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Original article

Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens

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Abstract

The aim of the present study was to determine the concentration of serum amyloid A (SAA) and the activity of ceruloplasmin (Cp) in milk from cows with subclinical mastitis caused by different pathogens. Eighty-four milk samples from cows with subclinical mastitis and fourteen milk samples from healthy cows were examined. SAA concentration was determined using the commercial ELISA kit (Tridelta Development Ltd., Greystones, Wicklow, Ireland). Cp activity was assessed spectrophotometrically, using the Rice method. The results reveal that the concentration of SAA (with exception of CNS) and activity of Cp in cow milk can be regarded as markers of subclinical mastitis, irrespective of the microorganism inducing the disease. In conclusion, measurement of SAA and Cp in milk samples could be a useful method in diagnosing subclinical mastitis in cows, but the method should be adapted for field use.

Key words: serum amyloid A, ceruloplasmin, subclinical mastitis, milk, cows

Introduction

Subclinical mastitis is common in dairy herds and causes diagnostic difficulties due to the lack of visible symptoms (Reneau and Packard 1991). The disease results in serious economic losses, mainly associated with reduced production of milk and lack of certificates for purchase (Seegers et al. 2003). The basic method for detection of mastitis in cows is determina-

tion of somatic cell count in milk. However, this method is not sufficiently efficient; therefore, alternative and more reliable markers are being sought.

Recently, many studies have been focused on the possible use of acute phase proteins as markers of mastitis in cows (Eckersall et al. 2001, 2006, Grönlund et al. 2003, Nielsen et al. 2004, O'Mahony et al. 2006, Akerstedt et al. 2007, Hiss et al. 2007, Kovac et al. 2007, Wenz et al. 2010, Pyörälä et al. 2011). Acute

phase proteins are blood proteins whose concentration changes in animals due to factors such as inflammation, tissue damage or stress (Murata et al. 2004, Petersen et al. 2004). Their blood concentration is related to the severity of disease and extent of tissue damage (Murata et al. 2004).

Serum amyloid A (SAA) belongs to the most important acute phase proteins in cattle (Kostro et al. 2001, Murata et al. 2004, Petersen et al. 2004). Although SAA is mostly produced during the acute phase response in the liver, its extra-hepatic production is also possible (Kostro et al. 2001, Murata et al. 2004). Numerous studies confirmed the production of SAA in the mammary gland (McDonald et al. 2001, Larson et al. 2005, Weber et al. 2006). The biological role of SAA has not been fully elucidated yet, but many data indicate its involvement in immune response modulation during infection (Petersen et al. 2004). The level of SAA increased rapidly in serum and milk following experimentally induced udder infection with *E. coli* (Suojala et al. 2008), *S. aureus* (Grönlund et al. 2003), *Str. uberis* (Pedersen et al. 2003) and intra-udder infusion of endotoxin (LPS) derived from the cell wall of *E. coli* (Lehtolainen et al. 2004). It was demonstrated that SAA levels increased much earlier in milk than in serum and preceded increased somatic cell count in milk (Pedersen et al. 2003).

Ceruloplasmin (Cp) belongs to the acute phase proteins whose serum level can increase by tens of percent due to infection or other tissue-damaging factor (Conner et al. 1986). The main source of Cp is hepatocytes yet it can also be synthesised in the mammary gland (Jaeger et al. 1991, Cerveza et al. 2000, Donley et al. 2002) and places of tissue damage (Kehrer 1993). Cp, as an extracellular antioxidant also present in colostrum and milk, is essential for anti-oxidative defence of the organism (Kim et al. 1998, Donley et al. 2002). Compared to other acute phase proteins, Cp is less frequently used as a diagnostic marker in cattle, nevertheless its value as a marker of various inflammations in cattle was confirmed (Conner et al. 1986, Chassagne et al. 1998, Sheldon et al. 2001). Increased activity of Cp in blood and milk was observed both in spontaneous (Conner et al. 1986, Szczubiał et al. 2008) and experimentally induced mastitis in the cow (Chacornac et al. 1986).

Many microorganisms, with staphylococci and streptococci being essential, can induce subclinical mastitis (Costa et al. 1998, Busato et al. 2000, Taponen and Pyörälä 2009). It is known that clinical symptoms and severity of the udder inflammatory reaction depend on the pathogenicity of a mastitis-inducing microorganism and extent of tissue damage (Pyörälä and Syvajarvi 1987). To date, only a few stu-

dies compared the levels of acute phase proteins in milk of cows with mastitis caused by various microorganisms.

The aim of the present study was to determine the concentration of SAA and the activity of Cp in milk from cows with subclinical mastitis caused by different pathogens.

Materials and Methods

The study material included milk samples collected from individual udder quarters of 84 cows (Polish Holstein-Friesian breed, 3-9 years of age) from the Lublin region herds. The cows were between 15 and 200 days after delivery. Subclinical mastitis was diagnosed based on clinical examinations of the udder, the TOK¹ test and bacteriological milk analyses (lack of clinical symptoms of mastitis, lack of changes in milk, positive (+, ++; +++) TOK results, presence of pathogens in milk were regarded as signs of subclinical mastitis). The study also involved 14 milk samples from healthy cows (Polish Holstein-Friesian breed, 2-6 years age) between 20 and 80 lactation day.

Milk, about 10 ml, was sampled into sterile test tubes. Bacteriological testing was carried out using phenotyping by colony morphology on agar with blood and on Sabouroud medium, Gram staining and cultures on TKT and Chapman media. The bacterial species were identified using API tests (20 Staph and 20 Strep, bioMerieux, France). After bacteriological testing, the samples were frozen at -76°C and kept for further analysis. The level of SAA and activity of Cp were determined.

Determination of SAA. SAA was determined using the commercial ELISA (Tridelta Development Ltd., Greystones, Wicklow, Ireland). Prior to testing, milk samples were diluted 1:50. The detection limit of the test was 0.9 µg/L. Results were expressed in µg/ml.

Determination of Cp activity. The activity of Cp was determined spectrophotometrically using the method described by Rice et al. (1963). The reaction mixture contained 1 ml of acetate buffer (300 mmol/l, pH 5.7) and 1 ml of *p*-phenylenediamine (0.2%). After the 5-minute incubation at 37°C, 100 µl of milk was added and the mixture incubated at 37°C for 30 min. To stop the enzymatic reaction, 2 ml of sodium azide (0.04%) was added. Following the 10-minute incubation at 20°C, the absorbance at 540 nm was measured. The control sample contained the same components but first 2 ml of sodium azide was added following 100 µl of milk after 30 minutes of incubation. The calculations were based on the formula:

¹ TOK test – the equivalent of California Mastitis Test

U/L = (absorbance of the examined sample – absorbance of control) x 137. Results were expressed in units per gram of protein (U/g protein).

Protein determination. The protein content was determined using the commercial kit (Total Protein Kit, Cormey, Lublin, Poland).

Statistical analysis. The data were statistically analysed by calculating the mean, standard deviation (\pm SD) and significance of differences. Inter-group differences were evaluated using the Statistica 5.0 software. The significance of differences was set at $P \leq 0.05$ and $P \leq 0.01$.

Results

The pathogens were found in all samples from cows with subclinical mastitis (Table 1). The most common bacteria isolated were coagulase-negative staphylococci (CNS) – 19 (22.6%), *Str. dysgalactiae* – 18 (21.4%) and *Str. uberis* – 18 (21.4%), followed by *S. aureus* – 12 (14.3%). *Str. agalactiae* were detected in 9 (10.7%) whereas *Candida spp.* in 8 (9.5%) cases. In the milk samples from healthy cows, no microorganisms were isolated.

Table 1. Pathogens isolated from milk of cows with subclinical mastitis.

Pathogens	Number (%)
<i>S. aureus</i>	12 (14.3)
<i>Str. Agalactiae</i>	9 (10.7)
<i>Str. Dysgalactiae</i>	18 (21.4)
<i>Str. Uberis</i>	18 (21.4)
CNS	19 (22.6)
<i>Candida spp.</i>	8 (9.5)
Total	84 (100.0)

The results of determinations of acute phase proteins are presented in Table 2. The concentration of SAA in milk from cows with subclinical mastitis ranged from 6.53 to 221.64 μ g/ml and in the milk from healthy cows – 5.24 – 19.04 μ g/ml. The comparison of mean values of SAA concentration in milk from cows with subclinical mastitis showed differences depending on the infecting pathogens. The highest mean concentration of SAA was found in the *Candida*-positive milk (101.52 ± 55.56 μ g/ml), whereas the lowest one in milk with CNS (12.47 ± 6.95 μ g/ml).

The concentration of SAA in *S. aureus*-positive milk was significantly higher than that in milk from cows with subclinical mastitis caused by CNS and significantly lower than in *Str. dysgalactiae*-, *Str. uberis*-

and *Candida*-induced inflammations. In milk from cows with mastitis induced by streptococci, the highest SAA concentration was detected in milk with *Str. uberis*. The statistically significant differences in SAA were found in milk with *Str. uberis* and *Str. agalactiae*. The SAA concentration in milk from cows with mastitis caused by CNS was significantly lower than in cases of mastitis induced by other bacteria and yeast-like fungi. Moreover, the concentration of SAA in milk with yeast-like fungi was significantly higher compared with milk with individual bacteria. Irrespective of the inducing pathogens, the mean SAA concentration was higher in milk from cows with mastitis compared with healthy cows (11.67 ± 7.40). The SAA differences in milk from healthy and affected cows were statistically significant, except for cows with mastitis caused by CNS.

Table 2. SAA concentration and Cp activity in milk from cows with subclinical mastitis depending on the isolated pathogens.

Pathogens	SAA (μ g/ml)	Cp (U/g protein)
<i>S. aureus</i>	** 41.75 ± 26.66^{aA} (8.76 – 74.75)	** 4.38 ± 0.36^{aA} (4.64 – 5.25)
<i>Str. agalactiae</i>	** 60.11 ± 17.53^b (9.78 – 111.26)	** 5.37 ± 1.17^A (4.23 – 8.02)
<i>Str. dysgalactiae</i>	** 72.43 ± 43.22^{Ac} (19.90 – 121.20)	** 5.55 ± 1.13^{ac} (3.71 – 7.24)
<i>Str. uberis</i>	** 92.23 ± 28.64^{abd} (8.58 – 221.64)	** 5.44 ± 0.94^{ad} (4.43 – 5.44)
CNS	12.47 ± 6.95^{abcde} (6.53 – 23.33)	** 4.51 ± 0.93^{cd} (3.91 – 5.10)
<i>Candida spp.</i>	** 101.52 ± 55.56^{ac} (9.61 – 199.36)	** 5.15 ± 1.09^A (3.35 – 6.78)
Pathogen-free milk	11.67 ± 7.40 (5.24 – 19.04)	1.20 ± 0.42 (0.73 – 2.11)

Explanations:

** – statistically significant differences compared to micro-organism-free milk at $P \leq 0.01$;

a, b, c, d, e – values in the same column denoted with the same letters are statistically significantly different at $P \leq 0.01$

A – values in the same column denoted with the same letters are statistically significantly different at $P \leq 0.05$

The activity of Cp in milk from cows with subclinical mastitis ranged from 3.35 to 8.02 U/g protein whereas in milk from healthy cows from 0.73 to 2.11 U/g of protein. Regardless of the inducing pathogens, the mean Cp activity in milk of cows with subclinical mastitis was significantly higher than in pathogens-free milk from healthy cows. A significantly lower Cp activity was observed in milk with *S. aureus*-

us compared to milk with streptococci and yeast-like fungi. Moreover, the mean Cp activity in milk from cows with mastitis caused by CNS was lower compared to milk from cows with mastitis caused by the remaining microorganisms; however, statistically significant differences were found in milk with *Str. dysgalactiae* and *Str. uberis*.

Discussion

The study revealed low SAA concentrations and low Cp activity in milk from healthy cows and their increased values in milk from cows with subclinical mastitis. The findings showed that acute phase reaction developed in cows with subclinical mastitis and confirmed earlier observations reported by other authors (Winter et al. 2003, Grönlund et al. 2005, Eckersall et al. 2006, Akerstedt et al. 2007, Hiss et al. 2007, Szczubiał et al. 2008, Pyörälä et al. 2011). Acute phase proteins are mainly produced in the liver, yet it was demonstrated that both SAA (McDonald et al. 2001, Weber et al. 2006) and Cp (Jaeger et al. 1991, Cerveza et al. 2000, Donley et al. 2002) could also be produced in the udder. The udder production of these proteins indicates the relation between their concentration and udder health conditions.

Besides haptoglobin, SAA is the most commonly determined acute phase protein to monitor the health of cows (Kostro et al. 2001, Petersen et al. 2004). Numerous studies have demonstrated low or undetectable levels of SAA in milk from healthy quarters and increased SAA levels in infected quarters of the same udder (Eckersall et al. 2001, Grönlund et al. 2003). Furthermore, increased SAA level is found to occur much earlier in serum and preceded increased somatic cell count (Pedersen et al. 2003). According to Nielsen et al. (2004), inflammatory processes outside the udder, which cause an increase in serum SAA levels, do not induce its significant increase in milk. Increased SAA levels have also been observed in collected milk from cows with subclinical mastitis (Akerstedt et al. 2007). The results reported by Grönlund et al. (2003), Pedersen et al. (2003) and Akerstedt et al. (2007) indicate that SAA is a better marker of subclinical mastitis than haptoglobin.

Cp is a ferroxidase involved in anti-oxidative defence of the organism (Murata et al. 2004). The studies carried out by Donley et al. (2002) demonstrate that the majority of Cp present in milk comes from the mammary gland and only a small proportion permeates to milk from blood. Cp exerts anti-inflammatory effects by reducing the adhesion of neutrophils to the endothelial cells and removal of free radicals (Murata et al. 2004). Increased Cp activity in milk was

detected both in clinical and subclinical mastitis in cows (Chasagne et al. 1998, Szczubiał et al. 2008) which suggests its usefulness as an early diagnostic marker of mastitis.

The SAA concentrations and Cp activity in milk from cows with subclinical mastitis disclosed significant differences depending on the aetiological factor. The highest SAA concentration was observed in milk with *Candida* and environmental streptococci (*Str. dysgalactiae*, *Str. uberis*), whereas the lowest one in milk with CNS. The highest activity of Cp was found in milk from cows with *Str. dysgalactiae*- and *Str. uberis*-induced mastitis; the lowest one in milk from cows with mastitis caused by *S. aureus* and CNS. Our findings correspond to the results reported by other authors (Hiss et al. 2007, Wenz et al. 2010, Pyörälä et al. 2011).

Hiss et al. (2007), who studied the concentration of haptoglobin in milk from cows with subclinical mastitis, demonstrated that mean levels of this acute phase protein in milk infected with the major pathogens (*E. coli*, *S. aureus*) were higher than in cases of mastitis caused by minor pathogens (*C. bovis*, CNS). The highest concentration of haptoglobin was observed in milk from cows with *E. coli* – induced subclinical mastitis and the lowest one in CNS infections. According to Wenz et al. (2010), the concentration of haptoglobin and lipopolysaccharides-binding protein (LBP) was higher in milk with Gram-negative bacteria compared to their concentrations in Gram-positive-infected milk. Likewise, Pyörälä et al. (2011) observed the highest concentrations of haptoglobin and SAA in milk from cows with *E. coli* – induced mastitis and the lowest concentrations in milk from cows with CNS-induced mastitis. Moreover, experimental studies revealed very high concentrations of acute phase proteins in milk infected with *E. coli* (Suojala et al. 2008) compared to *S. aureus* (Grönlund et al. 2003, Eckersall et al. 2006) and *Str. uberis*-induced udder infections (Pedersen et al. 2003). The high concentration of acute phase proteins in milk from cows with *E. coli*-induced mastitis reflects the strong inflammatory reaction caused mainly by LPS present in the cell membrane of Gram-negative bacteria (Larson et al. 2005). In our study, *E. coli* was not isolated in milk from cows with subclinical mastitis, therefore our results cannot be compared with the reported findings.

In our study, the majority of milk samples contained staphylococci, which confirms that these bacteria are most commonly responsible for subclinical mastitis in cows (Taponen and Pyörälä 2009). The staphylococci inducing mastitis in cows include coagulase-positive *S. aureus* and many coagulase-negative staphylococci, which are considered the normal skin flora (Devrise and De Keyser 1980). *S. aureus* belongs

to the group of major pathogens while CNS are considered minor pathogens (Taponen and Pyörälä 2009). Both induce mastitis of low severity, often chronic or subclinical (Sutra et al. 1999, Taponen and Pyörälä 2009). This may explain lower levels of acute phase proteins in milk from cows with mastitis caused by these bacteria, compared to environmental streptococci (*Str. dysgalactiae* and *Str. uberis*) found in our study and reported by other authors (Pyörälä and Syväjarvi 1987, Pyörälä et al. 2011). High concentration of SAA and activity of Cp in milk infected with environmental streptococci indicates their high pathogenicity, which confirms the fact that these streptococci cause mainly clinical, often acute mastitis (Pyörälä and Syväjarvi 1987, Pedersen et al. 2003).

Our findings showed particularly low concentrations of SAA in milk with CNS. Slight increases in SAA concentration in milk from cows with mastitis caused by CNS was also reported by other authors (Simojoki et al. 2009, Pyörälä et al. 2011).

The highest SAA concentration demonstrated in milk from cows with subclinical mastitis caused by *Candida spp.* is likely to indicate the highest intensity of the inflammatory reaction in quarters infected with these microorganisms and to reflect their high pathogenicity. Fungal mastitis caused by them can be clinical (acute, sub-acute and chronic) or subclinical (Krukowski 2001). In most cases, subclinical inflammations caused by yeast-like fungi are long-lasting. The literature provides no studies on the levels of acute phase proteins in milk from cows with fungus-induced subclinical mastitis, which makes the comparisons with our results impossible.

The differences in SAA concentration and activity of Cp observed in milk might have resulted from various amounts of pro-inflammatory cytokines released under the influence of individual pathogens. It is known that the production of acute phase proteins is stimulated by these cytokines (Murata et al. 2004). Riolett et al. (2000) demonstrated the differences in serum cytokine levels after experimental *E. coli* and *S. aureus*-induced udder infections. The differences in the levels of acute phase proteins in milk samples infected with different pathogens may also be explained by different duration of mastitis. In experimental *E. coli* mastitis, the concentration of SAA increased after 12 hours and its highest concentration was observed 60 hours after the infection (Suojala et al. 2008). The experimental *Str. uberis*-induced udder infection caused increased concentration of SAA in milk after 6 hours; after 12 hours the SAA concentration was ten-fold higher, compared to pre-infection values (Pedersen et al. 2003). In milk from sheep with experimental *S. epidermidis*-induced mastitis, the highest values of SAA concentration were found 24 hours

after the infection; the concentration decreased 144 hours after the infection (Winter et al. 2003). The above studies indicate that the level of acute phase proteins in milk with mastitis shows some dynamics and the time of sample collection affects the values determined. In our study, the duration of subclinical mastitis caused by individual pathogens was unknown, which might have affected the differences in SAA concentration and activity of Cp in milk from cows with subclinical mastitis caused by these microorganisms. Moreover, it cannot be excluded that the differences in levels of acute phase proteins were affected by the amount of microorganisms in milk, yet such determinations were beyond the scope of our study.

Our results indicate that SAA (with exception of CNS) and Cp determinations in cow milk may be the markers of subclinical mastitis, irrespective of the aetiological factor inducing the disease. In conclusion, measurement of SAA and Cp in milk samples could be a useful method in diagnosing subclinical mastitis in cows, but the method should be adapted for field use.

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