

# The diagnostic value of determination of positive and negative acute phase proteins in milk from dairy cows with subclinical mastitis

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**Abstract** Currently, somatic cell count (SCC) and bacterial culture are considered as the gold standard methods for the diagnosis of bovine subclinical mastitis. However, SCC has a low diagnostic accuracy. Therefore, for identification of infected animals, new biomarkers with high diagnostic accuracy are needed. Acute phase proteins (APPs) are proteins that are increased (positive APPs) or decreased (negative APPs) in response to inflammation. The objective of this study was to determine the diagnostic value of milk APPs for the diagnosis of subclinical mastitis in dairy cows. A total of 90 clinically healthy cows were randomly selected. Of these, 52 cows were considered subclinical

mastitic based on a SCC higher than  $130 \times 1,000$  cells/mL of milk and positive bacterial culture results of milk samples obtained from at least one of the quarters. Milk amyloid A (MAA) concentration was measured using a commercial ELISA kit and albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and immunoglobulin (Ig) were measured in the whey samples by the use of cellulose acetate electrophoresis. Diagnostic sensitivity and specificity and cutoff points for each test were determined via receiver-operating characteristics analysis. Significant ( $P < 0.001$ ) increases in the mean and median concentration of MAA, albumin,  $\alpha$ -lactalbumin, and Ig were found in the milk samples collected from cows with subclinical mastitis. MAA was the most accurate test with a diagnostic sensitivity of 92.3% and specificity of 92.1% at cutoff point of  $>1.6$  mg/L. The results of this study showed that determination of MAA can be used as a reliable method for the diagnosis of bovine subclinical mastitis.

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## Introduction

Mastitis is an inflammation of the mammary gland, predominantly caused by pathogenic bacteria (Stelwagen et al. 2009). Mastitis occurs in two different forms: clinical and subclinical mastitis. In clinical mastitis, abnormal milk is readily detected; however, in subclinical mastitis, no change in the milk is apparent. Mastitis, especially subclinical type, has been reported to be one of the most common diseases affecting the dairy industry worldwide (Seegers et al. 2003). Because subclinical mastitis is often

undetected, it has a great economic impact on dairy industry (Seegers et al. 2003; Bolourchi et al. 2008) and therefore, detection of cows with subclinical mastitis at an early stage is important.

Although, a number of diagnostic techniques for detection of subclinical mastitis have been used, the inflammatory reaction caused by infections in the mammary tissue is most commonly measured by somatic cell count (SCC) in milk (Sloth et al. 2003). Currently, SCC and bacterial culture are considered as the gold standard methods for diagnosing subclinical mastitis in dairy animals. To distinguish between healthy cows and cows with subclinical mastitis, a SCC at cutoff point of  $130 \times 1,000$  cells/mL has been recommended (Holdaway et al. 1996). However, it has been shown that the correlation between infection of the udder and SCC may not be always good (Schepers et al. 1997). In addition, SCC results may be affected by factors such as number and stage of lactation, level of milk production, season, and breed (Schepers et al. 1997). Although, using bacterial culture for the diagnosis of subclinical mastitis has the advantage of high diagnostic sensitivity and specificity, it is a labor-intensive and time-consuming technique. Therefore, for identification of infected animals, new biomarkers with higher diagnostic accuracy and faster turnaround times are needed (Pyörälä 2003; Åkerstedt et al. 2008).

Efforts to develop alternative methods to SCC have led to different methods measuring acute phase proteins (APPs) in milk samples from cows with subclinical mastitis. The APPs are proteins that are increased (positive APPs) or decreased (negative APPs) in response to stress or inflammation such as infection, injury, trauma, or other tissue necrosis (Murata et al. 2004). In the last several years, there has been considerable progress in studies on the milk APPs including milk amyloid A (MAA), milk albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and immunoglobulin (Ig).

Serum amyloid A (SAA), a major bovine positive APP is produced mainly by the liver. Serum amyloid A has antimicrobial functions through attaching to gram-negative bacteria and thereby facilitating phagocytosis (Larson et al. 2005). Amyloid A is also produced by the mammary gland and therefore it is present in milk from dairy animals. It has been suggested that intramammary synthesis of MAA is increased during mastitis (McDonald et al. 2001). MAA has been suggested as an early biomarker for mastitis (Eckersall et al. 2001; O'Mahony et al. 2006) and a marker for milk quality (Åkerstedt et al. 2008).

Serum albumin produced by the hepatocytes is a negative APP; however, synthesizing albumin by the mammary gland is increased in mastitis as part of the innate nonspecific defense system (Shamay et al. 2005). Therefore, determina-

tion of milk albumin may provide an additional tool for the detection of inflammatory process and can be used as a diagnostic marker for subclinical mastitis.

Although in an early study,  $\alpha$ -lactalbumin concentration in milk has been shown to be increased (Waite and Blackburn 1963), recent studies have shown decreased  $\alpha$ -lactalbumin concentration in milk from cows with mastitis and suggested that this protein should be considered as a negative APP (Ishikawa et al. 1982; Wickstrom 2009). Mammary gland inflammation may also result in decreasing local synthesis of  $\beta$ -lactoglobulin and therefore  $\beta$ -lactoglobulin is considered as a negative APP (Ishikawa et al. 1982). Decreased  $\beta$ -lactoglobulin in milk may also be due to destruction of blood–milk permeability barriers during mastitis (Ishikawa et al. 1982; Wickstrom 2009).

Immunoglobulins are the main immune components of the acquired immune system present in milk. The most abundant Ig class in bovine milk is IgG<sub>1</sub>. Other classes of Ig including IgG<sub>2</sub>, IgA, and IgM are present at much reduced concentrations in bovine milk (Barrington et al. 1997; Stelwagen et al. 2009). In mastitis, serum Igs pass into milk because of increased permeability of blood–milk barrier caused by inflammation (Khan and Khan 2006).

Although MAA has been suggested as a good biomarker for identification of subclinical mastitis (Grönlund et al. 2003; Safi et al. 2009; Gerardi et al. 2009), limited information is available on the diagnostic value of other APPs in milk from cows with subclinical mastitis. The objective of this study was to determine the diagnostic value of milk APPs including MAA, albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and Ig for the diagnosis of bovine subclinical mastitis.

## Materials and methods

### Animals and samples

From February to October 2010, a total of 90 multiparous Holstein cows were randomly selected from five commercial dairy herds in Tehran province, Iran. All animals were healthy based on physical examination and clinical pathology profiles. Cows in late pregnancy or early lactation were excluded from this study. The animals had no clinical signs of mastitis or other abnormalities in the udder or clots in the milk. Of these, 52 cows were considered subclinical mastitic based on an SCC higher than  $130 \times 1,000$  cells/mL of milk and positive bacterial culture results of milk samples obtained from at least one of the quarters. Thirty-eight cows were considered healthy based on an SCC lower than  $130 \times 1,000$  cells/mL of milk and negative bacterial culture results of milk samples obtained from all four udder quarters.

Analysis of milk samples for SCC, California Mastitis Test (CMT), and bacterial culture

For the SCC and APPs analysis, pre-milking quarter samples were collected after discarding the first few streams of milk. Samples were immediately transported to the clinical pathology laboratory in a cooler with ice packs. Several aliquots were prepared from each sample. One aliquot was used for the SCC analysis and whey fraction preparation; the other aliquots were kept at  $-70^{\circ}\text{C}$  until analysis. The SCC was performed using an electronic fluorescence-based cell counting technique (Fossomatic 5000; Foss Electric, Hillerød, Denmark). The CMT was performed at the collection site using quarter foremilk samples (Shirazma Co., Noor, Iran). For the bacteriologic examination, aseptic milk samples were collected in separate sterile plastic bags to prevent spilling and cross contamination, and immediately transported to the microbiology laboratory in a cooler with ice packs. The samples were processed immediately upon arrival using aseptic techniques. Identification of microorganisms was performed according to the National Mastitis Council guidelines (National Mastitis Council 1999).

#### Preparation of whey fraction

Whey fraction preparation was performed according to the method described previously (Ishikawa et al. 1982). Briefly, skim milk was prepared by centrifugation of the whole milk at  $1,000\times g$  for 20 min at  $4^{\circ}\text{C}$ . To precipitate casein, the pH of the skim milk was adjusted to 4.6 by slow addition of HCl (1 M). The supernatant fraction was separated from the casein by centrifugation at  $2,000\times g$  for 20 min at room temperature. To remove casein residues, the supernatant whey was filtered through Whatman no.1 filter paper. The filtered whey was concentrated using dialysis against polyethylene glycol and stored frozen at  $-70^{\circ}\text{C}$  until analysis.

#### Assays for determination of APPs

Samples were warmed at room temperature (about  $24^{\circ}\text{C}$ ) prior to analysis. MAA concentration was measured using a commercial ELISA kit (Mast ID RANGE Milk Amyloid A Assay, cat TP-807, Tridel Development Ltd, Wicklow, Ireland) according to the manufacturer's instruction. The optical density of samples was measured using an automated plate reader (Model ELX 800; Bio-Tek Inc., VT, USA) at 450 nm with a reference at 630 nm. The limit of detection of the ELISA was 0.1 mg/L according to the manufacturer.

Albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and Ig were measured in the whey samples by the use of cellulose acetate electrophoresis (Helena Laboratories, Beaumont,

Texas, USA) as described previously (Bell and Stone 1978). Each protein fraction was quantified by densitometry of cellulose acetate strips at a wavelength of 525 nm (Helena Laboratories). The concentration of total protein in the whey samples was determined by the biuret method (Johnson and Swanson 1952) and the concentration of each fraction was calculated from the total protein by proportion (Eckersall 2008).

#### Statistical analysis

All statistical analyses were performed using SPSS statistical software version 16 (SPSS Inc., Chicago, IL, USA). Data were analyzed for normality using the Kolmogorov–Smirnov test. The mean and median values of each protein were compared between the healthy cows and cows with subclinical mastitis using the Mann–Whitney test and the difference was considered statistically significant at  $P$  value of  $<0.05$ . The SCC and bacterial culture were considered as the gold standard tests. To achieve high diagnostic sensitivity and specificity for the diagnosis of subclinical mastitis, different cutoff points were selected for each protein using receiver-operating characteristic (ROC) analysis, and the area under the ROC curve (AUC) of  $>0.9$  was considered as high accuracy of a diagnostic test (Gardner and Greiner. 2006). The diagnostic sensitivity and specificity, and AUC of the all tests were compared by using the McNemar test.

## Results

Table 1 summarizes the results of MAA, albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and Ig concentrations in milk samples from healthy cows and cows with subclinical mastitis. Significant ( $P<0.001$ ) increases in the mean and median concentration of MAA, albumin,  $\alpha$ -lactalbumin, and Ig were found in the milk samples collected from cows with subclinical mastitis. Although a significant decrease in  $\beta$ -lactoglobulin concentration was not found in the milk samples collected from cows with subclinical mastitis, mean concentration of  $\beta$ -lactoglobulin was slightly lower in the milk samples collected from cows with subclinical mastitis compared to healthy cows.

Table 2 shows the diagnostic sensitivity and specificity of each test for the subclinical mastitis based on a cutoff value from ROC analysis. Overall, MAA concentration at the cutoff point of 1.6 mg/L showed the highest and  $\beta$ -lactoglobulin concentration at the cutoff point of  $<2,697.5$  mg/L showed the lowest diagnostic accuracy for the diagnosis of bovine subclinical mastitis. The McNemar test analysis revealed that the AUCs for MAA, albumin, and Ig concentration were significantly larger than the AUCs for  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin concentration.

**Table 1** Results of milk amyloid A, albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and immunoglobulin concentration in milk samples from healthy cows ( $n=38$ ) and cows with subclinical mastitis ( $n=52$ )

Analyte (mg/L)	SCC ( $\times 1,000$ cells/mL)	Bacterial culture	Mean $\pm$ SD	Median	Minimum–maximum	<i>P</i> value <sup>a</sup>
Milk amyloid A	<130	–	0.61 $\pm$ 0.5	0.44	0.1–1.9	<0.001
	>130	+	12.83 $\pm$ 12.8	7.88	0.84–50.5	
Albumin	<130	–	244.33 $\pm$ 167.0	208.6	77.4–952.5	<0.001
	>130	+	682.0 $\pm$ 310.9	635.8	188.5–1566.4	
$\alpha$ -lactalbumin	<130	–	1767.24 $\pm$ 378.7	1,736.5	1,020–2,512.5	<0.001
	>130	+	2,257.18 $\pm$ 48.0	2,186.6	1,540–3,411	
$\beta$ -lactoglobulin	<130	–	2,679.31 $\pm$ 457.2	2,686.6	1,933–3,888	0.193
	>130	+	2,566.1 $\pm$ 297.0	2,611.2	1,827.5–2,974.4	
Immunoglobulin	<130	–	690.87 $\pm$ 382.3	532.0	308.2–1,815	<0.001
	>130	+	1,953.09 $\pm$ 849.0	1,904.2	423.4–3,689	

Animals were considered healthy based on an SCC lower than  $130 \times 1,000$  cells/mL of milk and negative milk bacterial culture result, and were considered subclinical mastitic based on a SCC higher than  $130 \times 1,000$  cells/mL of milk and positive milk bacterial culture result

SCC somatic cell count

<sup>a</sup> Mann–Whitney test between healthy cows and cows with subclinical mastitis for each analyte

## Discussion

In the present study, the diagnostic accuracy of MAA, milk albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and Ig was evaluated compared to SCC and bacterial culture as the current gold standard tests for diagnosis of bovine subclinical mastitis.

MAA concentration in cows with subclinical mastitis was significantly higher than healthy cows. This result is in agreement with the results reported by other investigators (Grönlund et al. 2003; Safi et al. 2009; Gerardi et al. 2009). However, the mean and median concentration of MAA in healthy cows and cows with subclinical mastitis found in the present study were higher than those reported by Gerardi et al. (2009) and lower than those reported by Safi et al. (2009). The discrepancy between our results and results reported by other investigators could be due to several factors including biological differences of the animals, nature of the mastitic condition, and differences in quantitation techniques. The MAA test at the cutoff value of 1.6 mg/L showed high diagnostic sensitivity (92.3%) and specificity (92.1%) for the diagnosis of subclinical mastitis in dairy cows.

In the present study, the mean and median concentrations of milk albumin and Ig in cows with subclinical mastitis were significantly higher than healthy cows. These results are in agreement with the results reported by other investigators (Randolph et al. 1973; Ishikawa et al. 1982). The overall diagnostic accuracy of milk albumin and Ig for the diagnosis of bovine subclinical mastitis was high.

The concentration of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin in milk from cows with mastitis is a matter of controversy. Some investigators have reported a decrease in concentration of  $\alpha$ -lactalbumin (Ishikawa et al. 1982; Wickstrom 2009) and  $\beta$ -lactoglobulin (Ishikawa et al. 1982) in milk from cows with mastitis. However, a marked increase in the concentration of  $\alpha$ -lactalbumin (Waite and Blackburn 1963) and no change in the concentration of  $\beta$ -lactoglobulin (Nagasawa and Tanahashi 1963) in mastitic milk have also been reported. In the present study,  $\alpha$ -lactalbumin concentration was significantly increased in milk samples from cows with subclinical mastitis and although not statistically significant, the mean concentration of  $\beta$ -lactoglobulin in mastitic cows was lower than healthy cows. Although the reason for such variations in

**Table 2** Sensitivity, specificity, and area under the curve of milk amyloid A, albumin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and immunoglobulin concentration for diagnosis of bovine subclinical mastitis based on somatic cell count (SCC) and bacterial culture results

Analyte	Cutoff (mg/L)	Sensitivity (95% CI)*	Specificity (95% CI)*	AUC (95% CI)
Milk amyloid A	>1.6	92.3	92.1	0.978 <sup>a,b*</sup>
Albumin	>375.3	88.5	86.8	0.931 <sup>a,c</sup>
$\alpha$ -lactalbumin	>1,985.4	65.4	71.1	0.783 <sup>f</sup>
$\beta$ -lactoglobulin	<2,697.5	50.0	68.4	0.577 <sup>e</sup>
Immunoglobulin	>814.3	92.3	78.9	0.929 <sup>a,d</sup>

AUC area under the curve

\* Analytes with common superscript letters are not significantly different



the results of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin determination in milk remains unclear, the differences may partially be due to type and severity of udder infection (Randolph et al. 1973) and methods employed for  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin measurement.

## Conclusion

To the authors' knowledge, the diagnostic sensitivity and specificity, and cutoff values of whey proteins in bovine subclinical mastitis have never been reported. The results of the present study showed that determination of MAA concentration has a very high accuracy for the diagnosis of subclinical mastitis, and therefore, it can be used as a reliable method for the diagnosis of bovine subclinical mastitis. On the other hand, it can be used as a reliable alternative or complement test to the routine SCC in the diagnosis of subclinical mastitis. Furthermore, determination of whey proteins can be employed as potential biomarkers for diagnosis and monitoring subclinical mastitis in dairy cows.

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