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Milk lactate dehydrogenase and alkaline phosphatase as biomarkers in detection of bovine subclinical mastitis

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ABSTRACT

Currently, somatic cell count (SCC) and bacterial culture is considered as the gold standard of detecting subclinical mastitis. However, the above-mentioned tests have a low diagnostic accuracy. Therefore, for identification of infected animals, new biomarkers with high clinical accuracy are needed. The objective of this study was to determine the diagnostic value of milk lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) for the diagnosis of subclinical mastitis in dairy cows. The activities of these enzymes increase during mastitis, which make them to be the potential biomarkers for screening of mastitis. A total of 145 clinically healthy cows were randomly selected. Of these, 77 cows were considered to be affected by subclinical mastitis based on a SCC higher than 100×1000 cells/ml of milk and positive bacterial culture results of milk samples obtained from at least one of the quarters. Enzymes activities were measured in blood serum and defatted milk (centrifuged at 5000 g for 15 min at 4 ℃) using commercial kits. Diagnostic sensitivity and specificity and cutoff points for each test were determined via receiver-operating characteristics curve. Significant ($P<0.001$) increases in the mean and median activities of LDH and ALP were found in the milk samples collected from cows with subclinical mastitis. Milk LDH had the most clinical accuracy with 94.8% sensitivity and 94.1% specificity at cutoff point of 109 U/L. The results of the present study showed that the measurement of LDH and ALP activities in milk samples could be used as reliable method for detection of bovine subclinical mastitis.

Keywords: Lactate dehydrogenase, Alkaline phosphatase, Bovine, Milk, Subclinical mastitis

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INTRODUCTION

In spite of the great advances in sciences like genetics, nutrition, housing and milking conditions, there is a remarkable increase in a group of multifactorial diseases known as “production diseases” in most of high producing dairy farms [1]. Among the above-mentioned diseases, subclinical mastitis is the most economically important one, which result in reduced milk production, therapeutic expenses and milk disposal during the treatment period [2, 3], changes in milk hygiene and quality, reduced birth rate, increased mortality and early culling of superior cows [4, 5, 6]. In fact, subclinical mastitis is the most common and from economic point of view, is the most important disease in dairy industry [7]. Mastitis occurs in two different forms: clinical and subclinical mastitis. Clinical mastitis is recognized by abnormal udder appearance, changes in milk appearance and also systemic signs which might be seen

in affected cows and are easily diagnosed. Subclinical mastitis is more difficult to be recognized with no apparent change in udder or milk, which causes the disease to be unrecognized leading to drastic economic loss in most dairy breeds [8, 9, 10], so that 70-80% of the loss is attributed to subclinical mastitis [11]. Subclinical mastitis not only leads to reduced milk quality and quantity but also increases the risk of transferring of the disease to healthy cows. If subclinical mastitis is not recognized on time, the disease would be spread in the herd leading to an outbreak and consequently increased therapeutic expenses [2, 9, 10, 12]. Therefore using the effective techniques capable to diagnose the disease at early stages is of great importance [10, 13]. Although some diagnostic methods have been used for detection of subclinical mastitis, inflammatory reactions which are caused by infections in mammary glands are often examined by enumerating somatic cells in milk [14]. Now a day the cut-off point for detection of subclinical mastitis is considered as 100,000 cells/ml [8, 10, 11]. It should be noted that this approach lacks sufficient specificity because high somatic cell count doesn't necessarily indicate mastitis and other factors could influence SCC as well [10, 15, 16]. On the other hand SCC doesn't have enough sensitivity to be used as a screening test in detection of infected quarters [2, 12, 16]. Also the standard method for counting somatic cells is the Fussomatic electro optical method, which is limited to reference laboratories in most developing countries [10, 17]. At the present time, SCC together with bacterial culture is considered as the gold standard in diagnosis of subclinical mastitis[2, 10, 14, 18]. Also bacteriological tests are costly and time consuming and are not suitable to be used as routine tests [10, 12]. It is therefore of great importance to identify specific and sensitive new biomarkers that can be used for rapid detection of subclinical mastitis. The biomarkers preferably should be measured quickly and easily using routine techniques [2, 10]. During recent years, there has been an increased interest in the use of acute phase proteins in the monitoring and management of animal health [19, 20, 21]. also much research has been done in the field of acute phase proteins (APPs) in milk and it has been shown that measuring these proteins in milk has a diagnostic value but since the kits for measuring APPs are costly, the routine use of them takes time [8, 22, 23]. For years, the use of different enzymes in milk as biomarkers to identify mastitis has attracted attention and it has been shown that measuring enzyme activities in milk has a diagnostic potential for detection of mastitis [4, 24]. The concentrations of some milk enzymes such as lactate dehydrogenase and alkaline phosphatase increase during inflammation of mammary glands and the enzymes have the potential to be used as a screening test for detection of subclinical mastitis [1, 6, 11, 13, 25]. Infiltration of polymorphonuclear leukocytes and macrophages into mammary glands is one of the essential defense mechanisms against clinical and subclinical mastitis. During the inflammatory process, these cells and damaged cells of the udder's epithelial and interstitial cells, secrete products that contain hydrolytic enzymes. Some of these enzymes, such as lactate dehydrogenase (LDH) are among the non lysosomal enzymes and other enzymes are lysosomal ones [13, 26]. LDH is a cytoplasmic enzyme that has been proposed as a biomarker for udder health check [1, 6, 13, 15, 27]. Studies have shown that the activity of this enzyme significantly increase in milk obtained from quarters with subclinical mastitis [1, 6, 11,13, 27] and its activity has a high and positive correlation with SCC especially in infected quarters[11, 27]. Also, studies have shown that the activity of alkaline phosphatase in the milk of quarters with subclinical mastitis increases significantly compared to healthy quarters [20, 28, 29], and its activity has a positive correlation with SCC [28]. The origin of increased LDH is leukocytes found in mastitic milk [11] also the epithelial and interstitial cells, which have been damaged during the inflammatory process [13, 24], although the importance of epithelial cells for the activity of LDH in milk is not clear [11]. Also the origin ALP in subclinical mastitis milk is leukocytes and damaged mammary epithelial cells and interstitial cells during inflammation, particularly from disintegrated leukocytes [28, 29].

The objective of the present study was to determine the diagnostic value of LDH and ALP in cows with subclinical mastitis.

MATERIAL AND METHODS

Animals and sampling

A total of 145 Holstein cows were randomly selected from 7 industrial dairy farms in Tehran and Mazandaran provinces, Iran. The selected cows were in the lactation period and were milked three times a day using milking machines. A complete clinical examination and udder health check was performed on each cow and cows with no clinical sign of disease and no abnormality in udder and milk appearance were selected for this study. Cows in late pregnancy or early lactation were excluded from this study.

At first quarters were washed thoroughly with lukewarm water and dried. Then the teat end for each quarter were disinfected using ethanol 70% and allowed to dry. The first three streams were discarded and milk samples were taken from each quarter prior to the milking in three separate tubes. one tube was used to SCC analysis in milk lab.

The second tube was immediately transported to the clinical pathology laboratory in an icebox and was used for measuring the studied enzymes. All the milk samples for measurement of enzyme activity were skimmed by centrifugation at 5000g for 15 min at 4℃ and skim milk kept at -20 ° C until the results of microbial culture and SCC were ready. The third part of samples were taken into sterile flacons and immediately transported to microbiology laboratory in a cooler with ice packs for bacteriological culture. In order to measure the activities of studied enzymes in serum, a blood sample was taken from jugular vein of each cow into commercial Vacutainer tubes (Golden Vac^{TM}, China) on the same day as milk collection and immediately sent to the Clinical Pathology Lab, Saadat Abad Small Animal Polyclinic, Tehran, Iran, where the serum was separated and kept at -20 ° C until analysis. In this study, the cut-off point for SCC was chosen as 100×10^3 cells/ml to discriminate healthy cows from cows with subclinical mastitis. Cows with at least one quarter with SCC of more than 100×10^3 cells/ml and a positive bacterial culture were considered to be affected by subclinical mastitis and cows which had 4 quarters with SCC of $<100 \times 10^3$ cells/ml and with negative bacteriologic results were considered as healthy cows (control group). Milk samples (from 4 quarters) from healthy cows were mixed together and enzyme activities were measured in the composite milk samples.

Somatic cell count and bacterial culture

SCC in milk samples were performed using a Fossomatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark) in the milk center of agricultural organization, Shahriar & Sari, Iran. Blood agar, McConkey agar and CHROMagar^{TM} Mastitis (GP&GN) media were used for routine bacteriologic examination in Microbiology laboratory, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran, according to the National Mastitis Council guidelines [30].

Assays of enzyme activity

Milk samples were skimmed by centrifugation at 5000 g for 15 min at 4℃. Blood serum and defatted milk were used for determination of enzyme activities. DGKC method was used for measurement of LDH activity as follows:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

ALP activity was measured by DGKC method as follows:

$$\text{p-Nitrophenylphosphate} + \text{H}_2\text{o} \xrightarrow{\text{ALP}} \text{p - Nitrophenol} + \text{Phosphate}$$

using commercial kits (Farasamed, Iran) and Autoanalyzer (BT 1500, Roma, Italy) by the Department of Clinical Pathology, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran. Those samples which had the enzyme activities out of the linear range of the kits were diluted by saline solution (9 gr/L NaCl) at 1:10 dilution, according to the manufacturer's instructions. The result was then multiplied by 10.

Statistical analysis

All statistical analyses were performed using SPSS statistical software version 20 (IBM SPSS Statistics 20). Data were analyzed for normality using the Kolmogorov–Smirnov test. The mean and median values of each parameter were compared between the healthy cows and cows with subclinical mastitis using the Mann–Whitney test for nonparametric data and the independent samples t Test for serum LDH activities with normal distribution. 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 cut-off 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 accurate [31]. The diagnostic sensitivity and specificity, and AUC of the all tests were compared by using the McNemar test.

RESULTS

77 out of 145 selected cows had SCC higher than 100×10^3 cells/ml and positive culture results in at least one quarter and therefore, were considered as cows with subclinical mastitis. 68 cows had SCC lower than 100×10^3 cells/ml and negative culture results in all four quarters were considered as healthy cows. Out of 77 cows, Streptococcus uberis was isolated from 20 samples (25.97%), Streptococcus agalactiae from 14 samples (18.18%), Staphylococcus aureus from 18 samples (23.38%), E.coli from 14 samples (18.18%) and Streptococcus dysgalactiae

from 11 samples (14.29%). Descriptive statistics of the studied parameters in serum and milk samples of healthy and affected cows are shown in Table (1). The median and mean LDH activities in cows with subclinical mastitis were significantly ($P < 0.001$) higher than for healthy cows, while the mean LDH and ALP activities in the serum samples of the affected cows had no significant difference compared to those of healthy cows ($P = 0.775$ and $P = 0.873$ respectively). There was also a significant positive correlation ($P < 0.001$) between milk LDH and ALP activities and SCC. Sensitivity (Se), Specificity (Sp), clinical accuracy and cut-off points of the studied parameters are shown in Table 2. The results of the present study showed that the Se and Sp of LDH activity at cut-off point of 109 U/L were 94.8% and 94/1%, respectively for the diagnosis of bovine subclinical mastitis. The Se and Sp of ALP activity at cut-off point of 409 U/L was recorded as 83.1% and 77.9%, respectively. Also McNemar Test showed agreement (P = 0.597) between the two above-mentioned cut-off points. Also serum LDH and ALP activities had a low clinical accuracy for detection of subclinical mastitis and so they are not suitable markers for detection of subclinical mastitis.

Table 1. Descriptive statistics for milk lactate dehydrogenase (MLDH), milk alkaline phosphatase (MALP), serum lactate dehydrogenase (SLDH) and serum alkaline phosphatase (SALP) in healthy cows (n = 68) and cows with subclinical mastitis (n = 77)

Analyte (U/L)	SCC (×1000 cells/ml)	Bacterial culture	Mean ± SE	Median	Minimum−Maximum	*P*−Value
MLDH	< 100	−	54 ± 4	46	14 − 212	< 0.001[a]
	> 100	+	839 ± 113	504	80 − 4855	
MALP	< 100	−	300 ± 23	259/5	64 − 937	< 0.001 [a]
	> 100	+	918 ± 69	793	173 − 3980	
SLDH	<100	−	1602 ± 440	1587	752 − 2411	0.775[b]
	> 100	+	1623 ± 446	1611	679 − 2451	
SALP	< 100	−	121 ± 7	106	55 − 405	0.873[a]
	> 100	+	119 ± 7	107	54 ± 476	

Animals were considered healthy based on an SCC lower than 100×10^3 cells/ml of milk and negative milk bacterial culture result, and were considered subclinical mastitis based on a SCC higher than 100×10^3 cells/ml of milk and positive milk bacterial culture result
[a]Mann−Whitney test and [b]independent samples t test between healthy cows and cows with subclinical mastitis for each analyte

Table 2. Proposed cut-off value and resulting sensivity, specificity, and area under the curve (AUC) of milk lactate dehydrogenase (MLDH), milk alkaline phosphatase (MALP), serum lactate dehydrogenase (SLDH) and serum alkaline phosphatase (SALP) activities for diagnosis of bovine subclinical mastitis based on somatic cell count and bacterial culture tests

Analyte	Cut-off (U/L)	Sensitivity (95% CI)	Specificity(95% CI)	AUC (95% CI)
MLDH	109.5 [a]	94/8	94/1	0.992
MALP	409.5[a]	83/1	77/9	0.895
SLDH	1285.5[b]	74	29/4	0.520
SALP	86.5[b]	74	26/5	0.492

CI, confidence interval
Cut-offs with common superscript letters, agree together diagnostically by using the McNemar test

DISCUSSION

The bacteriologic results from the cows in this study with subclinical mastitis reflected the usual pathogenic bacteria, isolated from quarters affected with subclinical mastitis in Iran. The contagious pathogens causing subclinical mastitis were Streptococcus agalactiae (18/18%) and Staphylococcus aureus (23/38%). Also, the environmental pathogens were Streptococcus uberis (25.97%), E.coli (18.18%) and Streptococcus dysgalactiae (14.29%). The results of the present study showed that the mean LDH activities in milks from cows with subclinical mastitis were significantly ($P < 0.001$) higher than those from healthy cows. Our finding is consistent with the results of other researchers' studies [1, 6, 11, 13, 27]. Mean LDH activity in milk and SCC showed a positive significant ($P < 0.001$) correlation, which was reported by other researchers [11, 27]. Also mean ALP activities in milk of cows with subclinical mastitis were significantly ($P < 0.001$) higher than those from healthy cows and there was a positive significant ($P < 0.001$) correlation between milk ALP and SCC. The findings were consistent with the results of other researchers [6, 28, 29, 32]. The mean LDH and ALP activities in serum samples of cows with subclinical mastitis and healthy cows didn't show any significant difference ($P = 0.775$ and $P = 0.873$ respectively). Batavani et al., reported the same results [1, 32]. At the present study, clinical accuracy of LDH and ALP in serum and milk samples in detection of subclinical mastitis, considering SCC and bacterial culture as the gold standard,

were determined. Milk LDH at cut-off point of 109 U/L had the highest Se and Sp (94.8% and 94.1%, respectively). Se and Sp of milk ALP at cut-off point of 409 U/L were 83.1%, 77.9%, respectively. Symons and Wright proposed that milk LDH could be used as a sensitive marker for inflammatory changes of mammary glands [33]. Katsoulous et al., reported that LDH activity at cut-off point of 197 U/L for sheep and 185 U/L for goats had the Se and Sp of 92.8%, 98.2% and 95.4% and 96.3%, respectively and declared that LDH activity is sensitive and reliable marker for detection of subclinical mastitis [12]. Chagunda and colleagues reported that LDH activity had a high correlation with SCC, especially in milk samples of cows with subclinical mastitis. They also reported that LDH had a higher Se than NAGase in detection of subclinical mastitis [27]. Akerstedt and colleagues showed that LDH among all biomarkers of mastitis had the least variation [15]. Hiss et al., reported that LDH is a useful marker for detection of subclinical mastitis [11]. Yang et al., reported that measuring LDH and ALP activities in milk could be a useful diagnostic marker in detection of subclinical mastitis [6]. These findings are different from findings of Babai et al., which reported that LDH was not a sensitive marker for early detection of subclinical mastitis and only ALP had a high sensitivity in this regard [13]. The findings of the present study indicate that serum LDH and ALP had a low clinical accuracy for detection of subclinical mastitis and therefore, could not be used as a reliable marker for study of udder inflammation.

Inflammation of mammary gland can affect the milk composition in several ways. Due to increased permeability of blood-milk barrier, the serum proteins can leak into the milk. Also the damaged epithelial cells result in intracellular components release into milk and finally synthesis of milk-specific components produced in the mammary epithelium is reduced [8, 34]. Intramamary infection can increase the permeability of small vessels through secretion of chemical mediators such as histamine, prostaglandins, kinine, and oxygen free radicals from inflammatory cells [35]. The origin of increased LDH is the leukocytes in the milk from affected quarters [11] or the damaged epithelial mammary and interstitial cells during inflammatory processes [13, 24]. The increased ALP in the milk of cows with mastitis originates from mammary leukocytes and epithelial cells and also from damaged interstitial cells during inflammation, especially from damaged leukocytes [28, 29].

Since the blood-milk barrier is damaged, so it is also possible that the blood LDH or ALP may be transferred to milk [13]. While Batavani et al., showed that blood serum was not a significant source of these enzymes in the milk [32]., but it is likely that the damaged leukocytes and parenchymal cells of the breast can release the enzymes. However, the importance of damaged epithelial cells for LDH activity in milk is unknown [11]. Our research also showed that there is a significant positive correlation between LDH and ALP in milk and somatic cells and on the other hand no significant increase was seen in the activity of these enzymes in the blood serum of dairy cows with subclinical mastitis compared to the healthy cows.

Today, SCC together with bacterial culture are considered to be the gold standard in the diagnosis of subclinical mastitis [8, 12, 14, 18]. It should be noted that SCC lacks the needed specificity because its high levels may not necessarily reflect mastitis. In other words, the number of somatic cells in mastitis is affected by many other factors such as the number of lactations, stage of lactation, level of milk production, season, age and breed of cattle [2, 10, 16]. SCC also lacks enough sensitivity to be used as a screening test in detection of infected quarters because in the early stages of mastitis somatic cell count may not be elevated [2, 16]. Meanwhile the number may be increased in the first few days of lactation and remain high until the first month of lactation [8, 36]. The standard method for SCC is Fossomatic electro optical method, which is limited to reference laboratories in many developing countries [10, 17]. Also, bacteriological test are not suitable to be used as a routine test in the diagnosis of subclinical mastitis because of being costly and time consuming. Abstinence from infection during sampling is difficult and on the other hand there is the possibility of false negative results in quarters, which are chronically infected [13].Therefore, early detection of subclinical mastitis in milk requires inflammatory markers, which are reliable and fast enough to be used routinely [10]. Much research has been performed for the diagnosis of acute phase proteins in milk and it has been shown that measurement of these proteins in milk has a diagnostic value but since the APP diagnostic kits are expensive, their use as routine tests takes time [8, 22, 23].

It has been many years that measuring different enzymes in milk has drawn attention as biomarkers for detection of mastitis and it has been shown that milk enzymes have diagnostic potentials for detection of clinical and subclinical mastitis [4, 24]. The present study showed that measuring LDH and ALP activities in milk which is both easy and low cost compared to other methods could be used as a diagnostic test with acceptable sensitivity and specificity for detecting of quarters with subclinical mastitis. Moreover, unlike the other methods for routine diagnosis of mastitis,

measurement of the above-mentioned enzymes is also appropriate to be used during early lactation and the dry period in order to selective treatment [13].

CONCLUSION

Our investigation showed that measurement of LDH has high clinical accuracy, sensitivity and specificity in the detection of subclinical mastitis and could be used as a reliable method in dairy cows. So we propose that measurement of LDH and ALP, especially LDH has the potential to substitute SCC or to be used as a complementary test combined with SCC for early diagnosis of subclinical mastitis to reduce the enormous losses to the dairy industry greatly.

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