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Original article

Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis

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Abstract – New tools are needed to detect chronic sub-clinical mastitis, especially in automatic milking systems. Haptoglobin and serum amyloid A (SAA) are the two most sensitive bovine acute phase proteins, and their concentrations increase in milk from cows with clinical mastitis and in milk from cows with experimentally induced chronic sub-clinical *Staphylococcus aureus* mastitis. The aim of this study was to further evaluate the potential for haptoglobin and SAA in milk as indicators of chronic sub-clinical mastitis. Quarter milk samples were collected from 41 cows with a mean composite milk somatic cell count (CSCC) above 300 000 cells/mL during at least two months prior to sampling. Quarter milk samples were also taken from eleven cows with a mean CSCC below 80 000 cells/mL during at least two previous months. These samples were analysed for haptoglobin, SAA, adenosine triphosphate (ATP) activity and bacterial growth. The samples were grouped according to their ATP, haptoglobin and SAA status. ATP+ samples had ATP > 2×10^{-10} mol/mL, Hp+ and SAA+ samples had detectable levels of haptoglobin (≥ 0.3 mg/L) and SAA (≥ 0.9 mg/L), respectively. In udder quarter samples from healthy cows, 42 out of 44 samples belonged to the ATP–Hp–SAA– group. Among cows with chronic sub-clinical mastitis, the ATP+Hp+SAA+ group contained 66 out of 164 samples while 44 samples belonged to the ATP+Hp–SAA– group. Detectable levels of haptoglobin and SAA were found in 92 and 80 samples, respectively. Growth of udder pathogens was detected in 28 samples and *Staphylococcus aureus* was the most common bacteria. In conclusion, haptoglobin and SAA concentrations below the detection limit were considered as good indicators of healthy udder quarters. A substantial variation in haptoglobin and SAA concentrations in milk was observed in udder quarters with chronic sub-clinical mastitis.

serum amyloid A / haptoglobin / chronic / sub-clinical / mastitis

1. INTRODUCTION

Chronic sub-clinical mastitis is a common problem in dairy herds with considerable economic consequences, mainly due to reduced milk production and discarded milk [20]. For successful mastitis control in a herd, rapid and accurate diagnosis of mastitic cows is crucial. Detection of clinical mastitis by visual inspection and palpation is relatively

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easy, but diagnostic problems arise when dealing with sub-clinical mastitis, where an increased somatic cell count (SCC) is the only finding. Today, sub-clinical mastitis is mostly diagnosed by cow-side tests like California Mastitis Test (CMT), or by laboratory analyses of SCC using automatic cell counters. SCC can also be determined indirectly by measuring the adenosine triphosphate (ATP) [4, 5, 14] or N-acetyl-ß-D-glucosaminidase [10] activity in milk. In addition, electrical conductivity is relatively commonly used in some milking systems for on-line detection of mastitis. However, this analysis is less efficient in detecting chronic sub-clinical mastitis than for acute clinical cases [12]. Therefore, it is of importance to investigate alternative parameters and methods of detection.

The acute phase response (APR) is triggered when an animal is subjected to external or internal challenges, such as infection, inflammation, trauma or stress. A prominent event in the APR is the increase in acute phase proteins (APP). The APP are considered to be non-specific innate immune components involved in the restoration of homeostasis and restraint of microbial growth before animals develop acquired immunity to a challenge (reviewed by [16]). The two most sensitive bovine APP, haptoglobin and serum amyloid A (SAA), have been well studied in serum [2, 3, 6–8, 13, 15, 21], and in milk during acute clinical mastitis [3, 6, 15]. However, to our knowledge there are few reports on APR during episodes of sub-clinical mastitis. In a previous study, we found elevated levels of both haptoglobin and SAA in milk from udder quarters with sub-clinical mastitis 21 to 35 days after experimental infection with *Staphylococcus aureus,* but only SAA was significantly different from pre-infection levels [6]. Winter et al. [22] also described elevated levels of SAA during sub-clinical experimentally induced *Staphylococcus epidermidis* mastitis in ewes. Several studies have also stated that healthy cows have very low, or undetectable, serum levels of SAA and haptoglobin [1–3, 6], and this is also the case in milk from healthy quarters [3, 6]. The latter studies also report that the rise in haptoglobin and SAA levels is specific for the infected udder quarter, and that milk from healthy quarters in cows with mastitis have low, or undetectable, contents of these APP.

In the study by Grönlund et al. [6], the APP response to one strain of *S. aureus* was tested under experimental conditions in a small number of animals during a limited time period after infection. During field conditions, several bacterial species and strains can cause sub-clinical mastitis of varying degree and duration. Therefore, the aim of this study was to examine if haptoglobin and SAA can be detected in quarter milk samples from cows with naturally occurring chronic sub-clinical mastitis. To further evaluate the potential for milk haptoglobin and SAA in mastitis diagnostics, comparisons were also made between APP concentrations in udder quarter milk samples and in composite milk samples.

2. MATERIALS AND METHODS

2.1. Animals and samples

Udder quarter milk samples were collected from 41 dairy cows (13 herds) with a cow composite milk SCC (CSCC) above 300 000 cells/mL for at least two consecutive monthly recordings previous to the sampling, i.e. chronic sub-clinical mastitis. The cows were in lactation numbers 1 to 8 (median of 2), lactation months 2 to 13 (median of 5) and produced between 16.8 to 48.0 kg milk per day (median of 30.2). Udder quarter milk samples were also collected from 11 healthy control cows with a CSCC below 80 000 cells/mL for at least two consecutive monthly recordings before the sampling. Their lactation numbers were 1 to 5 (median of 2), their lactation month 2 to 13 (median of 6) and their milk production was from 21.3 to 44.6 kg milk per day (median of 28.5). The cows were of the two main

Table I. Concentrations (mg/L) of haptoglobin and serum amyloid A (SAA), and bacteriological growth in relation to ATP ($\times 10^{-10}$ mol/mL) interval in 164 udder quarter milk samples from 41 cows with chronic sub-clinical mastitis in at least one udder quarter.

ATP[a]	No. (%) of quarters	No. (%) bact+[b]	Haptoglobin[c]		SAA[c]	
			Range	Median	Range	Median
≤ 2	21 (13)	1 (4)	< 0.3–1.2	< 0.3	< 0.9	< 0.9
> 2–≤ 5	49 (30)	2 (7)	< 0.3–3.7	< 0.3	< 0.9–15.6	< 0.9
> 5–≤ 15	44 (27)	5 (18)	< 0.3–132	1.0	< 0.9–22.7	1.3
> 15–≤ 50	36 (22)	10 (36)	< 0.3–232	5.4	< 0.9–22.3	3.1
> 50	14 (8)	10 (36)	< 0.3–358	21.6	< 0.9–151	3.5
Total	164 (100)	28 (100)	< 0.3–358	0.75	< 0.9–151	< 0.9

[a] ATP (adenosine triphosphate) intervals used correspond to CMT scores.
[b] Bact+ = bacteriologically positive, i.e. > 1 colony forming unit of an udder pathogen.
[c] The detection limits for haptoglobin and SAA were 0.3 mg/L and 0.9 mg/L, respectively.

Swedish breeds, Swedish Red and White, and Swedish Holstein.

Milk samples for bacteriology and ATP as an estimation of SCC [4, 5, 14] were collected aseptically using Mastistrip™ (Department of Mastitis and Diagnostic Products, National Veterinary Institute, Uppsala, Sweden) and sterile polystyrene tubes (Nunc™ Brand Products, Nalge Nunc International, Naperville, USA) at one occasion from each cow. The tube milk samples were frozen at –20 °C for later analysis of haptoglobin and SAA.

2.2. Analyses

Bacterial growth and ATP were analysed according to accredited methods at the Department of Mastitis and Diagnostic Products, National Veterinary Institute, Uppsala, Sweden. ATP levels $\leq 2 \times 10^{-10}$ mol/mL are equal to a negative CMT reaction. Thus, ATP concentrations $> 2 \times 10^{-10}$ mol/mL were considered to be elevated.

After thawing and gentle mixing of the udder quarter milk samples, composite cow milk samples were produced by pooling 200 µL milk from each udder quarter. Udder quarter and composite milk samples were analysed, in duplicate, for haptoglobin and SAA using commercially available ELISA (Bovine Hapto Assay and Phase™ Serum Amyloid A Assay, Tridelta Development Ltd., Greystones, Wicklow, Ireland) with addition to the SAA standard curve of some extra data points. The detection limits (DL) for haptoglobin and SAA were 0.3 and 0.9 mg/L, respectively.

2.3. Statistics

Descriptive statistics for ATP, haptoglobin and SAA contents in quarter and composite samples were produced. Since the parameters had a skewed distribution, range and median were used. The Mann-Whitney test was used to analyse differences in haptoglobin and SAA levels between the five ATP categories, specified in Table I, and the control group. Differences between the ATP-categories were not analysed statistically due to dependent samples within cow. For the same reason, correlation tests were only done within udder quarter (right fore, right hind, left hind, left fore), using the Spearman rank test to investigate associations between haptoglobin, SAA and ATP. A p-value < 0.05 was considered significant. The given DL of the analyte was used when the measured values were below DL.

Table II. Spearman rank test correlation coefficients between ATP[a], haptoglobin and serum amyloid A (SAA) within an udder quarter.

Udder quarter	Haptoglobin/SAA[b]	Haptoglobin/ATP[b]	SAA/ATP[b]
Right fore	0.72	0.85	0.74
Right hind	0.71	0.87	0.59
Left hind	0.78	0.71	0.52
Left fore	0.69	0.65	0.49

[a] Adenosine triphosphate.
[b] All correlation coefficients tested were significant ($p < 0.05$).

3. RESULTS

3.1. Udder quarter milk samples

All samples from the control cows had ATP levels below 2×10^{-10} mol/mL. They were bacteriologically negative, and the haptoglobin and SAA levels were below DL, except for one sample that contained 0.75 mg/L of haptoglobin and another sample that contained 2.3 mg/L of SAA.

In total, 164 udder quarter samples were obtained from cows with chronic sub-clinical mastitis. In 143 (87%) of the samples, the ATP content was $> 2 \times 10^{-10}$ mol/mL, ranging from 2.1 to 191.3×10^{-10} mol/mL (median of 10.2), while it was $\leq 2 \times 10^{-10}$ mol/mL in 21 (13%) samples. Twenty-eight of 41 cows (68%), had ATP $> 2 \times 10^{-10}$ mol/mL in all four udder quarters, seven cows (17%) had ATP $> 2 \times 10^{-10}$ mol/mL in three quarters, four cows (10%) had ATP $> 2 \times 10^{-10}$ mol/mL in two quarters, and two cows (2%) had ATP $> 2 \times 10^{-10}$ mol/mL in one quarter.

In 99 (60%) quarter samples from cows with chronic sub-clinical mastitis, one or both APP was above DL. Out of the 164 samples, haptoglobin was above DL in 92 (56%) and SAA was above DL in 80 (49%) of the samples. Haptoglobin ranged from 0.52 to 358 mg/L (median of 3.8 mg/L), and SAA ranged from 0.9 to 151 mg/L (median of 2.9 mg/L). In Table I, APP contents and bacteriological growth are given in relation to five ATP categories, equivalent to CMT scores negative, trace, weak, moderate and strong reaction, respectively. Compared to the healthy control cows, the haptoglobin and SAA levels were significantly higher in all ATP categories, except for SAA in ATP group $> 5 - \leq 15$. ATP, haptoglobin and SAA were significantly correlated, but the correlation coefficients varied between udder quarters as shown in Table II.

In Table III, the samples were grouped according to their ATP, haptoglobin and SAA status. The APP cut-off values used were based on results from the control group. Most of the samples were ATP+Hp+SAA+, and in this group, the majority of the bacteriologically positive samples were found. The second largest group was ATP+Hp–SAA–. These samples were always from an udder quarter in an udder where one or more of the other quarters were ATP+Hp+SAA+, ATP+Hp+SAA– or ATP+Hp–SAA+.

In 20 (48%) of the cows, udder pathogens were found in one or two udder quarters, while the other cows were bacteriologically negative. Udder pathogens were found in 28 of 164 udder quarter samples (17%), and mixed flora was found in 10 samples (6%). Penicillinase negative *S. aureus* were found in 13 samples, *Streptococcus dysgalactiae* in six samples, and *Streptococcus uberis* and coagulase negative staphylococci (CNS) were found in four samples each. One sample contained *Escherichia coli.* In

Table III. The distribution of 164 udder quarter milk samples from 41 cows with chronic sub-clinical mastitis based on ATP (adenosine triphosphate), haptoglobin (Hp) and serum amyloid A (SAA) status. The numbers of bacteriologically positive samples in each group are also given.

Status[a]	No. (%) of udder quarters	No. (%) of bact+[b] samples	ATP		Haptoglobin		Serum amyloid A	
			Range	Median	Range	Median	Range	Median
ATP+Hp+SAA+	66 (40)	20 (71)	2.3–191.3	21.0	0.6–358.0	5.3	0.9–151.0	3.4
ATP+Hp+SAA–	25 (15)	4 (14)	3.0–179.0	7.7	0.5–11.0	1.1	NA	NA
ATP+Hp–SAA+	7 (4)	1 (4)	4.7–12.6	8.7	NA	NA	1.3–3.5	1.8
ATP+Hp–SAA–	45 (27)	2 (7)	2.1–106.3	3.5	NA	NA	NA	NA
ATP–Hp–SAA–	19 (12)	1 (4)	NA[c]	NA	NA	NA	NA	NA
ATP–Hp+SAA+	1 (1)	0 (0)	NA	NA	NA	NA	NA	NA
ATP–Hp+SAA–	0 (0)	0 (0)	NA	NA	NA	NA	NA	NA
ATP–Hp–SAA+	1 (1)	0 (0)	NA	NA	NA	NA	NA	NA

[a] ATP+ = ATP > 2×10^{-10} mol/mL; ATP– = ATP ≤ 2×10^{-10} mol/mL; Hp+ = haptoglobin level ≥ 0.3 mg/L; Hp– = haptoglobin level < 0.3 mg/L; SAA+ = serum amyloid A level ≥ 0.9 mg/L; SAA– = serum amyloid A level < 0.9 mg/L.
[b] Bact+ = bacteriologically positive, i.e. > 1 colony forming unit of an udder pathogen.
[c] NA = not applicable; ATP ≤ 2×10^{-10} mol/mL, or haptoglobin and SAA below detection limit; or only one APP value at or above DL.

the 28 samples positive for udder pathogens, ATP ranged from 0.8 to 191.3×10^{-10} mol/mL (median of 28.3), haptoglobin from < 0.3 to 358 mg/L (median of 6.5) and SAA from < 0.9 to 151 mg/L (median of 2.6).

3.2. Composite milk samples

One, or both, of the APP were detected in 34 (83%) of 41 composite milk samples. Haptoglobin and SAA were above DL in 31 (76%) and 23 (56%) samples, respectively. Haptoglobin ranged from < 0.3 to 101 mg/L (median of 2.5), and SAA ranged from < 0.9 to 25.8 mg/L (median of 0.9). The relationships between contents in composite samples and numbers of quarters with haptoglobin or SAA above DL are given in Table IV. If the cow had three or more quarters with haptoglobin levels above DL, the composite sample had a detectable content. For SAA, a cow had to have at least two quarters with SAA levels above DL to achieve an SAA positive composite sample.

4. DISCUSSION

This study shows that an increase in the milk concentrations of APP, indicating an activation of the APR, occurred in cows with chronic sub-clinical mastitis, but that the contents of haptoglobin and SAA varied markedly. However, significant correlations were found between haptoglobin, SAA and ATP. An important finding was that almost all udder quarter samples from healthy control cows had undetectable levels of haptoglobin and SAA. From these results, we consider that haptoglobin and SAA levels above DL indicate an abnormal udder quarter. This was correlated with the fact that only 12% of the udder quarters from cows with chronic sub-clinical mastitis belonged to the ATP–Hp–SAA– group, and thus could be regarded as healthy. However, the haptoglobin and SAA content associated with most ATP intervals, including that corresponding to a negative CMT score, were significantly higher than the content in udder quarters from healthy control cows.

Table IV. The number of cows with a composite milk sample containing detectable or not detectable concentrations of haptoglobin or serum amyloid A (SAA), in relationship to the number of udder quarters (0–4) within cow with detectable haptoglobin or SAA concentration. All cows (n = 41) had chronic sub-clinical mastitis in at least one udder quarter.

Haptoglobin/SAA (mg/L) in composite samples	Number of udder quarters within cow with a haptoglobin/SAA concentration above DL					
	0	1	2	3	4	Total (%)
Haptoglobin						
< 0.3	1	6	3	0	0	10 (24)
≥ 0.3	0	9	3	10	9	31 (76)
Total (%)	1 (2)	15 (37)	6 (15)	10 (24)	9 (22)	41 (100)
SAA						
< 0.9	6	10	1	1	0	18 (44)
≥ 0.9	0	3	7	4	9	23 (56)
Total (%)	6 (15)	13 (32)	8 (19)	5 (12)	9 (22)	41 (100)

The true health status of an udder quarter is not often known, thus making the evaluation of mastitis markers difficult. At present, SCC measurement is the most common tool used in mastitis diagnostics, and gives one aspect of the inflammatory status of the mammary gland. However, the SCC remains elevated for weeks after the infection is eliminated and the gland is recovering [17]. To accurately diagnose udder infection, bacterial growth of specific udder pathogens must be demonstrated in an udder quarter. However, due to a variation in numbers of bacteria present at different time points during mastitis, negative bacteriological results are common, especially if the quarters are only examined once [18, 19]. In this study, only 17% of the udder quarters were bacteriologically positive, but this was probably an underestimation since it was based on only one sampling occasion. The bacteriological findings reflected the usual types of bacteria found in sub-clinical mastitis in Sweden (Department of Mastitis and Substrate Production, National Veterinary Institute, Uppsala, Sweden). However, the numbers of cases were too small to allow for statistical analysis on APP levels in relation to different udder pathogens.

The three inflammatory markers showed agreement as to whether an udder quarter should be classified as normal, or abnormal, in only 52% of the quarters from cows with chronic sub-clinical mastitis. In the rest of the quarters, the three parameters disagreed, and as many as 25% of all 164 quarters belonged to the ATP+Hp–SAA– group. These quarters were always found in udders where at least one more quarter was ATP+ and Hp+ or/and SAA+. Since most of the ATP+Hp–SAA– quarters had low ATP and were bacteriologically negative, it may be speculated that they were affected by a general recruitment and influx of leukocytes to the udder.

The discrepancy between SCC and APP could also be due to different mechanisms for their entry into the udder. Leukocytes migrate actively by means of adhesion molecules, while APP are considered to leak passively from blood to milk due to increased permeability in the inflamed area [3], and/or to be locally produced in the mammary gland [11]. At present, it is not possible to determine the origin of SAA and haptoglobin found in milk. As inflammation diminishes, the permeability decreases, and the leakage of APP from the blood stream

is likely to decline. Therefore, a more sensitive ELISA, or an ELISA that specifically measures the local isoforms, would perhaps give a better agreement between APP and ATP results.

The haptoglobin and SAA levels were correlated, but not totally unanimous. In most of the samples, both haptoglobin and SAA levels were above DL, and in samples where only one of the APP was above DL, haptoglobin was more often detected than SAA. This was in contrast to our earlier findings [6], whereby SAA, but not haptoglobin, was significantly elevated during chronic sub-clinical *S. aureus* mastitis. This discrepancy may be due to different amounts and/or combinations of cytokines released due to variations in bacterial virulence and the host response of the cows. It is likely that the duration of disease also has an impact on APP production. In the present study, the duration of mastitis was longer than in the experimental study by Grönlund et al. [6]. Different phases of inflammation may require the functions of different types of APP. As an example, α_1-acid glycoprotein is attributed as an APP expressed mainly during chronic conditions in cattle [9]. However, other factors, like milk production, stage of lactation, lactation number and the oestrus cycle, may also affect the APR, and should be investigated further.

Based on the monthly-recorded CSCC, all the cows were considered diseased, but only 83% of the cows had detectable levels of haptoglobin and/or SAA in the simulated composite milk samples. Haptoglobin was detected more frequently than SAA. The data also showed that a cow had to have detectable levels of haptoglobin or SAA in at least two udder quarters for elevated levels to be found in composite samples. Thus, it can be concluded that analysis on quarter level is preferable.

In conclusion, the results indicate that haptoglobin and SAA analyses in udder quarter milk samples may be potential indicators of udder health. However, more studies are needed to fully evaluate their usefulness in mastitis diagnosis. In addition, more research is needed to better understand the relationship between milk SCC and APP, and the true inflammatory status of the udder quarter. Present methodology for APP analysis is costly and time-consuming, and is not suitable for on-line measurements during milking. Thus, alternative methods of analysis are warranted.

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