAA was significantly different from the control group only in clinical mastitis. The results showed that TSA and LBSA were significantly different in control group compared with clinical and subclinical mastitis ($P < 0.001$). Protein bound sialic acid did not change in subclinical mastitis in comparison with control group ($P = 0.86$). There was positive correlation between LBSA and PBSA in clinical mastitis ($r = 0.72$, $P = 0.02$) whereas significant negative correlation was observed between LBSA and PBSA in subclinical mastitis ($r = -0.62$, $P < 0.001$). Results also showed no correlation between Hp and SAA with each other or with any other parameters in study groups.

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INTRODUCTION

Mastitis affects the quality of milk and is a potential health risk for the other cows (Yousaf *et al.*, 2010). In a well managed dairy herd, in addition to clinical mastitis, subclinical mastitis should be efficiently detected. Detection of clinical mastitis by visual inspection and palpation is relatively easy, but diagnostic problems arise when dealing with subclinical mastitis, where an increased somatic cell count (SCC) is the only finding. Today, subclinical mastitis is mostly diagnosed by cow-side tests like California mastitis test (CMT), or by laboratory analysis of SCC using automatic cell counters. This analysis is less efficient in detecting chronic subclinical mastitis than for acute clinical cases (Nielen *et al.*, 1995).

Plasma proteins are mainly synthesized by the liver. Analysis of total protein concentrations and percentage of protein fractions are important in various disease states (Kaneko, 1997). Glycoproteins are defined as proteins which contain glycan chains linked glycosidically to selected amino acid residues. Monosaccharides commonly found in the glycans of the glycoproteins including N-acetylneuraminic acid and sialic acid (Hemming, 1991). Sialic acids are one of the most important molecules of life, since they occupy the terminal position on macromolecules and cell membranes and are involved in many biological and pathological phenomena. The majority of sialic acids are found in either protein (PBSA) or lipid-bounded (LBSA) forms, while little amount is in the free forms. In addition, sialic acid is localized at the end chain of many acute phase proteins (Haq *et al.*, 1993; Thougard *et al.*, 1998). Sialic

acids usually occupy exposed terminal positions on the oligosaccharide chains of glycoconjugates and frequently serve as ligands for receptors such as selectins and siglecs, which mediate a variety of cell-cell adhesion processes in the inflammation and in the immune response (Malykhy *et al.*, 2001). Sialic acids are often involved in important cell surface communications and infection processes. The detection of sialic acid may be a valuable indicator for diagnosis and prognosis of inflammatory diseases (Motoi *et al.*, 1984). Serum sialic acid values were analyzed in many inflammatory and infectious diseases in cattle (Citil *et al.*, 2004; Karagenc *et al.*, 2005; Karapehivan *et al.*, 2007). The mechanism including sialic acid increase is not clearly understood. However, investigators have reported that SA localized at the end chain of many acute phase proteins can be used as marker for the determination of acute phase protein concentrations (Ekin *et al.*, 2003), because serum acute phase proteins, especially α 1-acid glycoprotein, are sialylated glycoproteins. The acute phase proteins (APPs) are a group of blood proteins that their concentrations change in animals subjected to external or internal challenges such as infection, inflammation, surgical trauma or stress (Murata *et al.*, 2004; Gruys *et al.*, 2005). Acute phase proteins and their changes due to various inflammatory and non-inflammatory conditions have been studied intensively in many animal species (Kaneko, 1997; Murata *et al.*, 2004; Murata, 2007).

There are no published reports about the concentration of serum sialic acid and their correlation with acute phase proteins in clinical and subclinical mastitis of cattle. Therefore, the present study was conducted to evaluate the concentrations of sialic acid and their correlation with acute phase proteins (Hp and SAA) in bovine clinical and subclinical mastitis.

MATERIALS AND METHODS

Experimental Animals

Thirty subclinical mastitic cows with positive CMT test and no clinical signs of mastitis, 10 clinical mastitic cows and 10 healthy cows with negative CMT test and normal somatic cell count (SCC) were selected. All cows were selected from one farm with the same management, lactation period, reproduction stage, nutrition and body condition score (BCS). Cows thoroughly examined and appropriate blood and milk samples were collected for bacteriology, hematology, clinical biochemistry and other relevant analysis.

Milk and blood sampling

Milk and blood samples were collected after confirmation of clinical and subclinical mastitis by somatic cell count and bacterial identification. Somatic cell count was determined using an electronic cell counter (Fossomatic 90; Fosso Electric, Denmark). Milk samples were collected for bacteriology and bacterial analysis were done according to routine validated methods (Quinn *et al.*, 1994). Blood samples were taken from tail vein into tubes without EDTA. The sera were separated by centrifugation at 750 g for 15 min and stored at -20°C until used.

Measurements

Haptoglobin (Hp) was measured according to prevention of the peroxidase activity of hemoglobin, which is directly proportional to the amount of Hp. The analytical sensitivity of this test in serum has been determined as 0.0156 mg/mL for Hp by the manufacturer (Tridelta Development Plc, Wicklow, Ireland). Serum amyloid A (SAA) was measured by a solid phase sandwich ELISA. The analytical sensitivity of this test in serum has been determined as 0.3 μ g/mL for SAA by the manufacturer (Tridelta Development Plc, Wicklow, Ireland). Serum total sialic acid concentration was determined by thiobarbituric acid method previously described by Warren (1959). The amount of total sialic acid was determined by use of a standard curve developed from a standard sample of N-acetyl neuraminic acid. Lipid bound sialic acid concentration was determined by the method described by Katopodis *et al.* (1982). The amount of lipid bound sialic acid was determined by use of a standard curve developed from a standard sample of N-acetyl neuraminic acid. Protein bound sialic acid concentration was measured by subtracting serum total sialic acid from lipid bound sialic acid.

Statistical analysis

Due to deviation of data from normality ($P < 0.05$ in Kolmogorov-Smirnov test) and inequality of variances, nonparametric Kruskal-Wallis and Mann-Whitney test was used for statistical comparisons. To find an index for better diagnosis of subclinical mastitis, ratio of Hp to SAA, and LBSA to PBSA were calculated and compared between study groups. Spearman's rank correlation coefficients were calculated to determine relationship between variables. All statistical analysis was performed using SPSS software (version 11.5). P-value less than 0.05 were considered as statistically significant.

RESULTS

Frequency of isolated bacteria of milk in cattle with clinical and subclinical mastitis and control group are presented in Table 1. The most dominant isolated bacterium from clinical and subclinical samples was *Staphylococcus aureus* ($n=17$; 20%). The most dominant isolated bacteria from clinical (60) and subclinical (63) samples were *Staphylococcus* spp (Table 1). There was no bacterial growth in 3% ($n=2$) of cattle with subclinical mastitis. In cattle with clinical mastitis, 66.7% ($n=19$) of isolated bacteria of milk was *S. aureus*. *S. dysgalactiae* is the most dominant bacterium of *Streptococcus* genus isolated from milk in cattle with clinical mastitis.

Haptoglobin and SAA increased significantly in both types of mastitis compared with control group ($P < 0.001$). However, the ratio of HP/SAA was significantly different from the control group only in clinical mastitis. The results showed that TSA and LBSA were significantly different in control group compared with clinical and subclinical mastitis ($P < 0.001$). Protein bound sialic acid did not change in subclinical mastitis in comparison with control group ($P=0.86$) (Table 2).

There was positive correlation between LBSA and PBSA in clinical mastitis ($r=0.72$, $P=0.02$) whereas significant negative correlation was observed between LBSA and PBSA in subclinical mastitis ($r=-0.62$,

$P < 0.001$). Results also showed no correlation between Hp and SAA with each other or with any other parameters in study groups (Table 3).

Table 1: Frequency of isolated bacteria from milk in different groups of cattle (control and clinical and subclinical mastitis) (n=85).

Bacteria	C	SC	CL	Total	%
Staphylococci					
<i>S. aureus</i>	0	13	4	17	20.00
<i>S. hyicus</i>	0	11	0	11	12.94
<i>S. epidermidis</i>	0	8	0	8	9.41
CNS	0	7	1	8	9.41
CPS	0	1	1	2	2.35
<i>S. intermedius</i>	0	1	0	1	1.18
Streptococci					
<i>S. dysgalactiae</i>	0	2	3	5	5.88
<i>S. uberis</i>	0	2	0	2	2.35
<i>Streptococcus spp.</i>	0	4	1	5	5.88
Corynebacteria					
<i>C. bovis</i>	0	3	0	3	3.53
<i>C. renale</i>	0	1	0	1	1.18
<i>Corynebacterium spp.</i>	0	1	0	1	1.18
<i>Rhodococcus equi</i>	0	7	0	7	8.23
<i>Enterobacter spp.</i>	0	1	0	1	1.18
<i>E. coli</i>	0	1	0	1	1.18
No growth	10	2	0	12	14.12
Total	10	65	10	85	100

C=Control; SC=Subclinical; CL=Clinical; CNS=Coagulase Negative Staphylococci; CPS=Coagulase Positive Staphylococci

Table 2: Comparison of study parameters (Mean \pm SE) between control and clinical and subclinical mastitis groups

Parameters	Control (n=10)	Clinical Mastitis (n=10)	Subclinical Mastitis (n=30)
TSA (mmol/L)	2.52 \pm 0.03 ^a	3.27 \pm 0.10 ^b	2.68 \pm 0.01 ^c
LBSA (mmol/L)	1.18 \pm 0.02 ^a	1.64 \pm 0.05 ^b	1.35 \pm 0.02 ^c
PBSA (mmol/L)	1.34 \pm 0.03 ^a	1.63 \pm 0.07 ^b	1.32 \pm 0.01 ^a
Hp (g/L)	0.09 \pm 0.00 ^a	0.96 \pm 0.03 ^b	0.59 \pm 0.03 ^c
SAA (μ g/mL)	5.13 \pm 0.20 ^a	129.08 \pm 6.92 ^b	47.33 \pm 4.55 ^c
HpSAA	0.017 \pm 0.001 ^a	0.007 \pm 0.000 ^b	0.016 \pm 0.002 ^a
LBSA/PBSA	0.89 \pm 0.04 ^a	1.01 \pm 0.03 ^b	1.02 \pm 0.02 ^b

Note: Different letters (a,b,c) in each row shows significant difference ($P < 0.05$). TSA: total sialic acid; LBSA: lipid bound sialic acid; PBSA: protein bound sialic acid; Hp: haptoglobin; SAA: serum amyloid A.

DISCUSSION

In the present study, the most dominant isolated bacterium from clinical and subclinical samples was *Staphylococcus aureus*, the most important bacterial agent of bovine mastitis (Lipman *et al.*, 1996; Phuektes *et al.*, 2001; Melchior *et al.*, 2007). The most dominant isolated bacterium from subclinical (41/65) mastitis samples was *Staphylococcus spp* of which 31.71% (n=6) and 19.51% (n=5) were *S. aureus* and *S. epidermidis*, respectively. Barkema *et al.* (1997) reported that the most bacterial agents in bovine subclinical mastitis were *S. aureus*,

Streptococcus agalactiae and *Corynebacterium bovis*. Shitandi and Kihumbu (2004) reported that *S. aureus* is the most bacterial agent of bovine subclinical mastitis.

As it was demonstrated, serum total sialic acid, lipid-bound sialic acid, Hp and SAA concentrations were significantly higher in diseased animals compared to healthy ones. The results showed that TSA and LBSA were significantly different in control group compared with clinical and subclinical mastitis ($P < 0.001$). Protein bound sialic acid did not change in subclinical mastitis in comparison with control group ($P = 0.86$).

Table 3: Spearman's rank correlations coefficients between study variables in control and clinical and subclinical mastitis groups

Group/Parameters	TSA	LBSA	PBSA	Hp
Control (n=10)				
LBSA	-0.09	-	-	-
PBSA	0.86**	-0.50	-	-
Hp	0.11	0.45	0.00	-
SAA	-0.14	-0.54	0.20	0.24
Clinical mastitis (n=10)				
LBSA	0.91**	-	-	-
PBSA	0.85**	0.72*	-	-
Hp	0.04	-0.06	0.28	-
SAA	-0.58	-0.58	-0.23	0.50
Subclinical mastitis (n=30)				
LBSA	0.70**	-	-	-
PBSA	-0.02	-0.62**	-	-
Hp	-0.03	0.07	-0.18	-
SAA	-0.14	0.06	-0.30	0.18

* $P < 0.05$, ** $P < 0.01$; TSA: total sialic acid; LBSA: lipid bound sialic acid; PBSA: protein bound sialic acid; Hp: haptoglobin; SAA: serum amyloid A.

Serum sialic acid values were analyzed in many inflammatory and infectious diseases in cattle (Karagenc *et al.*, 2005; Karapehlihan *et al.*, 2007) to which the results of this study are in agreement with. Citil *et al.* (2004) evaluated changes in sialic acid concentrations as an indicator for the determination of inflammatory process associated with TRP; it was observed that total, lipid- and protein-bound sialic acid concentrations in cattle with TRP were higher than those of healthy cattle. At the beginning of inflammatory reactions or in injury, serum SAA concentrations increase rapidly. However, the underlying mechanism that causes increase in serum SAA has not been clearly defined. Serum sialic acid may be a marker of the acute-phase response, since serum concentrations were significantly related to established acute-phase proteins such as alpha-1 acid glycoprotein (Haq *et al.*, 1993). Acute phase reactants influence total sialic acid concentrations due to their glycoprotein structure (Taniuchi *et al.*, 1981). The increase in PBSA levels may be attributable to elevated serum acute phase proteins during inflammation. It is demonstrated that SAA concentration increase rapidly following the inflammatory and injury process (Citil *et al.*, 2004). The increase level of sialic acid may alter receptor-ligand interactions, which are known to play an important role in inflammation and immune response (Karagenc *et al.*, 2005). On the other hand, increase of TSA and LBSA during inflammation and tissue damage is attributed to liberation of sialic acid

from cell membrane into circulation as sialic acid is abundantly present in all biological membranes (Haq *et al.*, 1993; Thougard *et al.*, 1998).

There was positive correlation between LBSA and PBSA in clinical mastitis ($r=0.72$, $P=0.02$) whereas significant negative correlation was observed between LBSA and PBSA in subclinical mastitis ($r=-0.62$, $P<0.001$). Results also showed no correlation between Hp and SAA with each other or with any other parameters in study groups.

SAA and Hp were significantly higher in diseased animals compared to healthy ones. Both SAA and Hp are among the major positive APPs in cattle and can increase several-fold from baseline levels after tissue injury (Murata *et al.*, 2004; Petersen *et al.*, 2004). SAA reflected the course of inflammation and its level correlated with the clinical severity of the inflammation. SAA has the greatest role in bacterial and pyogenic infections and increases in common infectious diseases such as metritis, haematologic, respiratory and digestive infections and TRP (Nazifi *et al.*, 2008a). The results of the present study is consistent with the study of Nazifi *et al.* (2008a), which reported that the concentration of SAA in cows with some inflammatory diseases such as TRP and acute metritis was higher compared to healthy ones. Hp is a prominent acute phase protein in most species studied, and statistically significant increase in Hp was observed between clinically healthy and diseased cows in similar researches with the same inflammatory diseases (Kato and Nakagawa, 1999; Ganheim *et al.*, 2003). Nazifi *et al.* (2008b) revealed that Hp concentration increase in some inflammatory diseases such as acute diffuse traumatic reticuloperitonitis and acute respiratory infections and the results of this study are in agreement with the previous ones. In another study done by Nazifi *et al.* (2009) on cattle with traumatic reticuloperitonitis, a significant increase and strong correlation between SAA and Hp were shown, indicating a very similar pattern of changes for these two APPs in various internal disorders in cattle including TRP.

The results of this study revealed that sialic acid (TSA, LBSA) and acute phase proteins (SAA and Hp) can be a suitable indicator of clinical and subclinical mastitis. However, mentioned parameters in this study increase in diseased cattle and it may be a useful indicator in differentiating diseased cattle from healthy ones.

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