Study on the accuracy of milk amyloid A test and other diagnostic methods for identification of milk quality

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Article Info	Abstract
Article history: Received: 22 July 2017 Accepted: 24 October 2017 Available online: 15 June 2018 **Key words:** Acute phase proteins Amyloid A Milk quality Sensitivity Specificity	Bulk tank somatic cell count (BTSCC) is a gold standard test for identification of milk quality, but its results are influenced by several interventional factors. Recently, application of acute phase proteins and especially milk amyloid A (MMA) has been considered as accurate parameters for milk quality study. The current research was done to evaluate the accuracy of MMA, BTSCC, fat, protein and lactose for identification of milk quality. Ninety bulk tank milk samples were collected from 30 randomly selected dairy herds and classified into two groups of samples with BTSSC > 200000 cells per mL and those with BTSSC < 200000 cells per mL. Protein, fat, lactose and MAA contents of samples were analyzed. Average amount of the MAA in healthy and mastitic milk samples were 5.15 and 504.35 ng mL^{-1}, respectively. Statistically significant difference was seen for MAA and total protein contents between two groups. Clinical accuracy of MAA-, total protein-, fat- and lactose–based methods was 0.937, 0.757, 0.665 and 0.547, respectively. The MAA method at concentration of 20.78 ng mL^{-1} had the highest sensitivity (97.30%) and specificity (46.70%). Evaluation of MAA is recommended as a rapid and accurate method for determination of the unfavorable changes in milk quality and early subclinical mastitis diagnosis.

مطالعه دقت آزمون آمیلوئید A شیر و سایر روش های تشخیصی برای شناسایی کیفیت شیر

چکیده

شمارش سلول های سوماتیک تانک سردکننده شیر (BTSCC) یک استاندارد طلایی برای شناسایی کیفیت شیر می باشد، اما نتایج آن تحت تاثیر عوامل مداخله ای متعددی قرار می گیرد. اخیراً، استفاده از پروتئین های فاز حاد و خصوصاً آمیلوئید A شیر (MAA) به عنوان فراسنجه های دقیقی جهت مطالعه کیفیت شیر در نظر گرفته شده است. تحقیق حاضر به منظور مطالعه دقت BTSCC، MAA، چربی، پروتئین و لاکتوز برای شناسایی کیفیت شیر انجام پذیرفت. نود نمونه شیر تانک سردکننده شیر از ۳۰ گله شیری به صورت تصادفی جمع آوری و به دو گروه نمونه های با BTSCC<۲۰۰۰۰۰ و BTSCC >۲۰۰۰۰۰ سلول در هر میلی لیتر تقسیم بندی شدند. محتوی پروتئین، چربی، لاکتوز و MAA نمونه ها ارزیابی شد. میزان میانگین MAA در نمونه های شیر سالم و ورم پستانی به ترتیب ۵/۱۵ و ۵۰۴/۳۵ نانوگرم بر میلی لیتر بود. اختلاف معنی دار آماری برای محتوی MAA و پروتئین تام بین دو گروه مشاهده شد. دقت بالینی روش های بر پایه MAA، پروتئین تام، چربی و لاکتوز به ترتیب ۰/۹۳۷، ۰/۷۵۷، ۰/۶۶۵ و ۰/۵۴۷ بود. روش MAA در غلظت ۲۰/۷۸ نانوگرم بر میلی لیتر بیشترین حساسیت (۹۷/۳۰ درصد) و ویژگی (۴۶/۷۰ درصد) را دارا بود. ارزیابی MAA به عنوان یک روش سریع و دقیق جهت تعیین تغییرات نامطلوب در کیفیت شیر و تشخیص زود هنگام ورم پستان تحت بالینی پیشنهاد می شود.

واژه های کلیدی: آمیلوئید A، پروتئین های فاز حاد، حساسیت، کیفیت شیر، ویژگی

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Introduction

Mastitis, inflammation of the udder tissue, is the most common and costly multi-etiologic disease in dairy industry affecting the yield, composition, quality and processing properties of the milk.[1,2] Mastitis costs the United States dairy industry approximately 2 billion dollars annually and has a similar impact in European union.[3] Mastitis causes severe economic costs due to milk production reduction, milk condemnation due to antibiotic residues and culling of chronically infected animals.[1-3] Mastitis also causes unfavorable changes in the milk quality which made it non-consumable for human.[1-3]

Subclinical form of mastitis is routine in dairy herds and causes diagnostic difficulties due to the lack of visible symptoms. Moreover, subclinical mastitis has a serious zoonotic potential associated with shedding of dangerous bacteria and their toxins in the milk. It is a serious public health threat because of affected cow's milk entrance into the bulk tanks.[4] It is therefore of great importance to identify specific and sensitive biomarkers that can be used for rapid detection of unfavorable changes in milk quality as a consequence of mastitis.[4,5]

Somatic cells are part of the natural defense mechanism of the body and include lymphocytes, macrophages and polymorphonuclear and epithelial cells.[6] The bulk tank somatic cell count (BTSCC) is considered as an indicator of the average udder health and is also the standard method for detection of subclinical mastitis and also milk quality. The BTSCC is influenced by several factors such as age, breed, milking management, physiological stage of the animal (stage of lactation, numbers of lactation and dry period), season, numbers of lambs born and stressors.[6,7] Previous researches have showed that BTSCC is an expensive, time-consuming, insensitive and nonspecific diagnostic method for identification of subclinical mastitis with poor correlation with bulk tank milk quality.[6,7] Therefore, researchers have attempted to find alternative biomarkers with higher sensitivity and specificity for rapid identification of subclinical mastitis.

Total protein test is another routine parameter used for evaluation of the milk quality and especially the quality of its proteins. This parameter can be used as an effective factor for milk price determination. These cases can be generalized to measure lipid and lactose levels of milk.[6,7]

Acute phase proteins (APPs) have been well investigated as markers of milk quality and subclinical mastitis in cows.[8] Milk amyloid A (MAA) is an APP produced by the mammary glands epithelial cells. It is one of the first proteins generated in milk following mammary glands infection.[8-10] The MAA concentration may increase up to 1000-fold following localized inflammations of udder tissue and decline rapidly following recovery from the diseases. It is a highly specific biomarker for subclinical mastitis identification.[8-10]

Comparison between the MAA and fat, protein and lactose content of milk represents the accuracy and capacity of MAA in the evaluation of milk quality. However, the comparison between MAA and BTSCC is more practical.[8-10]

The biological roles of MAA as an indicator of udder subclinical edema and milk quality have not been fully elucidated. Therefore, the current research was done to study the accuracy of MAA test in comparison with other diagnostic methods for identification of cow's milk quality.

Materials and Methods

Ethical consideration. The content of the current research was approved by the Ethical Council of Research of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran (Consent Ref Number 8823). Verification of this research project and the licenses related to sampling process were approved by the Prof. G. Karim and Prof. S. Safi (License number 2016-39).

Samples. The present study was performed on the bulk tank milk of the 30 randomly selected dairy farms in Tehran province, Iran. Three bulk milk samples at three different times were collected to consider the risk of any probable changes in the sampling procedure. Therefore, a total of 90 bulk tank milk samples were randomly collected. Contents of each bulk tank milk were mixed before sampling via blowing air into tank's upper inlet. A total amount of 300 mL milk was taken from each bulk tank. Collected milk samples were divided into 6 equal parts and used for identification of the MAA, BTSCC, total protein, casein, total fat and lactose. All tests were performed on the fresh milk samples. Identification of MAA was done on the frozen milk at −70 °C. [11-13]

Identification of BTSCC. Identification of BTSCC was done using the electronic fluorescence based cell counting (Fossomatic 5000; Foss, Hillerod, Denmark). [14]

Identification of Milk Amyloid A. Sandwich enzyme linked immunosorbent assay (ELISA, Tridelta Development Ltd., Wicklow, Ireland) was used for identification of MAA. All the test reagents and samples were allowed to reach room temperature before use. In brief, 50 μL of diluted biotinylated monoclonal antibody was added to each well. After vortexed for 2 min, milk samples were diluted 1:50 in 1x diluent buffer and added in duplicate to each well (50 μL). Following incubation for 1 hr at 37 °C, the plate was washed four times with diluted wash buffer. After the last washing, the plates were dried using an absorbent paper and 100 μL of streptavidin-horseradish peroxidase (HRP; Merck, Darmstadt, Germany) was added to each well. The plate was incubated at room temperature in the dark for 30 min. After washing the plate for four times, 100 μL of TMB substrate solution (Thermo Fisher Scientific, Darmstadt,

Germany) specify at 37 °C was added to them. After incubation in the dark condition at room temperature for 30 min, 50 µL of stop solution was added to each well and absorbance was read at 450 nm. For interpretation of results, mean absorbance for each sample and standards was calculated. Absorbance values of standards against the calibrator concentration were studied using the spectrophotometer (Shimadzu, Tokyo, Japan). Best smooth curve was drawn through these points to construct the calibration curve. Test samples concentrations were determined through multiplying the interpolated value by the appropriate dilution factor. Samples having a signal greater than the top calibrator or fall on the non-linear part of curve were further diluted in 1x diluent buffer and reanalyzed.[9] Figure 1 shows the standard curve of the MAA in the ELISA method.

Analysis of chemical properties of bulk tank milk samples. The percent of fat in milk samples was evaluated using the Gerber method (Gerber butyrometer).[15]

The nitrogen content in the milk samples was estimated by the Kjeldahl method and crude protein content was calculated as Nitrogen × 6.25.[16]

Lactose content of milk samples was determined using commercial Boehring kit (Roche, Mannheim, Germany).[17] The detection limit of the kit was 0.035 g per 100 g.

Statistical analysis. SPSS (version 21.0; SPSS Inc., Chicago, USA) software was used for statistical analysis of data collected from this study. At first, all data were subjected to Kolmogorov-Smirnov test to study their distribution. Data taken from total fat and total protein had normal distribution, while those of MAA, BTSCC and lactose hadn't normal distribution. Independent sample *t*-test was used for statistical analysis of data taken from total fat and total protein in milk samples with BTSSC > 200000 cells per mL and those with BTSSC < 200000 cells per mL milk. Mann-Whitney test was applied for comparison of MAA and lactose in milk samples with BTSSC > 200000 and BTSSC < 200000 cells per mL milk. The Receiver Operating Characteristic (ROC) analysis was performed to compare clinical accuracy of each milk quality parameter considering BTSCC as a gold standard. The ROC curve demonstrated quantity of true positive (sensitivity) and false positive (specificity) samples in potential cut off points. The area under the curve (AUC) was utilized to evaluate clinical accuracy of each test. The AUC of 0.50 to 0.70 indicated low clinical accuracy that of 0.70 to 0.90 represented moderate clinical accuracy and the quantities higher than 0.90 demonstrated high clinical accuracy for each test.[18]

Results

Table 1 represents the numbers of BTSCC and MAA concentration in milk samples. The MAA was detectable in 81 out of 90 bulk tank milk samples (90.00%). Average amounts of MAA, total protein, total fat and lactose in healthy and mastitic bulk tank milk samples were 5.15 and 504.35 ng mL^{-1}, 2.98 ± 0.05 and 3.05 ± 0.09 percent, 3.25 ± 0.29 and 3.41 ± 0.27 percent and 4.71 ± 0.17 and 4.72 ± 0.01 percent, respectively. There were no statistically significant differences for fat and lactose contents between two groups of bulk tank milk samples ($p > 0.05$). Statistically significant difference was seen for the contents of MAA ($p < 0.001$) and total protein ($p < 0.006$) between two groups of bulk tank milk samples.

Figure 2 represents the ROC curve analysis of the MAA, total fat, total protein and lactose contents in two groups of bulk tank milk samples. Analysis of the AUC showed that the MAA method had the highest levels of clinical accuracy (0.93). Clinical accuracy of total protein, total fat and lactose methods were 0.75, 0.66 and 0.54, respectively.

The MAA has exhibited the highest sensitivity (97.30%) and moderate specificity (46.70%) at a concentration of 20.78 ng mL^{-1}. Total protein, total fat and lactose levels were 2.93% (sensitivity of 89.30% and specificity of 20.00%), 2.91% (sensitivity of 96.00% and specificity of 13.30%) and 4.63% (sensitivity of 85.30% and specificity of 13.30%), respectively. The MAA has exhibited the lowest sensitivity (72.00%) and highest specificity (100%) at a concentration of 283.32 ng mL^{-1}.

Table 1. Descriptive statistics of quality parameters in two groups of bulk tank milk samples based on the BTSCC concentration lower and higher than 200000 cells per mL.

Parameter	Group	No. of samples	Mean ± SD*	Median	Minimum - Maximum	*p* Value
MAA** (ng mL^{-1})	1****	15	59.69 ± 20.67	50.15	0.40 - 278.30	<0.001
	2*****	75	551.83 ± 47.96	504.35	0.40 - 2305.70	
Total protein (%)	1	15	2.98 ± 0.05	2.98	2.88 - 3.08	0.006
	2	75	3.05 ± 0.09	3.04	2.84 - 3.36	
Total fat (%)	1	15	3.25 ± 0.29	3.32	2.69 - 3.69	0.036
	2	75	3.41 ± 0.27	3.46	2.85 - 4.04	
Lactose (%)	1	15	4.71 ± 0.17	4.71	4.61 - 4.82	0.566
	2	75	4.72 ± 0.01	4.74	4.48 - 4.94	
BTSCC*** (cells mL^{-1})	1	15	157.60 ± 22.78	162	120.00 – 194.00	<0.001
	2	75	425.81 ± 306.14	320	202.00 – 2450.00	

*Standard deviation; **Milk amyloid A; ***Bulk tank somatic cell count; ****Samples with BTSCC < 200000 cells mL^{-1}; and *****Samples with BTSCC > 200000 cells mL^{-1}.

Total protein, total fat and lactose levels were 3.05% (sensitivity of 46.70% and specificity of 93.30%), 3.68% (sensitivity of 21.30% and specificity of 93.30%) and 4.78% (sensitivity of 25.30% and specificity of 80.00%), respectively. The MAA has also exhibited the high sensitivity (88.00%) and specificity (86.70%) at a concentration of 137.12 ng mL^{-1}. Total protein, total fat and lactose levels were 3.00% (sensitivity of 74.70% and specificity of 60.00%), 3.34% (sensitivity of 64.00% and specificity of 66.70%) and 4.71% (sensitivity of 61.30% and specificity of 53.30%), respectively (considering BTSCC as a milk quality gold standard).
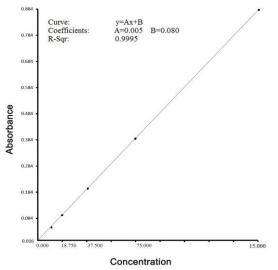

Fig. 1. Standard curve of the milk amyloid A in the ELISA method. Increase in the absorbance rate caused increase in the concentration of the milk amyloid A. All points were located accurately on the curve which represented its high accuracy.
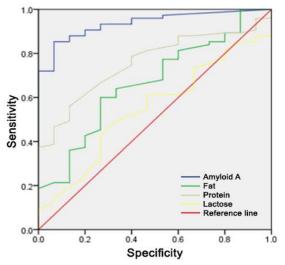

Fig. 2. The ROC curve analysis of milk amyloid A, total fat, total protein and lactose contents in two groups of bulk tank milk samples. As it shows milk amyloid A test had the highest sensitivity and specificity. Its specificity and sensitivity was higher than reference line in the figure.

Discussion

Clinical mastitis diagnosis is based on the appearance of abnormally appearing milk. Milk may be off color, watery, bloody and/or have the appearance of serum. Subclinical mastitis diagnosis is more problematic since the milk appears normal but usually has an elevated somatic cell counts. The BTSCC is performed routinely as an indication of milk quality, but it can be used to monitor the level of udder health when the bulk tank scores are monitored over time. The BTSCC has also a lot of deficiency such as low sensitivity and specificity, time consuming and its high cost.[4,19-21]

The current research showed the correlation between BTSCC and other qualitative markers with MAA indicating its comprehensiveness in milk quality assessment. Consistent with the results of several studies,[10,22-25] findings of the current investigation revealed that MAA test is an accurate, sensitive, specific and rapid method for milk quality diagnosis prior to subclinical mastitis. We found relatively high sensitivity and specificity for MAA test in milk quality determination compared to protein, fat and lactose based methods.[26]

Changes in the MAA levels can also be used as a biomarker for subclinical mastitis identification. The MAA was detected and measured in bulk tank samples in accordance with findings of earlier investigations.[27] Elevated level of MAA has previously been shown in milk from cows with clinical mastitis as a result of this protein leakage from the blood to the milk.[27-29] On the other hand, MAA is synthesized directly in the mammary epithelia of the udder in response to infection.[27-29] Therefore, MAA is believed to be a more sensitive indicator of mastitis.

Considering BTSCC as a gold standard for subclinical mastitis identification, MAA concentration in milk samples with BTSCC higher than 200000 cells per mL was significantly higher than those with BTSCC lower than 200000 cells per mL ($p < 0.001$). Similar results have been reported previously.[27,28] The median MAA concentrations from milk samples with BTSCC higher than 200000 per mL and those with BTSCC lower than 200000 per mL were 50.15 ng mL^{-1} and 504.35 ng mL^{-1}, respectively. Median serum amyloid A (SAA) concentration of 1300.00 ng mL^{-1} has been determined previously in bulk tank milk samples [27]which was higher than the content of MAA showed in our study. This difference may be due to the fact that aforementioned researchers[27] have detected all isotypes of amyloid, while the current study was focused only on the detection of MAA. It is because of that the MAA has been reported as the most accurate marker for milk quality identification. Proportion of healthy and mastitic milk in bulk tank milk samples and of healthy and mastitic milk in bulk tank milk samples

(46.70%) and considerable increase of false positive cases (53.30%). This phenomenon imposes severe economic losses to suppliers and dairies due to healthy milk samples condemnation. Using MAA test at concentration of 283.32 ng mL^{-1} (with 100% specificity, 72.00% sensitivity and 0.93 clinical accuracy) is an accurate and applicable method for milk quality identification. This phenomenon has the lowest false positive results (0.00%) and is more suitable to meet higher milk quality and food safety standards for consumers. Using MAA test at concentration of 137.31 ng mL^{-1} (with 86.70% specificity and 88.00% sensitivity) is an appropriate method especially in dairy industry.

Utilization of cutoff points with highest specificity or moderate values of sensitivity and specificity seems to be more practical, while reducing cutoff value as a pure screening test resulted in increased sensitivity followed by considerable false positive cases and decreased specificity.[8-10, 22,23]

The main evaluating factor in determination of different tests accuracy is statistical analysis. For this purpose, ROC analysis with the aim of comparing the efficacy of each test represents the true positive (sensitivity) and false positive (specificity) values at all cutting points. The sub-curved surface was used to evaluate the efficacy (sensitivity, specificity and clinical accuracy) of each test. In total, values higher than 0.90 showed the highest clinical accuracy for each diagnostic test.

However, increase in the BTSCC higher than 200000 cells per mL causes MAA clinical accuracy reduction (0.723 µg mL^{-1}), but it remains higher than other studied milk quality parameters. It seems that MAA is more sensitive than SAA for mastitis diagnosis in cow.[8-10,22,23] It is because of that the SAA enhancement can be induced also by other factors than disease, such as stress.[34] Lack of the correlation between SAA in milk and serum and expression of serum amyloid protein homologue in the mammary gland indicate that SAA is also produced locally in the mammary gland[35] supporting the higher accuracy of MAA than SAA. Therefore, application of MAA as an accurate biomarker for bovine subclinical mastitis diagnosis has a clinical importance. It has been reported that MAA ranges from 0.49 to 2.07 µg mL^{-1} with a mean value of 1.22 ± 0.44 in milk samples and significant increase can be seen in concentrations of MAA in quarter milk samples with subclinical mastitis compared to healthy animals. Further, it has been shown that the MAA at concentrations of 0.264 µg mL^{-1} and 39.41 µg mL^{-1} had the highest sensitivity (100%) and specificity (100%), respectively.[35] Also, earlier and higher peak of MAA compared to SAA has been reported suggesting that acute phase responses occurred during mastitis were more accurately detected in milk rather than serum.[36] Haghkhah *et al.* have recorded highest sensitivity and specificity (100%) using MAA as a diagnostic parameter compared to other acute phase proteins.[33] They have reported that the sensitivity of MAA test is higher than other diagnostic methods. It is because of that the MAA increases only in the developmental phase of mastitis. Therefore, MAA is an appropriate diagnostic method in the early stages of the mastitis. Safi *et al.* have compared the accuracy of measurement of APPs in milk and serum.[8] They have showed that MAA analysis at concentrations >16.40 mg L^{-1} had the highest sensitivity (90.60%) and specificity (98.30%). Thomas *et al.* have reported that the concentration of median SAA in milk samples is 3.87 µg mL^{-1}.[37] They have showed that the lack of correlation among SCC, median SAA and c-reactive protein can result from the higher sensitivity of median SAA. Miglio *et al.* have reported that the concentrations of MAA in healthy udders and those with subclinical mastitis are 29.68 ± 27.98 and 114.37 ± 41.14 µg mL^{-1}, respectively which were lower than our findings.[9] They have also showed that MAA concentration is affected by the udder health status and is a useful indicator for subclinical mastitis identification in sheep.

In conclusion, the high importance of MAA as an accurate, rapid, sensitive and specific marker for identification of milk quality and also subclinical mastitis has been reported. Furthermore, we considered that the concentration of MAA can be a useful indicator of mammary gland inflammation and unfavorable changes in milk quality in cow; although a clear and standardized cut-off value for the healthy udder-half should be confirmed. These results encourage further study of the MAA physiology in other animal species. Using three different concentrations of MAA is practical approach to study the quality of bulk tank milk samples. The advantage of MAA over other mastitis markers is attributable to the fact that it is not present in the milk of healthy animals and is not influenced by factors other than mastitis. Therefore, MAA estimation in milk is a useful diagnostic method to detect subclinical mastitis especially in bulk tank milk samples to monitor herd health. Therefore, the online measurement of MAA with automated milking systems can enable early detection of mammary inflammation and infection, reduce the economic loss and improve the health and welfare of dairy herds as well as public health.

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Conflicts of Interest

Authors declare that they have no conflict of interest.

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