Interrelationship between Somatic Cell Count and Acute Phase Proteins in Serum and Milk of Dairy Cows

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Abstract

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The aim of the present study was to compare the concentrations of two acute phase proteins, haptoglobin (Hp) and serum amyloid A (SAA), in serum and milk of Holstein-Friesian dairy cows grouped according to somatic cell count thresholds (< 100,000 < 400,000 > 400,000 cells/ml) in composite milk samples. The SCC was assessed quantitatively by FOSSOMATIC 90 analyser; serum and milk Hp and SAA concentrations were determined using commercial ELISA kits (Tridelta Development, Ltd., Wicklow, Ireland). We have found a significantly higher Hp and SAA concentrations in the group with SCC > 400,000 cells/ml compared to groups with lower SCC thresholds in composite milk samples. In contrast, significant differences were not demonstrated between the groups in Hp and SAA concentrations determined in serum. The concentrations of Hp in serum and milk were found to be correlated ($r = 0.69$, $P = 0.0003$), whereas there was a lower correlation trend in the case of serum versus milk concentrations of SAA ($r = 0.43$, $P = 0.0478$). High significant correlations were observed either between milk haptoglobin values and SCC or milk serum amyloid A concentrations and SCC ($r = 0.83$, $r = 0.81$, $P < 0.0001$; respectively). An increase in SCC in cows suffering from mastitis has been accompanied by strong elevation of the milk Hp and SAA, significantly correlated with serum Hp ($r = 0.64$, $P = 0.0014$) and poorly correlated with SAA values determined in serum ($r = 0.43$, $P = 0.0478$). According to the results obtained in this study we can conclude that measurements of the acute phase proteins, haptoglobin and serum amyloid A predominantly in milk, may be a useful tool in diagnosing mastitis and may be a useful marker of milk quality.

Haptoglobin, serum amyloid A, mastitis

Mastitis affects the milk quality and production of the cow and may spread to other cows in the herd. In a well-managed dairy herd, in addition to clinical mastitis, subclinical mastitis should be efficiently detected. Indicators of inflammation in the milk, which can be determined using rapid, reliable and easy routine techniques, should be used for the early detection of mastitis.

The golden standard to measure inflammation of the mammary gland is the cytological investigation, milk somatic cell count (SCC), and other methods are compared with this variable (Hamann 2002). Recently an SCC limit of the 100,000 cells/ml was suggested for a healthy quarter (Hillerton 1999). For an udder with four healthy quarters, the count for the composite milk should not exceed 100,000 cells/ml (Laevens et al. 1997; Ma et al. 2000). If a somatic cell count exceeds 200,000 cells/ml in a composite sample of a cow, there is a 60% probability that this cow is infected in one or more quarters (Mellerberger 1999). In Europe, the Directive 92/46/EEC in 1992 stated that bulk milk samples with SCC over 400,000 cells/ml may not be used for fluid milk and since 1998 not even for human consumption.

Mastitis changes the composition of milk and the degree of changes depends on the pathogenicity of the mastitis-causing bacteria and the amount of affected tissue in the gland,

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especially the affected epithelial area. The main changes in the udder include leaking the ions, proteins and enzymes from the blood into the milk due to increased permeability, invasion of phagocyting cells into the milk compartment, and a decrease of the systemic capacity of the gland, resulting in decreased concentrations of certain milk constituents (Pyörälä 2003). The role of Hp is to bind haemoglobin released by damaged erythrocytes, and to help restrict the ability of free iron to invading bacteria. No biological function has been firmly established for SAA yet.

The affected quarter may also produce substances related to the inflammatory reaction such as acute phase proteins (Eckersall et al. 2001). The hypothesis that the major acute phase proteins in cows, haptoglobin (Hp) and serum amyloid A (SAA), may be transferred into milk during the acute phase response caused by mastitis was therefore investigated by adapting the assays to measure the concentration of proteins in milk. Furthermore, the acute phase protein, SAA3 has been identified in milk from cows with clinical mastitis and has significant potential as an early diagnostic marker for clinical and sub-clinical mastitis (Eckersall et al. 2005). Therefore acute phase proteins, haptoglobin and serum amyloid A, are also potential candidates for monitoring mastitis.

The objective of this experiment was to evaluate and compare the serum and milk concentrations of Hp and SAA in dairy cows selected on the basis of somatic cell count in composite milk samples.

Materials and Methods

Animals and samples

Samples were collected from 22 Holstein-Friesian dairy cows from a commercial dairy farm with 368 cows. The cows were lactating, primi- and multiparous, and had calved more than 10 days previously. They were selected according to general clinical examination (to detect visible inflammatory processes) and examination of the udder and milk samples. Cows with clinical symptoms of inflammatory processes were not included to the study.

Blood samples were taken from *v. caudalis mediana*, allowed to clot at room temperature, stored overnight at 4 °C and then centrifuged at 2,500 *g* for 15 minutes. Serum was separated and stored at -18 °C until analyses for Hp and SAA could be conducted.

Milk samples were collected into sterile plastic vials by hand-stripping. The surface of the teat end was disinfected by wiping it with clean cotton dipped in 95% alcohol. Forestrips were milked out and then 5 to 10 ml was milked into the vials, which were held almost horizontally to avoid bacterial contamination. The milk samples were taken from all four quarters for each cow where following variables were determined. The milk samples were examined by bacteriological cultivation, using standard methods (cultivation on the Columbia blood agar, Baird-Parker agar and Endo agar). The somatic cell counts were assessed quantitatively by FOSSOMATIC 90 analyser. Samples of whole milk were stored at -18 °C until they were analysed for Hp and SAA. The composite milk samples were prepared on the day of Hp and SAA detection as follows; prior to mixture all quarter milk samples were vortexed vigorously and afterwards an equal part (1 ml) of milk from each quarter was taken and mixed together.

After the aforementioned determinations, cows were divided into three groups according to the somatic cell count (SCC) in composite milk samples. Group I contained 7 cows with SCC < 100, 000 cells/ml on a composite milk sample, while SCC from each quarter did not exceed limit 100,000 cells/ml. Group II contained 7 cows with SCC < 400,000 cells/ml on a composite milk sample, while SCC from each quarter did not exceed limit 400,000 cells/ml but was higher than 100,000 cells/ml at least in one quarter. Group III contained 8 cows with SCC > 400,000 cells/ml on a composite milk sample (at least in two quarters of each cow the higher SCC score than 400,000 cells/ml was detected).

Serum and milk Hp concentrations

Serum and milk Hp concentrations (µg/ml) were determined using commercial ELISA kits (Tridelta Phase Range kit, Tridelta Development, Ltd., Wicklow, Ireland), and performed according to the manufacturer's instructions. Serum and milk samples were initially diluted 1 : 500 and 1 : 50, respectively, and all samples including the standards, were tested in duplicate. Samples with an optical density outside the range of the standard curve were diluted further and re-analyzed. The maximum dilution was 1 : 2000 for the serum samples and 1 : 80 for the milk samples. The optical densities were read on an automatic plate reader (Dynatech Laboratories, GB) at 450 nm. Lower limit of detection declared by manufacturer was 15.6 µg/ml for serum and 0.3 µg/ml for milk.

Serum and milk SAA concentrations

Serum and milk SAA concentrations (µg/ml) were determined using commercial ELISA kits (Tridelta Phase Range kit, Tridelta Development, Ltd., Wicklow, Ireland), first described by McDonald et al. (1991), and performed according to the manufacturer's instructions. Serum and milk samples were initially diluted 1: 500 and

1 : 50, respectively, and all samples including the standards, were tested in duplicate. Samples with an optical density outside the range of the standard curve were diluted further and re-analyzed. The maximum dilution was 1 : 2000 for the serum samples and 1 : 1500 for the milk samples. Optical densities were read on an automatic plate reader (Dynatech Laboratories, GB) at 450 nm. Lower detection limit declared by manufacturer was of 0.3 μg/ml for serum and 0.3 μg/ml for milk.

Statistical analysis

Hp and SAA concentrations for all three groups were compared by using a Kruskall-Wallis ANOVA. Differences were considered significant at $P < 0.05$. Spearman correlation coefficients were used to identify associations between serum and milk Hp and serum and milk SAA concentrations. The correlations between SCC and concentrations of Hp and SAA in serum or milk were tested by Spearman correlation as well. All statistical analyses were carried out in Graph Pad Prism, version 3.00.

Results

Bacteriological examination of milk samples

In total, 88-quarter milk samples were examined bacteriologically and following pathogens were identified: *E. coli* and *Staphylococcus aureus.* Of the 7 cows in the first group, 2 had sterile samples from all quarters, 5 had positive samples from one to three quarters. Of the 7 cows and 8 cows in the second and third group, respectively, 3 cows in each group had sterile samples from all four quarters; the rest ones had positive samples from one to three quarters.

Concentrations of haptoglobin in serum and milk

None of the cows from group I (SCC < 100,000 cells/ml) had a detectable concentration of haptoglobin either in serum or milk samples. In 2 of the 7 cows from group II (SCC < 400,000 cells/ml) the serum concentration of haptoglobin was detected, milk haptoglobin concentration was determined in one sample; the rest of them were under the detection limit. In the cows from group III (SCC > 400,000 cells/ml) 5 from 8 serum samples were detected; milk haptoglobin samples were all determined except one (Table 1, Table 2).

Table 1. Median and ranges of haptoglobin and SAA concentrations in the serum of dairy cows grouped according to SCC score in composite milk samples

SCC	Haptoglobin (μg/ml)			SAA (μg/ml)		
	Samples detectable* (> 15.6 μg/ml)	Median	Range	Samples detectable* (> 0.3 μg/ml)	Median	Range
< 100.000/ml	0/7	0	0-0	7/7	35.9	5.81-69.0
< 400.000/ml	2/7	0	0-500	7/7	93.54	13.53-376.0
> 400.000/ml	5/8	73.76	0-500	8/8	81.68	14.02-529.4

Number of samples with a concentration greater than the lower limit of detection of the assay used/total number of samples. NS (Kruskall-Wallis ANOVA).

Table 2. Median and ranges of haptoglobin and SAA concentrations in composite milk samples of dairy cows grouped according to SCC score

SCC	Haptoglobin (μg/ml)			SAA (μg/ml)		
	Samples detectable* (> 0.3 μg/ml)	Median	Range	Samples detectable* (> 0.3 μg/ml)	Median	Range
< 100.000/ml	0/7	0[a]	0-0	5/7	0.67[a]	0-1.79
< 400.000/ml	1/7	0[a]	0-0.68	5/7	1.52[a]	0-7.15
> 400.000/ml	7/8	6.76[b]	0-20.0	8/8	26.54[b]	1.81-54.28

Number of samples with a concentration greater than the lower limit of detection of the assay used/total number of samples. Within the columns the numbers with different superscripts differ significantly (Kruskall-Wallis ANOVA; $P < 0.05$)

The serum concentrations of haptoglobin did not differ significantly among the groups (Table 1). Milk haptoglobin concentrations were significantly different ($P < 0.05$) between three groups, with group III (SCC > 400,000 cells/ml) having the highest concentrations (Table 2, Fig 1a). Non-parametric analysis showed that there was a significant correlation ($r = 0.69$, $P = 0.0003$) between the serum and milk haptoglobin concentrations.

Serum concentrations of haptoglobin showed significant correlation with SCC ($r = 0.64$, $P = 0.0014$), whereas a higher significant correlation ($r = 0.83$, $P < 0.0001$) was found between milk haptoglobin and SCC.
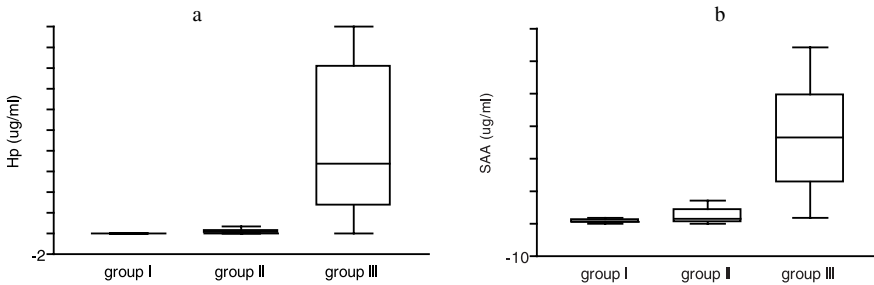

Fig. 1. Milk concentration of (a) Haptoglobin (Hp) and (b) serum amyloid A (SAA) in the three somatic cell count (SCC) score groups; group I (SCC < 100,000 cells/ml), group II (SCC < 400,000 cells/ml), group III (SCC > 400,000 cells/ml)

Concentrations of serum amyloid A (SAA) in serum and milk

The entire group I (SCC < 100,000 cells/ml) had a detectable concentration of serum SAA, while 2 from 7 milk samples had lower values of SAA than the detection limit of the assay (< 0.3 μg/ml). In the cows from group II (SCC < 400,000 cells/ml) the serum concentrations of SAA ranged from 13.53 to 376.0 μg/ml and milk SAA concentration was determined in 5 from 7 samples. The concentrations of SAA in the serum of the cows from group III (SCC > 400,000 cells/ml) ranged from 14.02 to 529.4 μg/ml and all milk samples had detectable concentrations of SAA (Table 1, Table 2).

The serum concentrations of SAA did not differ significantly among the groups (Table 1). The concentrations of SAA in milk were significantly different ($P < 0.05$) between three groups, with group III (SCC > 400,000 cells/ml) having the highest concentrations (Table 1, Fig. 1 b). Non-parametric analysis revealed that there was a lower correlation trend ($r = 0.43$, $P = 0.0478$) between serum and milk SAA concentrations.

Serum concentrations of SAA showed slight significant correlation with SCC ($r = 0.45$, $P = 0.034$), whereas a closer correlation ($r = 0.81$, $P < 0.0001$) was found between milk SAA and SCC.

Discussion

The diagnosis of mastitis is based predominantly on a clinical examination, measurements of somatic cell count and the cultivation of pathogens from the milk, but the demand for objective and rapidly assessable markers of udder health has increased. The acute phase proteins have recently been suggested as such indicators of inflammation in the mammary gland (Hirvonen et al. 1999; Eckersall et al. 2001, 2005; Nielsen et al. 2004). For them to be useful as specific indicators of mastitis it is therefore essential that they accumulate in milk only during episodes of mammary inflammation. The results of the study by Nielsen et al. (2004) show that the concentrations of Hp and SAA were higher in milk from infected quarters and those extramammary inflammatory processes that induced

increases in the serum concentration of Hp and SAA were not accompanied by increases in its concentration in milk.

In our study significant differences were not demonstrated in serum Hp and SAA concentrations in the groups divided according to SCC thresholds. Haptoglobin serum concentrations increased in experimentally induced mastitis (Hirvonen et al. 1996, 1999) and in field infections of different etiology (Ohtsuka et al. 2001). Serum Hp concentrations were higher in cows suffering from mastitis compared to healthy cows, but no difference was observed between the cows suffering from mild and moderate mastitis (Eckersall et al. 2001). However, others consider Hp as indicating the severity of infection and predicting the fatal outcome in heifers with experimentally induced mastitis (Hirvonen et al. 1996). The reason for this difference remains unexplained, but the use of different infection models might be a plausible cause. Serum amyloid A has also been found to be a marker of experimentally induced (Pedersen et al. 2003) and naturally occurring mastitis (Eckersall et al. 2001). In experimentally induced *E. coli* FT238 mastitis, the serum SAA response was found to relate with the severity of infection (Hirvonen et al. 1999). In cows suffering from mild to moderate clinical mastitis caused by field infections of different origins, higher SAA serum concentration was found compared to healthy cows, but no correlation between SAA and disease severity was found (Eckersall et al. 2001).

It seems that the acute phase response occurred during mastitis followed by an increase of the acute phase proteins is more accurately detectable in milk rather than in serum. In our study, milk Hp and SAA accurately reflect the degree of inflammation so that in cows with mastitis, determined by exceeded somatic cell count (> 400,000 cells/ml), milk Hp and SAA levels were significantly higher than in cows with SCC less than 400,000 cells/ml in composite milk samples. The milk Hp concentrations were defined as zero in the group of cows with SCC lower than 100,000 cell/ml because all of them were under the detection limit. In the case of milk SAA in the total of five samples from seven were detected, although the mean value was not higher than 1 µg/ml. The advantage of haptoglobin and SAA over other proteins as markers of mastitis is attributable to the fact that they are not present in the milk of healthy dairy cows. In the study by Eckersall et al. (2001), both tests measured the milk haptoglobin and SAA concentrations, had a high specificity (100%) with no false positive results, and a reasonable sensitivity (86 and 93%) for the diagnosis of mastitis. The assays for haptoglobin and SAA have the disadvantage of being ELISA tests, whereas the biochemical assays are faster and may be more easily adapted to automatic analysis systems. On the other hand, an ELISA can detect lower concentrations and is less sensitive to disturbing factors than the biochemical test (Pyörälä 2003).

Although the acute phase proteins have been conventionally thought to be synthesised in the liver, there have been reports of the expression of the messenger RNA for these proteins during the acute phase response in extrahepatic tissues such as lung (Yang et al. 1995), intestinal epithelium (Vreugdenhil et al. 1999) and endometrium (Sharpe-Timms et al. 1998). A lack of correlation between SAA in milk and serum (Eckersall et al. 2001), the expression of a serum amyloid protein homologue in the mammary gland (Molenaar et al. 2002, 2005) and the finding of a milk-specific form of bovine SAA (McDonald et al. 2001) indicate that SAA is also produced locally in the mammary gland and not just present in milk due to disruption of the blood-milk barrier. The concentration of Hp in serum and milk was found to be correlated, indicating that Hp in milk may originate from serum (Petersen et al. 2004).

It has been shown that in some cases acute phase proteins appear in the milk of cows with clinical mastitis just prior to or at the same time as SCC starts to rise (Hogarth et al. 2002). The concentration of SAA in milk samples increased prior to the increase in SCC in cows

experimentally infected with *Streptococcus uberis* (Pedersen et al. 2003). In the present study, the milk concentrations of the acute phase proteins increased significantly with increasing SCC thresholds, indicating that haptoglobin and SAA may be valuable as an indicator of the mastitis severity. A high correlation trend between SCC and milk values of Hp and SAA indicating, that grouping the cows according to SCC in milk samples can predict the variations of Hp and SAA concentrations in milk.

Practically, SCC cannot be used alone as a marker to identify infected quarters (Zecconi and Piccinini 2002). This represents a real risk for farmers or practitioners that use SCC as the only marker to select "suspicious" infected cows. Frequently, *Staphylococcus aureus* intramammary infections are characterized by low SCC (< 200,000 cells/ml), particularly at the beginning of lactation. These cases, if undetected, have a high probability to develop towards a chronic subclinical infection. In our study, we have confirmed the presence of positive milk samples in all three detected groups. The presence of positive *Staphylococcus aureus* milk samples and in one case *E. coli* sample were not accompanied by a high APP milk increase, neither by SCC elevation. It seems that bacteriological findings in our study had no interference with the inflammatory processes and acute phase response in the udder.

If APPs are produced locally in the udder as a response to mastitis might be more rapid and sensitive markers of acute inflammation than the somatic cell count. Furthermore, by testing milk, a large number of samples are easily obtained in a way that is less stressful than obtaining a blood sample (Petersen et al. 2004).

The results of the present study showed that measurements of the acute phase proteins, haptoglobin and serum amyloid A predominantly in milk, may be useful in the monitoring of udder health and have a high significant correlation with the SCC in milk.

Vzťah medzi somatickými bunkami a proteínmi akútnej fázy v sére a mlieku dojníc

Cieľom prezentovanej práce bolo porovnať koncentrácie dvoch proteínov akútnej fázy, haptoglobínu (Hp) a sérového amyloidu A (SAA) v sére a mlieku Holštýnsko-Frízskych dojníc zoskupených podľa počtu somatických buniek (< 100 000, < 400 000, > 400 000 buniek/ml) v zmesných vzorkách mlieka. Počet somatických buniek bol hodnotený kvantitatívne pomocou analyzátora FOSSOMATIC 90. Koncentrácie Hp a SAA v sére a mlieku boli stanovené použitím komerčných ELISA kitov (Tridelta Development, Ltd., Wicklow, Írsko). Zistili sme signifikantne vyššie koncentrácie Hp a SAA v skupine s počtom somatických buniek > 400 000 buniek/ml v porovnaní so skupinami s nižším rozpätím somatických buniek v zmesných vzorkách mlieka. Naopak, medzi skupinami sa neprejavili signifikantné rozdiely v koncentráciách Hp a SAA stanovenými v sére. Koncentrácie Hp v sére a mlieku sa ukázali ako korelujúce (r = 0,69; *P* = 0,0003), zatiaľ čo v prípade koncentrácie SAA v sére a mlieku bol korelačný trend nižší (r = 0,43; *P* = 0,0478). Vysoká signifikantná korelácia bola zaznamenaná buď medzi hodnotami haptoglobínu v mlieku a somatickými bunkami alebo medzi koncentráciou SAA v mlieku a somatickými bunkami (r = 0,83; r = 0,81; *P* < 0,0001; resp.). Nárast somatických buniek u kráv s mastitídou bol sprevádzaný výrazným vzostupom Hp a SAA v mlieku, signifikantne koreloval so sérovým Hp (r = 0,64; *P* = 0,0014) a slabo koreloval s hodnatami SAA stanovenými v sére (r = 0,43; *P* = 0,0478). Na základe výsledkov získaných v tejto práci môžeme uzavrieť, že meranie proteínov akútnej fázy, haptoglobínu a sérového amyloidu A prevažne v mlieku, môže byť užitočným nástrojom v diagnostike mastitídy a užitočným ukazovateľom kvality mlieka.

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