Diagnostic evaluation of milk lactate dehydrogenase and alkaline phosphatase activities by receiver operating characteristic analysis curve in early lactation of ewes with subclinical mastitis

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Article Info	Abstract
Article history: Received: 27 June 2017 Accepted: 07 November 2017 Available online: 15 December 2018 **Key words:** Alkaline phosphatase Early lactation Ewe Lactate dehydrogenase Subclinical mastitis	The aim of the present study was to investigate the diagnostic evaluation of milk lactate dehydrogenase (mLDH) and alkaline phosphatase (mALP) activities by receiver operating characteristic (ROC) analysis curve in early lactation of ewes with subclinical mastitis (SCM) and determine the correlation between number of somatic cell count (SCC) and mLDH and mALP activities. A total of 196 udder half milk samples were collected within the first 6 weeks of lambing. The SCM was determined by positive milk bacterial culture and positive California mastitis test (CMT); SCC was determined by fossomatic method and enzyme activities were determined spectrophotometrically. The mLDH and mALP of SCM cases were positively correlated with SCC values. Values of mLDH, mALP and SCC were significantly higher in SCM than non-SCM udder halves. The optimum cut-off points of mLDH and mALP activities for SCM diagnosis were determined at 203.61 (U L^{-1}) and 329.84 (U L^{-1}), respectively. In conclusion, SCC has positive correlation with mALP and mLDH activities in SCM ewes and mLDH and mALP activities could be considered as reliable indicators for intra-mammary inflammation diagnosis. © 2018 Urmia University. All rights reserved.

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ارزیابی تشخیصی فعالیت های لاکتات دهیدروژناز و آلکالین فسفاتاز شیر با استفاده از منحنی آنالیز مشخصه عملکرد سیستم در اوایل شیرواری میش های مبتلا به ورم پستان تحت بالینی

چکیده

هدف مطالعه حاضر بررسی ارزیابی تشخیصی فعالیت های لاکتات دهیدروژناز و آلکالین فسفاتاز شیر با استفاده از منحنی آنالیز مشخصه عملکرد سیستم در اوایل شیرواری میش های مبتلا به ورم پستان تحت بالینی و تعیین همبستگی بین تعداد شمارش سلول سوماتیک و فعالیت های لاکتات دهیدروژناز و آلکالین فسفاتاز شیر بود. در مجموع ۱۹۶ نمونه شیر نیمه پستان در طول شش هفته نخست بره زایی اخذ شد. ورم پستان تحت بالینی از طریق مثبت بودن کشت باکتریایی شیر و آزمون ورم پستان کالیفرنیایی تعیین شد؛ شمارش سلول سوماتیک از طریق روش فوسوماتیک مشخص گردید و فعالیت های آنزیمی از طریق اسپکتروفوتومتری تعیین شدند. مقادیر لاکتات دهیدروژناز و آلکالین فسفاتاز شیر موارد مبتلا به ورم پستان تحت بالینی همبستگی مثبتی با مقادیر شمارش سلول سوماتیک داشتند. مقادیر لاکتات دهیدروژناز، آلکالین فسفاتاز و شمارش سلول سوماتیک شیر در نیمه های پستان مبتلا به ورم پستان تحت بالینی به صورت معنی داری بیشتر از نیمه های پستان غیر مبتلا بودند. نقاط برش مطلوب فعالیت های لاکتات دهیدروژناز و آلکالین فسفاتاز شیر جهت تشخیص ورم پستان تحت بالینی به ترتیب ۲۰۳/۶۱ و ۳۲۹/۸۴ واحد بر لیتر تعیین شدند. در نتیجه گیری می توان اذعان داشت که تعداد سلول سوماتیک همبستگی مثبتی با فعالیت های لاکتات دهیدروژناز و آلکالین فسفاتاز شیر در میش های مبتلا به ورم پستان تحت بالینی دارد و فعالیت های لاکتات دهیدروژناز و آلکالین فسفاتاز شیر می توانند به عنوان شاخص‌های قابل اعتمادی جهت تشخیص آماس داخل پستانی مدنظر قرار گیرند.

واژه های کلیدی: آلکالین فسفاتاز؛ اوائل شیرواری؛ لاکتات دهیدروژناز؛ میش؛ ورم پستان تحت بالینی

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Introduction

Subclinical mastitis (SCM) is one of the most economically important infectious diseases in dairy small ruminants. Animals with SCM represent a constant risk of infection for the whole stock.[1] The gold standard for the diagnosis of intra-mammary infection (IMI) in dairy species is bacterial culture.[2] However, bacteriology has limited value because of the requirement for laboratory support and delayed results. A number of indirect tests such as somatic cell count (SCC) and California mastitis test (CMT) have been developed diagnosing intra-mammary inflammations efficiently and quickly. In general, CMT and SCC are accepted as valid tests for SCM detection in ewes,[3] but they could be subjected to different errors; therefore other inflammation markers should be used for SCM diagnosis.

Since the diagnosis of early lactation SCM by SCC and CMT can be challenging due to concurrent physiological increase in milk cellular content, the aim of the present study was to assess the validity of complementary enzymatic tests to distinguish IMI. The specific objectives of this study were to (a) investigate the variation in milk lactate dehydrogenase (mLDH) and alkaline phosphatase (mALP) activities of early lactation ewes with SCM and compare them with non-SCM ones and (b) evaluate the correlation between milk SCC, mALP and mLDH activities.

Materials and Methods

Animals and management. A total of 196 milk samples from one udder half of 196 Sangsari breed ewes were randomly gathered from twelve commercial out-door flocks that were undergone parturition between February and April 2015 in Semnan province, Iran. Animals included in the study were sampled once. Mean parity was 3.50 ± 0.30. Neonatal lambs were kept with ewes for approximately one month and the excess milk was manually milked by assigned shepherds.

Collection of samples. Milk samples were collected aseptically from each gland within six weeks post-partum. Ewes were restrained in a sitting position and the teat end of half udder was scrubbed thoroughly using cotton wool soaked in 70% ethyl alcohol. The first three streams were discarded, the teat orifice was disinfected again as described and 20 mL milk samples were taken in two sterile tubes held horizontally. All samples were kept in cold during transportation and delivered to the research laboratory of University of Semnan for examination within 2 hr after collection. The first sample was used for SCC and bacteriological examination and the other one was used for the milk enzymes determination.

California mastitis test. The CMT was performed on-site by one expert person using the method described by Schalm *et al.*[4] In brief, after discarding first three streams,3.00 mL milk was milked from aseptic half to CMT plate and mixed with 3 mL of reagent and agitated for 15 sec. According to the reactions obtained, the results were classified as follows: negative, traces, 1, 2 and 3, recorded as −, ±, +, ++ and +++, respectively. Samples with CMT grade 1, 2 or 3 were considered positive.

Bacteriology. Milk samples were cultured by standard loopful (0.01 mL) from each milk sample on blood agar medium (Bacto-Agar; Carolina biological supply Co., Burlington, USA) containing 5.00% of defibrinated sheep red blood cells and MacConkey agar (Merck, Darmstadt, Germany). All plates were incubated aerobically at 37 ℃ and examined for growth after 24 hr. If there was no growth, the plates were re-incubated and the final assessment was made after 48 hr. A gland was defined as bacteriological infected if five or more colonies of one or two types of bacteria were isolated. Samples from which less than five colonies were isolated or greater than two different colony types were cultured were defined as non-infected and contaminated, respectively. Bacteria were identified using colony morphology, hemolytic pattern on blood agar, Gram staining and standard biochemical methods as described by Sears *et al.*[5]

Somatic cell count. Milk was preserved with one drop of 2-bromo-2-nitropropane-1, 3-diol (D&F Control System Inc., San Francisco, USA) and stored for < 24 hr at 4 ℃. Then, SCC was determined using 500 μL of this preserved milk (Fossomatic-90 A/S N; Foss Electric, Hillerød, Denmark).

Enzyme assay. Milk from all samples was centrifuged at 15000 g at 4 ℃ for 30 min to separate the milk serum (middle layer). The activities of LDH and ALP enzymes in milk serum were determined spectrophotometrically using enzymatic kinetic methods.[6,7]

Case definition. Mammary glands without clinical abnormalities and with apparently normal milk that were CMT and bacteriologically positive were considered as SCM.[8,9] Samples from glands with gross clinical abnormalities and/or with abnormal milk appearance in strip cup (clot, pus or discoloration) were considered as clinical mastitis and accordingly omitted from the study.

Statistical analysis. Data were organized in Excel worksheets (version 15.0; Microsoft Corporation, Redmond, USA) and then, statistically analyzed by SPSS package (Version 19.0; IBM Corp. Armonk, USA). Specificity and sensitivity of CMT were calculated based on bacteriological culture**.** Agreement between bacteriological and CMT results for milk samples was investigated using Kappa statistics. The mLDH, mALP, SCC and parity data were assessed for normality with Kolmogorov–Smirnov test. The mean ± standard error of the mean of each parameter was compared between non-infected ewes and ewes with SCM using Mann-Whitney

test for mALP and with the independent *t*-test for mLDH and SCC. The means were considered as significantly different or tended to be significant when the *p*-values were less than 0.05 or less than 0.10, respectively. Pearson correlation test was used to determine the correlation between SCC and mLDH and mALP**.** The optimal cut-off values for SCC and evaluated enzymes were used to obtain the highest sum of diagnostic sensitivity (DSn) and specificity (DSp) and the greatest area under the curve (AUC). The DSn, DSp, AUC and its 95.00% confidence interval (CI), positive (PPV) and negative predictive values (NPV) and accuracy for ALP, LDH and SCC were determined by receiver operating characteristic (ROC) analysis by XLSTAT software (version 2015; Addinsoft, New York, USA).

Results

Since parity can contribute to significant changes of SCC in dairy sheep milk, the parity distribution in our study was normally distributed both for infected and non-infected ewes according to Kolmogorov–Smirnov test.

Distributions of microbial isolates from subclinical udder infection were as follows: *coagulase negative Staphylococci* (CNS), (64.00%), S*taphylococcus aureus* (31.00%) and *Bacillus spp* (5.00%).

Positive CMT was recorded in 89/196 (45.40%) and bacteria were isolated from 92/196 (46.90%) of milk samples. According to the aforementioned definition of SCM, 78 (39.70%) glands were considered to be affected. The specificity and sensitivity of CMT test in IMI detection were calculated as 89.40% and 84.80%, respectively. In early lactation ewes, kappa value for agreement of CMT and culture is 0.74.

The values for mLDH, mALP and SCC in normal milk samples and samples with SCM are presented in Table 1. The mean mLDH, mALP and SCC in milk from SCM udder halves were significantly higher than those with no SCM ($p < 0.01$).

Table 1. Mean values with standard error of the mean for milk serum lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities and somatic cell count (SCC) in milk samples obtained from non-infected (no-infection, California mastitis test (CMT) and bacteriologically negative) and subclinically infected (subclinical infection, CMT and bacteriologically positive) udder halves in ewes.

Parameters	No Infection (n=104)	Subclinical Infection (n=92)
LDH (U L^{-1})	168.50 ± 6.80[a]	329.70 ± 12.00[b]
ALP (U L^{-1})	297.80 ± 10.20[a]	362.60 ± 4.30[a]
SCC (× 10^3)	61.20 ± 7.20[a]	423.40 ± 74.40[b]

[a,b] Values within a row with different superscript letters are significantly different ($p < 0.01$).

Table 2 displays the correlation between SCC and mLDH and mALP. As it can be conferred, the SCC value was positively correlated with enzyme activities.

The optimum cut-off point of SCC for SCM diagnosis was $98 × 10^3$ (Cells per mL). The corresponding values of DSn, DSp, PPV, NPV and accuracy for this cut-off point were 97.50%, 80.00%, 90.70%, 94.10% and 91.70%, respectively (AUC 0.94, 95.00% CI: 0.89-1.00; Fig. 1A). The optimum cut-off point of mLDH for SCM diagnosis was 203.60 (U L^{-1}) with DSn, DSp, PPV, NPV and accuracy of 100%, 95.00%, 97.60%, 100% and 98.30%, respectively (AUC 0.99, 95.00% CI: 0.98-1.00; Fig. 1B) and for mALP it was 329.80 (U L^{-1}) with DSn, DSp, PPV, NPV and accuracy of 97.50%, 95.00%, 97.50%, 95.00% and 96.70%, respectively (AUC 0.99, 95% CI: 0.97-1.00; Fig. 1C).

Table 2. Correlation (Pearson) between the number of somatic cell count (SCC) and milk lactate dehydrogenase (mLDH) and alkaline phosphatase (mALP) activities in ewes.

Parameters	SCC	mLDH	mALP
SCC	1.00	0.70*	0.80*
mLDH	0.70*	1.00	0.90*
mALP	0.80*	0.90*	1.00

*Correlation is significant at the 0.01 level (2-tailed).
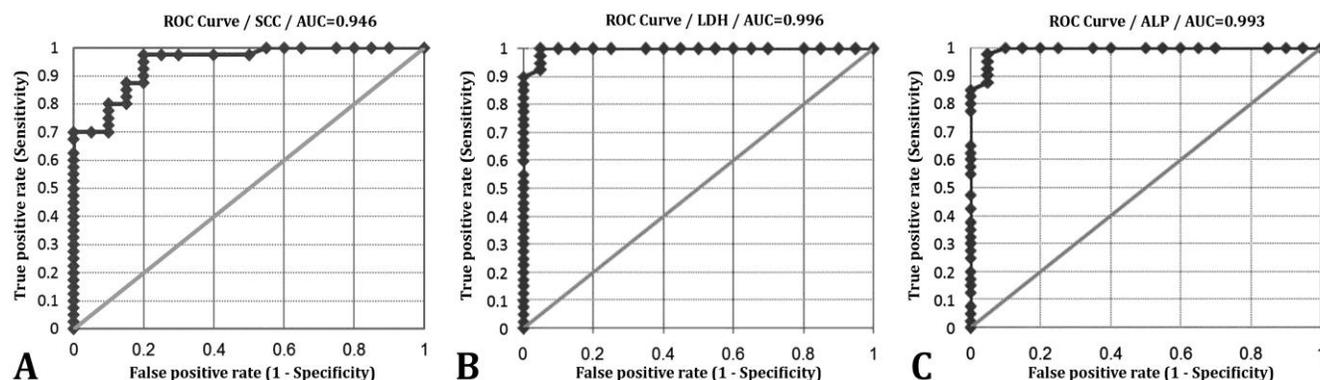
Fig. 1. Receiver operating characteristic (ROC) analysis curve for number of A) somatic cell count (SCC) in milk, B) lactate dehydrogenase (LDH), and C) alkaline phosphatase (ALP) in milk serum from 104 milk samples obtained from sheep udder halves with no infection and 92 milk samples obtained from subclinically infected udder halves. AUC: Area under the curve.

Discussion

To the best of our knowledge, no studies have been conducted in sheep for SCM detection in early lactation period using milk enzyme activities as IMI indicators. The use of SCC is one of the most established methods for udder health diagnosis in cows.[10] Unfortunately, SCC could not yet be established as a proven marker for SCM diagnosis in goats. Factors like parity, stage of lactation, estrus and breed cause significant changes of SCC in dairy goats milk.[11,12] The SCC is also affected by the nature of infection with minor or major pathogen organisms.[11,12] Although a moderately related study in sheep has shown that mLDH activity can be used as the most reliable indicator for SCM detection among the evaluated enzymes (LDH, ALP and aspartate aminotransferase)[13]. Results were in contrast with similar studies on dairy cow and buffalo.[14-17] For the mentioned bovids, it has been shown that not the mLDH but the mALP is a more reliable indicator for subclinical IMI detection.

During period of our study, the results showed that the main isolated bacteria from SCM cases are CNS that was in agreement with previous study.[3,13]

Although previously some researchers have believed that the stage of lactation cannot affect SCC values in sheep, other studies have shown that SCC is significantly higher in early lactation period. It is now known that SCC in non-infected ewes is higher in early lactation than mid-lactation and end-lactation counterparts.[15] Also, previous studies have concluded that breed, parity, stage of lactation, type of birth, estrus, diurnal variation, monthly variation and seasonal variations can contribute to significant changes of SCC in dairy sheep and goats milk.[10,18-20] Also, another study have showed that there are no reliable thresholds values for SCC in goat milk for SCM diagnosis. Depending on the individual study, goat milk has a significantly higher cell count than milk from cows and a higher variability in SCC.[1] While the health of udder quarters of cows is confirmed by SCC up to 100×10^3 cells per mL, the maximum SCC for goats ranges from 200×10^3 cells per mL up to a few million cells per mL.[10] On the other hand, McDougall *et al.* have showed that infection significantly increases SCC and CMT in sheep.[21] In another study, it was also found that CMT score is positively correlated with SCC and infection status.[22] The present study revealed that both CMT and SCC values were significantly higher in early lactation ewes with SCM than those without SCM. Therefore, CMT could be considered as an effective screening test for SCM in early lactation ewes; values for specificity and sensitivity were over 80.00%. These results were in agreement with other study.[3,9,23] On the other hand, other researchers have reported that use of CMT for IMI detection in sheep is less reliable.[8,24] These different results can be explained by the prevalence of SCM. Hueston *et al.* have proposed that specificity and

sensitivity of CMT would be much less reliable in the case of flock with low prevalence of SCM.[24]

In spite of discrepancies, we were also able to demonstrate that SCC can be used for SCM detection in first weeks of post-partum period. The ROC analysis showed that SCC was a good indicator when 98×10^3 Cells per mL cut-off point for detection of SCM with DSn and DSp more than 80.00% was defined. Other studies have reported higher cut-off point for SCC range between 300 and 1700×10^3 for SCM diagnosis.[22] In Churra breed ewes, the SCC thresholds for SCM diagnosis ranged between 150 and 700×10^3 cells per mL. It has been noted that type of milk record, infection criteria and prevalence of infection can be reasons for these SCC thresholds.[21] Also, these different thresholds have been proposed depending on the nature of the infections (mostly minor versus major pathogens), period of detection (early or mid-lactation and drying off) or even sampling methodology.[25]

Our findings showed that mLDH and mALP were statistically higher in SCM cases in comparison with milk samples from normal ewes. Similarly, higher mLDH and mALP in SCM cases have been reported in dairy sheep,[8,13] cows and buffaloes.[14,26]

Significant elevation of mALP in SCM might be due to both mammary epithelial damage and a breach in the blood-milk barrier selectively damaged by bacterial toxins. The origin of LDH in SCM milk is attributed to the presence of leukocytes and epithelial cells from the udder. Origin of elevated LDH and ALP activities was from leukocytes and mammary epithelial and interstitial cells damaged during inflammation, particularly from disintegrated leukocytes.[17] Therefore, on farm rapid test using for mLDH in dairy cows may be applicable in sheep for SCM detection. Of course, there is a rapid test for mALP determination which is used as an indicator of proper milk pasteurization. In attention to our results, these rapid tests should be evaluated for on farm sheep SCM detection and subsequent comparison with CMT.

Our findings showed that there is a good correlation between mALP and mLDH and SCC in SCM ewes in early lactation. In cows, there is a substantial agreement between CMT scores and mLDH and mALP with threshold of >180 and >40 IU L^{-1}, respectively, but only mALP showed proper sensitivity and reliability for the early diagnosis of SCM.[5] Other studies have showed that tissue disturbances of the mammary gland in SCM were accompanied by marked increase of mLDH activity in cow.[27-29] Also, regarding the results of enzymatic analysis in milk analysis of previous study, it has been observed that LDH and ALP activities are significantly increased in mastitis milk compared to normal milk in cow.[30] In buffalos, despite the significant increase in the concentration of mLDH and mALP, the authors have reported that SCM diagnosis individually by mALP is not completely satisfactory and it has been recommended

that mALP and zinc should be measured together in milk for screening large herds for SCM.[17] It has also been suggested that threshold value for mALP is fixed at 811.12 U L^{-1}. Although, other study has found that LDH amount and SCC have significant relationship in buffalos.[31]

In a similar study in sheep, subclinical IMI was shown to be associated with increased activities of mLDH and mALP values, but according to the results of ROC analysis, mLDH activity was identified as the most reliable indicator for SCM detection among the evaluated enzymes.[13] Using the proposed cut-off points of 197 U L^{-1} for sheep, the diagnostic sensitivities and specificities were higher than 92.00% Katsoulos *et al.*[13] In contrast, our results suggest that both milk enzyme (LDH and ALP) activities are acceptable indicators for SCM diagnosis at 203.61 (U L^{-1}) as cut-off point for mLDH and at 329.84 (U L^{-1}) as cut-off point for mALP, with DSn and DSp more than 95.00% for both of them. The differences between our findings and Katsoulos *et al.* results in mLDH and mALP cut points may be attributed to the stage of lactation and higher SCC in this period.[13] According to our ROC analysis, mLDH and mALP are more reliable indicators of SCM in early lactation ewes.

It can be concluded that in early lactation ewes, mLDH and mALP activities were higher in SCM ewes compared with healthy ones and had positive correlation with SCC. Therefore, evaluation of aforementioned parameters can be a reliable method for SCM detection.

Acknowledgments

Conflict of interest

There is no conflict of interest.

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