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Milk amyloid A and selected serum proteins in cows suffering from mastitis

Gabriel Kováč, Csilla Tóthová, Oskar Nagy, Herbert Seidel

University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic

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Abstract

The aim of this work was to evaluate the influence of clinical and sub-clinical mastitis on the concentrations of mammary associated isotype of serum amyloid A (M-SAA) in milk samples, as well as on the concentrations of haptoglobin (Hp) and serum amyloid A (SAA), and some other biochemical variables in blood serum of dairy cows (n = 41). The concentrations of aforementioned variables were measured in 4 groups of cows divided according to the results of the clinical examination of the udder and to the results of California Mastitis Test (CMT): group 1 – cows without clinical changes on the mammary gland and with negative CMT, group 2 – cows without clinical changes on the mammary gland and with weakly positive CMT, group 3 – cows without clinical changes on the mammary gland and with strongly positive CMT and group 4 – cows with clinical changes on the mammary gland and changes in milk appearance. The concentrations of M-SAA were analyzed also in 145 quarter's milk samples which were categorized according to the same criteria as cows used in the study. By the evaluation of M-SAA concentrations in composite milk samples we found significantly the highest mean value in cows with clinical signs of mastitis. Similar findings were recorded in the M-SAA concentrations in quarter's milk samples. Moreover, higher concentrations of M-SAA were found also in samples from mammary quarters without clinical changes and positive CMT. The analyses of Hp and SAA concentrations showed a trend of higher values in cows with clinical mastitis. The lowest mean concentration of albumin we found in cows with clinical signs of mastitis. Our results indicate elevated production of M-SAA in cows with clinical changes on mammary gland, and suggest the usefulness of this indicator also in the diagnosing of sub-clinical mastitis.

Acute phase proteins, mammary gland, dairy cattle, biochemical variables

Despite world-wide efforts, mastitis has remained economically the most important disease in dairy cattle, and despite different mastitis control programs it is still a major challenge for the dairy industry (Bradley 2002). While clinical mastitis is often easy to detect, sub-clinical mastitis, on the other hand, is a larger problem for the dairy industry since this condition shows no visible changes in the udder or in the milk (Sandholm et al. 1995). Sub-clinical mastitis is frequently diagnosed by California Mastitis Test (CMT), which may suffer from a lack of reproducibility, and the cell count (as CMT gives an indirect measure of the number of somatic cells present) is not useful in discriminating between the clinical and sub-clinical form of mastitis (Gerardi et al. 2009). Therefore, it is of great importance to investigate biomarkers that could be used for rapid detection of sub-clinical mastitis. One of the ways to identify cows with sub-clinical mastitis would be the measuring of concentrations of acute phase proteins (APPs). Many researches proved an increased production of major APPs in cattle, haptoglobin and amyloid A in serum of dairy cows with mastitis (Hirvonen et al. 1999; Eckersall et al. 2001). Further investigations showed an extrahepatic synthesis of specific isoform of serum amyloid A directly from mammary epithelial cells (M-SAA) (McDonald et al. 2001). Several studies were focused on the evaluation of amyloid A in serum and milk of dairy cows with clinical and sub-clinical mastitis, and stated that M-SAA could be a more reliable indicator of mastitis than systemically produced APPs (Grönlund et al. 2005; Nazifi et al. 2008).

Address for correspondence:
Gabriel Kováč, Prof., DVM, DrSc.
Clinic for Ruminants
University of Veterinary Medicine and Pharmacy
Komenského 73, 041 81 Košice
Slovak Republic

Phone: +421 915 984 597
Fax: +421 55 67 11 674
E-mail: kovac@uvm.sk
http://www.vfu.cz/acta-vet/actavet.htm

Bovine mastitis is an inflammatory reaction of one or more quarters of the mammary gland to bacterial, chemical, thermal or mechanical injury (Haghkah et al. 2009). It is often affecting not only the mammary gland, but the whole organism systemically manifested by decreased appetite, depression, increased body temperature, and alteration of the whole metabolism. The inflammatory response of the body may result in an increase of blood proteins and changes in other biochemical indicators (Kaneko et al. 1997).

Therefore, this work was aimed at evaluation of the influence of clinical and sub-clinical inflammatory diseases of the mammary gland on the concentrations of mammary associated isoform of serum amyloid A in composite and quarter's milk samples, as well as on the concentrations of selected acute phase proteins – haptoglobin and serum amyloid A, and other variables related to protein metabolism in blood serum of dairy cows.

Materials and Methods

Animals

In total 41 dairy cows, clinical cases hospitalized on the Clinic for Ruminants of the University of Veterinary Medicine and Pharmacy in Kosice (Slovak Republic) with various clinical findings on the mammary gland were included in our study. The animals were of the low-land black spotted breed and its crossbreeds. The cows were in the 3–4th lactation, but not in the period shortly after parturition. Clinical examination of the mammary gland was performed by visual inspection and palpation, using standard physical methods of examination. Clinical mastitis was diagnosed by the presence of observable signs of inflammation in the infected quarter such as swelling, heat, pain or redness, and by the presence of clots and flakes in the milk, or by its abnormal color or consistency. To detect sub-clinical mastitis, the California Mastitis Test (CMT) was performed (Jackson and Cockcroft 2002). According to the results of the clinical examination of the udder and to the results of CMT, the animals were divided into 4 groups: group 1 – cows without clinical changes on the mammary gland and with negative CMT (n = 7), group 2 – cows without clinical changes on the mammary gland and with weakly positive CMT (n = 12), group 3 – cows without clinical changes on the mammary gland and with strongly positive CMT (n = 13) and group 4 – cows with clinical changes on the mammary gland and changes in milk appearance (n = 9).

Analysis of milk and blood

The analyses of selected variables were performed in milk and blood samples. Milk samples were collected into plastic tubes by hand-stripping. Blood samples were collected by direct puncture of v. jugularis. Milk samples were analyzed for mammary associated isoform of serum amyloid A (M-SAA, ng/ml). Blood serum was analyzed for selected acute phase proteins – haptoglobin (Hp, mg/ml) and serum amyloid A (SAA, µg/ml), and for some variables related to protein metabolism – total proteins (TP, g/l), albumin (Alb, g/l), and total immunoglobulins (TIg, U ZST – units of zink-sulphate turbidimetric test).

The concentrations of M-SAA were measured in 41 composite milk samples, as well as in 145 separate milk samples from each lactating quarter. The obtained results from these mammary quarter's milk samples were categorized according to the same criteria as for cows used in the study: group 1 – quarters without clinical changes and with negative CMT (n = 42), group 2 – quarters without clinical changes and with weakly positive CMT (n = 65), group 3 – mammary quarters without clinical changes and with strongly positive CMT (n = 29), group 4 – quarters with clinical changes and changes in milk appearance (n = 9). Within the total 164 quarters 19 were non-lactating quarters.

The California Mastitis Test was performed using equal volumes of milk and alkyl-aryl-sulphonate by the same person in each cow, and the results were evaluated according to Jackson and Cockcroft (2002). The concentrations of M-SAA were determined by ELISA method using commercial diagnostic kits (Tridelta Development, Ireland). Haptoglobin in blood serum was assessed using a commercial colorimetric kit (Tridelta Development, Ireland) based on Hp-haemoglobin binding and preservation of the peroxidase activity of the bound haemoglobin at low pH. SAA was analysed by method of sandwich enzyme linked immunosorbent assay using commercial ELISA kit (Tridelta Development, Ireland). The reading of absorbancies and the consecutive calculation of final concentrations of M-SAA, Hp and SAA were performed on automatic microplate reader Opsys MR (Dynex Technologies, USA). Concentrations of TP and Alb were determined using commercial diagnostic kits (Randox) on automatic biochemical analyser ALIZE (Lisabio, France). Total immunoglobulins were analysed by spectrophotometric turbidimetric method (zinc-sulphate test) (Slanina et al. 1976).

Statistics

The evaluation of results was performed by assessment of means (x) and standard deviations (SD) for each evaluated variable. The significance of differences in means between the groups of cows both for variables measured in milk and blood samples was evaluated by Kruskal-Wallis nonparametric ANOVA test and Dunn's Multiple Comparisons Test. The same tests were used for the evaluation of the significance of differences in means for M-SAA between samples from separate mammary quarters. Statistical analyses were performed using programme GraphPad Prism V3.06 (GraphPad Software Inc.).

Results

The results obtained in the examined groups of cows characterised by means, standard deviations, and evaluation of the significances of differences in means are presented in Table 1 and Table 2.

Table 1. Concentrations of mammary associated serum amyloid A (ng/ml) in composite and quarter's milk samples from cows / quarters with various clinical findings and Californian Mastitis Test score

Variable		Groups of cows / quarters				K-W
		1	2	3	4	*P*
c-M-SAA ng/ml	x	325.7^{A}	1433.1^{a}	3910.4^{B}	$6073.8^{b,B}$	< 0.001
	± SD	173.8	949.2	2145.8	4414.0	
q-M-SAA ng/ml	x	473.7^{A}	1431.0^{B}	6528.6^{C}	10476.0^{C}	< 0.001
	± SD	548.6	1347.6	3823.2	5447.2	

Means with different superscripts within rows differ significantly (a, b – $P < 0.05$; A, B, C – $P < 0.001$)
K-W – Kruskal-Wallis analysis; *P* – significance of K-W analysis
c-M-SAA – concentration of mammary associated serum amyloid A in composite milk samples
q-M-SAA – concentration of mammary associated serum amyloid A in quarter's milk samples
Group 1 – cows / quarters without clinical findings and with negative CMT, group 2 – cows / quarters without clinical findings and with weakly positive CMT, group 3 – cows / quarters without clinical findings and with strongly positive CMT, group 4 – cows / quarters with clinical changes and changes in milk appearance.

Table 2. Concentrations of haptoglobin, serum amyloid A and variables related to protein metabolism in blood serum of cows with various clinical findings on the mammary gland and Californian Mastitis Test score (x ± SD)

Variable	Groups of cows				K-W
	1	2	3	4	*P*
Hp (mg/ml)	0.046 ± 0.053	0.122 ± 0.263	0.299 ± 0.314	0.329 ± 0.339	< 0.05
SAA (μg/ml)	29.7 ± 27.6	27.6 ± 28.0^{a}	48.2 ± 42.5	71.5 ± 31.5^{b}	< 0.05
TP (g/l)	75.5 ± 7.1	72.4 ± 7.3	78.1 ± 6.6	77.6 ± 14.0	n. s.
Alb (g/l)	37.7 ± 5.7	36.6 ± 6.3	40.0 ± 3.6^{a}	32.8 ± 5.4^{b}	< 0.05
TIg (U ZST)	28.7 ± 7.0	29.9 ± 3.3	31.1 ± 4.4	28.3 ± 5.1	n. s.

Means with different superscripts within rows differ significantly ($P < 0.05$);
Hp – haptoglobin, SAA – serum amyloid A, TP – total proteins, Alb – albumin, TIg – total immunoglobulins
K-W – Kruskal-Wallis analysis; *P* – significance of K-W analysis
Groups of cows: 1 – cows without clinical changes on the mammary gland and with negative CMT, 2 – cows without clinical changes on the mammary gland and with weakly positive CMT, 3 – cows without clinical changes on the mammary gland and with strongly positive CMT, 4 – cows with clinical changes on the mammary gland and changes in milk appearance.

M-SAA concentrations in milk samples differed significantly between groups ($P < 0.001$), with concentrations in samples from cows with clinical mastitis (group 4) being significantly higher than in samples from groups 1 and 2 ($P < 0.001$ and $P < 0.05$, respectively). Similar findings were recorded in the M-SAA concentrations in quarter's milk samples with the highest mean value in samples from mammary quarters with obvious clinical changes ($P < 0.001$). Moreover, the concentrations of M-SAA in quarter's milk samples from mammary quarters without clinical changes but with weakly or strongly positive CMT were also significantly higher than in samples from group 1 ($P < 0.001$). Except for group 2, the mean concentrations of M-SAA in the separate quarter's milk samples were markedly higher than in composite milk samples.

The serum concentrations of Hp also showed tendency of a gradual significant increase with increasing the CMT score and clinical changes on the mammary gland ($P < 0.05$, Table 2). The highest mean Hp concentration was found in cows with clinically manifested

signs of mastitis. Similarly, SAA concentrations differed significantly between the evaluated groups of cows ($P < 0.05$) with the highest mean concentration in animals with clinical signs of mastitis. The mean SAA concentrations found in cows from group 1 and group 2 were roughly uniform; significantly higher mean concentration of SAA was found in group 4 ($P < 0.05$).

No significant differences in mean concentrations were observed for total proteins, and total immunoglobulins (Table 2). However, total protein concentrations found in cows without clinical findings on the mammary gland, but with strongly positive CMT, as well as in cows with clinical signs of mastitis were higher than in cows from group 1 and 2. Significant differences in means between the examined groups of cows was found in the concentrations of albumin ($P < 0.05$), with the lowest mean concentration in group 4 being significantly lower compared to mean concentration recorded in cows from group 3 ($P < 0.05$). The mean serum concentrations of albumin found in cows from group 1 and group 2 were approximately uniform.

Discussion

The results of our study indicate that inflammatory diseases of the mammary gland lead to an increase in concentrations of M-SAA. Raised levels of Hp and SAA have previously been shown in milk from cows with clinical mastitis as a result of the leakage of these proteins from the blood to the milk (Hirvonen et al. 1999; Eckersall et al. 2001). On the other hand, M-SAA is synthesized directly in the mammary epithelia of the udder in response to infection (Jacobsen et al. 2005). Therefore, M-SAA is believed to be a more sensitive indicator of mastitis; it accumulates in milk only during mammary inflammation. Several studies were focused on the evaluation of M-SAA as a marker of inflammation in the mammary gland (Petersen et al. 2005; Nazifi et al. 2008). Petersen et al. (2005) reported that M-SAA concentrations, similarly to our results, were higher in quarters with mastitis compared to healthy quarters. According to Berry et al. (2005) M-SAA concentrations in uninfected mammary quarters are very low, and values higher than 500 ng/ml are indicative of an inflammatory response. In our study, analyses showed markedly higher mean M-SAA concentrations in milk samples from quarters with clinical changes, as well as from quarters without clinical signs of mastitis, but with strongly positive CMT. On the other hand, mean concentration of M-SAA found in samples from mammary quarters without clinical changes (473.7 ng/ml) was also relatively high compared to 500 ng/ml, which Berry et al. (2005) presented as threshold value for inflammatory processes in the mammary gland. These results suggest that some quarters might be affected by the inflammatory process, but still without positive reaction of CMT. In contrast, Nazifi et al. (2008) presented markedly higher mean M-SAA concentrations for clinically healthy cows and for cows with sub-clinical mastitis (6.96 and 54.53 μg/ml, respectively). Higher M-SAA concentrations for healthy cows and cows with clinical mastitis were reported also by Haghkhah et al. (2009) (9.90 and 105.12 μg/ml, respectively). These contradictory data indicate that further studies are necessary to deepen our knowledge about the behavior of M-SAA in such conditions.

Moreover, this response characterized by increased synthesis of M-SAA is specific for the quarter and does not necessarily result in detectable concentrations in composite milk samples. Grönlund et al. (2005) reported also some interpretative problems by the use of composite milk samples if only one quarter is sub-clinically infected. Our results showed similar findings in composite milk samples as in samples from separate quarters, but the mean concentrations of M-SAA were lower in composite milk samples, which suggest a diluting effect of milk from quarters with less marked changes. Therefore, it seems that composite milk samples are less suitable for detection of sub-clinical mastitis than samples from separate mammary quarters.

O'Mahony et al. (2004) reported a significant correlation between concentrations of M-SAA and both the SCC and CMT. In our study, the concentrations of M-SAA in composite, as well as quarter's milk samples increased with increasing CMT score and changes on the mammary gland. O'Mahony et al. (2004) stated that high M-SAA concentrations but low SCC may represent false-negative results for SCC, possibly attributable to active trafficking of leukocytes to areas of greatest pathophysiological need. Thus higher milk SAA concentrations with negative CMT found in cows with clinically healthy mammary gland and negative CMT may in fact indicate sub-clinically inflamed mammary quarter, but not expressing that mammary inflammation in the form of positive CMT. Raised concentrations of milk SAA may be interpreted also in light of the extremely rapid response of M-SAA following mammary infection, with changes occurring within a timeframe preceding raised SCC or changes in CMT (Sorensen et al. 2002). On the other hand, raised M-SAA concentrations and negative CMT may equally represent false-positive results for M-SAA. Therefore, further investigations are needed on a larger sample size of cows.

Mastitis can be caused by different microbial agents, mostly bacteria. Some bacteria invading a cow's mammary gland absorb milk nutrients, and some of them can produce endotoxins that destroy mammary tissue (Haltia et al. 2006). If these toxins escape the gland and spread throughout the cow's body, they may activate systemic inflammatory reactions. Moreover, other inflammatory mediators, e.g. cytokines released in response to infection and injury may activate systemic inflammatory reactions, including the induction of the synthesis of APPs by the liver (Baumann and Gauldie 1994). The results of our study showed that the concentrations of Hp and SAA were higher in serum from cows with clinical mastitis, and increased with increasing CMT score. The increases observed in the concentrations of these proteins in serum of cows with mastitis are in line with several previous studies (Hirvonen et al. 1999; Eckersall et al. 2001). It appears that localized severe inflammation of the udder is sufficiently intense to induce a measurable systemic acute phase response. The concentrations of measured APPs had a tendency to be higher in the serum from the cows with local signs of mastitis and also in cows without clinical changes on the mammary gland, but with positive CMT. However, the finding that the differences in Hp and SAA concentrations observed between the groups of cows were less significant than the differences in M-SAA concentrations means that the measuring of serum concentrations of some APPs would be less useful to the evaluation of the severity of mastitis than the measuring of the concentrations of M-SAA directly in milk samples. Moreover, Heegard et al. (2000) reported that SAA is more sensitive to stimulation, and its increase can be induced also by other factors than disease, such as stress. Therefore, a possible use of SAA in the diagnosis of mastitis is questionable.

Our results indicate that inflammatory diseases of the mammary gland may result not only in changes of serum concentrations of some APPs, but also in other biochemical and physiological changes, including the serum concentrations of some variables related to protein metabolism. Cebra et al. (1996) reported lower total protein values in cows with mastitis than in healthy animals. In contrast, our results indicated a tendency of total protein concentrations to increase with the increasing CMT score, and manifestation of clinical signs on the mammary gland. These higher concentrations of total proteins may reflect the obvious response of the organism to inflammation.

Several studies have observed lower serum concentrations of albumin in cows with mastitis compared to healthy cows (Katholm et al. 1992; Rişvanli et al. 1999). Our results showed the lowest mean serum albumin concentration in cows with clinical signs of mastitis. According to Toussaint et al. (2005) chronic inflammatory conditions are also responsible for lower albumin synthesis. On the other hand, Murray et al. (2001) reported that albumin concentration decreases also in acute inflammatory conditions. However,

further studies are needed to clarify the influence of inflammatory diseases of the mammary gland on the concentrations of serum biochemical indicators of protein metabolism.

Presented results indicate elevated production of M-SAA in cows with clinically manifested changes on the mammary gland and also in cows with sub-clinical mastitis. This suggests the usefulness of this indicator in the laboratory diagnosis of bovine sub-clinical mastitis. It appears that the measurement of some acute phase proteins in serum would be less useful to discriminate between cows with various signs of mastitis, mainly in sub-clinical cases. Because of the large number of sub-clinical mastitis cases, in which the diagnosis based on clinical signs is not possible, the diagnosis of mastitis can also depend on indirect tests. Our results suggest that the measurement of M-SAA concentrations may provide additional diagnostic information in the determination of the severity of mastitis.

Mliečny amyloid A a vybrané proteíny krvného séra u dojníc postihnutých zápalovými ochoreniami mliečnej žľazy

Zámerom práce bolo posúdenie vplyvu klinickej a subklinickej mastitídy na koncentráciu mliečnej izoformy sérového amyloidu A (M-SAA) vo vzorkách mlieka, ako aj na koncentráciu haptoglobínu (Hp) a sérového amyloidu A (SAA) a niektorých ďalších biochemických ukazovateľov v krvnom sére dojníc (n = 41). Koncentrácie uvedených ukazovateľov boli hodnotené v 4 skupinách dojníc rozdelených na základe výsledkov klinického vyšetrenia mliečnej žľazy a vykonanej rýchlej maštaľnej skúšky (NK-test): skupina 1 – dojnice bez klinicky zjavných zmien na mliečnej žľaze a s negatívnym NK-testom, skupina 2 – dojnice bez klinicky zjavných zmien na mliečnej žľaze a s mierne pozitívnym NK-testom, skupina 3 – dojnice bez klinicky zjavných zmien na mliečnej žľaze a s výrazne pozitívnym NK-testom, skupina 4 – dojnice s klinicky zjavnými zmenami na mliečnej žľaze a zmyslovo zmeneným mliekom. Koncentrácie M-SAA boli stanovené aj v 145 štvrťových vzorkách mlieka, ktoré boli rozdelené do skupín na základe rovnakých kritérií ako dojnice použité v sledovaní. Pri hodnotení koncentrácií M-SAA v štvrťových vzorkách mlieka signifikantne najvyššia priemerná hodnota bola zistená u dojníc s klinickými príznakmi mastitídy. Podobné výsledky boli zaznamenané aj pri hodnotení koncentrácií M-SAA v štvrťových vzorkách mliekai. Okrem toho, vyššie koncentrácie M-SAA boli zaznamenané aj vo vzorkách z mliečnych štvrtí bez klinicky zjavných zmien, ale s pozitívnym NK-testom. Hodnotením koncentrácií Hp a SAA bol zaznamenaný podobný trend vyšších hodnôt u dojníc s klinickou mastitídou. Najnižšia priemerná koncentrácia albumínu bola zistená u dojníc s klinickými príznakmi mastitídy. Dosiahnuté výsledky poukazujú na zvýšenú produkciu M-SAA u dojníc s klinicky zjavnými zmenami na mliečnej žľaze a naznačujú významnú úlohu uvedeného ukazovateľa aj v diagnostike subklinickej mastitídy.

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