



## Assessment of Lactate Dehydrogenase Enzyme Activity in Milk as a Marker for Detection of Subclinical Mastitis

Mahavir Singh<sup>1</sup>, Anshu Sharma<sup>1\*</sup>, Dinesh Mittal<sup>1</sup>, Poonam Yadav<sup>2</sup> and Gaurav Charaya<sup>3</sup>

<sup>1</sup>College Central Laboratory, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, INDIA

<sup>2</sup>Centre for Radioecology, GJUS & T, Hisar, Haryana, INDIA

<sup>3</sup>Department of Veterinary Medicine, LUVAS, Hisar, Haryana, INDIA

\*Corresponding author: A Sharma; Email: anshusharma\_dr@yahoo.com

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### ABSTRACT

Sub clinical mastitis is responsible for heavy economic losses throughout the world in dairy sector. The objective of present study was to investigate lactate dehydrogenase (LDH) enzyme activity in milk for detection of subclinical mastitis in buffaloes. Milk samples from 60 buffaloes, including 40 subclinical infected and 20 healthy were subjected to the estimation of LDH using a LDH activity assay kit. LDH concentration in milk of buffaloes having subclinical mastitis was reported to be high ( $1.88 \pm 0.91$   $\mu\text{mole/ml/min}$ ) as compared to healthy animals ( $0.26 \pm 0.10$   $\mu\text{mole/ml/min}$ ). The receiver operating characteristics analysis at cut off value of  $0.44$   $\mu\text{mole/ml/min}$  for LDH activity exhibited sensitivity and specificity of 95% each and area under curve was 0.959. Results of the correlation matrix revealed significantly strong positive interdependence of somatic cell count with enzyme LDH ( $r = 0.722^{**}$ ;  $P < 0.01$ ). Measuring LDH activity in milk was found both easy and cost effective with high sensitivity and specificity indicating that LDH activity has great potential as a diagnostic tool for detection of sub clinical mastitis in buffaloes.

**Keywords:** Buffalo, subclinical mastitis, lactate dehydrogenase enzyme, somatic cell count

Mastitis is most costly disease of dairy animals and responsible for huge economic losses in India (Sharma *et al.* 2012). Majority of intramammary infections result in subclinical mastitis (SCM), which shows no inflammatory signs in udder and no abnormalities in udder secretions. However, compositional quality of milk is severely impacted by subclinical infection. Animals with subclinical mastitis should be considered as a constant risk of infection within and between herds (Charaya *et al.* 2013).

Therefore, early detection of sub clinical mastitis is of utmost importance in order to take appropriate measures toward cure and prevention of transmission of infection to other healthy buffaloes in herd. Although several diagnostic techniques have been used for detection of SCM, they are of little importance because of less sensitivity and specificity. Advance tests like real time PCR and estimation of acute phase proteins are costly and their use as routine tests will take time. Therefore, other

inflammatory markers with high diagnostic value and faster turnaround times are required for early diagnosis of SCM.

For many years there has been an interest in using different enzymes in milk as biomarkers for mastitis. The present study intended to investigate the possibility of optimizing the determination of enzyme Lactate dehydrogenase (LDH, EC 1.1.1.27) activity in milk in order to evaluate its use as a potential marker for detection of subclinical mastitis.

### MATERIALS AND METHODS

#### Sample collection and bacteriological examination

Milk samples were collected aseptically from all the four quarters from apparently healthy buffaloes of an organized buffalo farm. Hands were properly washed with soap and water and teat apices swabbed with 70% alcohol. The first

few milk stripping's were discarded and nearly 15 ml of quarter milk sample was collected separately in sterilized test tubes. Samples were transported to laboratory on ice and subjected to bacteriological examination (Quinn *et al.* 2004) and somatic cell count (SCC) (Schalm *et al.* 1971) for detection of subclinical mastitis following International dairy federation (IDF) criteria. As per this criteria, milk samples showing SCC more than 5 lac/ml of milk with isolation of bacteria, were considered positive for SCM.

### Lactate dehydrogenase enzyme estimation

The concentration of milk lactate dehydrogenase enzyme was estimated using a LDH activity assay kit (Sigma-Aldrich). Milk samples were defatted by centrifugation at 12000 rpm for 30 min at 4°C. Each of defatted milk samples (200 µl) were homogenized with 500 µl of cold LDH assay buffer on ice. Samples were centrifuged at 12000 rpm for 20 min at 4°C to remove insoluble material. Only soluble fraction was used for assay. The NADH standards were made for colorimetric detection. For this, 0, 2, 4, 6, 8, and 10 µL of the 1.25 mM NADH standard were added in duplicate into a 96 well plate, generating 0 (blank), 2.5, 5, 7.5, 10, and 12.5 nmole/well standards and LDH assay buffer was added to a final volume of 50 µL. A total of 10 µl milk samples in duplicate were added in 96 well microtitre plate and 40 µl of LDH assay buffer was added to make it 50 µl. Master reaction mix (LDH assay buffer 48 µl + 2 µl LDH substrate mix) in each well was added. Contents were mixed well and the plate was protected from light. After 2-3 minutes, initial measurement at 450 nm wavelength was taken. The plate was incubated at 37°C taking measurements every 5 min. Measurements were taken continuously until value of most active sample was greater than the value of highest standard.

Final measurement was the final reading where most active sample was near or exceeds the end of linear range of standard curve. Background values were corrected by subtracting the final measurement ( $A_{450}$  final) obtained for the 0 (blank) NADH standards from the final measurement ( $A_{450}$  final) of the standards. LDH activity of milk samples were determined by the equation.

$$\text{LDH activity} = \frac{\text{Amount of NADH generated} \times \text{Dilution factor}}{\text{Time} \times \text{Sample volume}}$$

One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 µmol of NADH per minute at 37°C.

## RESULTS AND DISCUSSION

Certain enzyme levels increase in milk during intramammary infections (Zhao and Lacasse, 2008 and Anderi *et al.* 2011). LDH is one of the enzymes which have been used as an indicator of inflammatory conditions of the udder. It is a cytoplasmic enzyme involved in carbohydrate metabolism that gets released into milk from ruptured mammary epithelial cells, phagocytes and from serum resulting into change in the physical and chemical properties of milk during mastitis (Larsen and Aulrich, 2012).

In present study, milk samples from 60 buffaloes, including 40 subclinical infected and 20 healthy were subjected to the estimation of LDH to classify animals in udder health categories for early detection of mastitis. Staphylococci and streptococci were the microbes associated with subclinical mastitis. LDH concentration in milk ranged from 0.12 µmol/ml/min to 3.38 µmol/ml/min with a mean value of  $1.88 \pm 0.91$  in subclinical mastitis animals (Table 1 and Figure 1). LDH activity increased significantly in infected buffaloes as compared to healthy ones ( $*P < 0.001$ ). Numerically higher variation in LDH (CV= 48% v. CV=40%) activities had been observed in sub clinically infected buffaloes as compared to healthy ones. The negative skewness values of LDH in SCM group suggested that their distribution was in lower range while in healthy group, with positive skewness value enzyme exhibited distribution towards higher range. Negative kurtosis values for LDH in both groups exhibited platykurtic distributions in lower range. Lower IQR values for the enzyme in healthy group indicated that the data points are clustered somewhat around the mean value.

In line with our results, Many researchers (Batavani *et al.* 2003; Chagunda *et al.* 2006; Batavani *et al.* 2007; Zaki *et al.* 2008; Larsen *et al.* 2010; Mohammadian, 2011; Kalantari *et al.* 2013 and Sorensen *et al.* 2015) reported marked elevation in LDH activity in milk serum from subclinically infected bovine mammary gland, attributed to tissue disturbances of the udder parenchyma.

**Table 1.** Descriptive statistics of LDH activity in milk of healthy and subclinical mastitic buffaloes

Enzyme	Animal health status	Min	Max	Mean	±SD	IQR	Skewness	Kurtosis	CV %
LDH (µmol/ ml/min)	Subclinical	0.12	3.38	1.88	0.91	1.20	-0.16	-0.74	48
	Healthy	0.10	0.47	0.26	0.10	0.13	0.46	-0.56	40

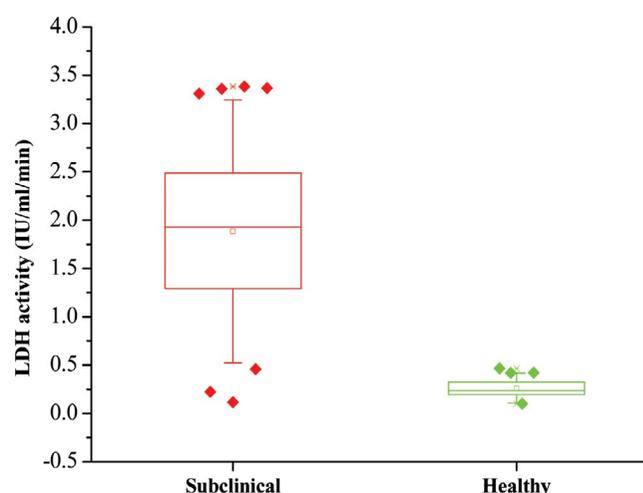
In contrast, Babaei *et al.* (2007) reported that LDH was not a sensitive marker and suspected that due to damage of blood-milk barrier, it is possible that the blood LDH may be transferred to milk. However, several other workers (Kato *et al.* 1989; Lauzon *et al.* 2005 and Batavani *et al.* 2007) reported that the higher level of LDH in mastitic milk than blood serum shows that blood serum was not the sole source of this enzyme in mastitic milk and it was probably also liberated from disintegrated leukocytes and the parenchymal cells of the udder. Kalantari *et al.* (2013) showed that there is a significant positive correlation between LDH 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.

In present study, ROC analysis at cut off value of 0.44 µmole/ml/min for LDH activity exhibited sensitivity and specificity of 95% each and area under curve is 0.959. The high degree of sensitivity and specificity of LDH activity as ascertained in this work supports the findings of Chagunda *et al.* (2006) who reported that the test had 73–95% sensitivity, 92-99% specificity. Among the enzymes, milk LDH was reported having highest clinical accuracy with respective sensitivity, specificity percentages of 94.8 and 94.1 (Kalantari *et al.* 2013).

**Associations among SCC and enzyme activity**

To explore the interdependence among LDH and SCC, Pearsons’ correlation technique was applied to the observed values. Results of the correlation matrix revealed significantly strong positive interdependence of SCC with enzyme LDH ( $r = 0.722^{**}$ ;  $P < 0.01$ ). In line with our results, significantly higher association of SCC with LDH ( $r = 0.76$ ) was also documented by Chagunda *et al.* (2006). Milk from subclinically inflamed udders from German Holstein cows was documented having positive correlations of LDH ( $r = 0.76$ ,  $P = 0.01$ ) activity with SCC (Hiss *et al.* 2007). In a recent study conducted on Swedish dairy herds having intramammary infection. Nyman *et al.*

(2014) reported positive correlations among udder-health indicators, SCC and LDH activities.



**Fig. 1.** Box whisker plots displaying distribution of LDH activity in milk of subclinical mastitic and healthy buffaloes

**CONCLUSION**

At present the SCC and bacteriological examination in milk has been used widely as an indicator for the detection of subclinical mastitis in animals as per IDF criteria but these tests incur high cost and labour. High correlation between LDH and SCC in sub clinically infected buffaloes in the present study suggests that LDH activity can substitute SCC to detect sub clinical mastitis in buffaloes. Lack of speed and high cost per sample limit the use of bacteriological examination as a screening test. Current study shows that measuring LDH activity in milk is both easy and cost effective with high sensitivity and specificity having great potential as a diagnostic tool for detection of sub clinical mastitis in buffaloes. Since the LDH test does not provide any information regarding etiology, it can be used for primary screening of large herds for identifying subclinically infected animals for subsequent bacteriological examination. The LDH activity can be

used to develop kit for diagnosing sub clinical mastitis in the field.

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