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ASSESSMENT OF LACTATE DEHYDROGENASE AND ALKALINE PHOSPHATASE AS BIOMARKERS FOR DETECTION OF SUBCLINICAL MASTITIS IN CATTLE

By

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ABSTRACT

Subclinical mastitis is a very important health problem affecting dairy cattle. It is associated with reduced milk quality and quantity and if not recognized and controlled in time it increases the risk of transferring of the infection to the healthy cows. Currently, somatic cell count (SCC), California mastitis test (CMT) and bacterial culture still are the standard gold tests used for detection of subclinical mastitis.The drawbacks of these conventional techniques necessitate the search of more sensitive biomarkers with high clinical accuracy and sensitivity. The activities of leukocyte enzymes including lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), which increase during mastitis, are potential biomarkers for detection of subclinical forms of mastitis. The objective of this study was to evaluate the diagnostic potential of milk LDH and ALP for diagnosis of subclinical mastitis in dairy cows as compared to SCC, CMT and bacterial culture. A total of 108 clinically apparently healthy cows were randomly selected in this work. Using SCC, CMT and bacteriological isolation, 50 cows (47%) were considered to be affected by subclinical mastitis. The following bacterial species were recovered from thesemilk sample; *Staphylococcus aureus*(18%), *Staphylococcus epidermidis* (14%), *E. coli* (14%), *Klebsiella* spp (36%), *proteus* spp (18%). The collected milk samples were examined for the LDH and ALP Enzymes activities. The mean and median activities of LDH and ALP were higher in the milk samples collected from cows with subclinical mastitis and reached to 818 ^{B}*± 38.2 and 123.4 B* ± 3.2 µ/L, respectively, as compared to **103** ± 2.5 and 30.3 ^{A} ± 1.3**,** respectively, in milk samples from normal cattle that proved free from subclinical mastitis. The obtained results revealed that the LDH and ALP activities in milk samples are reliable sensitive biomarkers for detection of bovine subclinical mastitis.

Keywords:

Bovine subclinical mastitis, SSC, CMT, Milk Lactate dehydrogenase, alkaline phosphatase.

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INTRODUCTION

Mastitis inflicts heavy economic losses on account of reduced milk production, treatment costs, increased labor, milk withheld from human consumption following treatment and premature culling. Therefore, early detection of mastitis at the subclinical stage is important for most dairy farmers to reduce production losses and to enhance prospects of recovery. Diagnosis of clinical mastitis is based on the local and systemic reactions and changes in milk (e.g. off color, watery, bloody appearance and presence of flakes, clots and pus). The diagnosis of subclinical mastitis is problematic since the milk appears normal but usually has an elevated somatic cell count **(Forsback *et al*., 2010)**. Subclinical mastitis has 15-40 times more prevalence than clinical mastitis. This might be attributed to the fact that most cases of subclinical mastitis are associated with no visible change in milk appearance or udder and the disease remains undetected. The compositional changes of milk due to subclinical mastitis reflect the degree of physical damage to the udder parenchyma **(Eshratkhah *et al*., 2012).** Diagnosis of mastitis at subclinical stage is vital because changes in the udder tissue take place much earlier than they become apparent. The drawback of the currently available subclinical mastitis diagnostic tools, namely, somatic cell count (SCC), CMT and bacterial culture necessitate the search for a more sensitive accurate approach **(Gera *et al.*, 2006)**. According to **(Kalantari *et al*., 2013)** the concentrations of some milk enzymes such as lactate dehydrogenase (LDH) and alkaline phosphatase (AP) increase during inflammation of mammary glands and the enzymes have the potential to be used as a screening test for detection of subclinical mastitis**.** Infiltration of polymorph nuclear 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 including LDH and ALP **(Babaei *et al*., 2007)**. The present work aimed to correlate between SCC, CMT, microbiological examination and LDH and ALP enzymatic activities in diagnosis of subclinical mastitis in cattle.

MATERIAL AND METHODS

Samples:

A total of 108 milk samples (50ml/ each) were collected aseptically in sterile McCartney bottles as described by **(Blowey, 2010)**. Each sample was divided into three parts. One was used for bacteriological examination, the second part one for SCC and CMT determination and the third portion was used for measurement of LDH and ALP enzymatic activities. All samples were sent immediately to the laboratory in ice box for examination.

California mastitis test (CMT):

California mastitis test (CMT) was carried out according to **Schalm and Noorlander, (1957)**. The results were recorded after 10 seconds and judged as follows. Negative (-ve), where the mixture remains liquid with no evidence a precipitate formation. Positive (+ve) reaction is associated with transformation of the mixture into gel.

Somatic cell count (SCC):

Somatic cell count (SCC) was measured by NucleoCounter® NC-100™ in Animal Reproduction Research Institute, Giza.

Bacteriological examination of samples:

Milk samples were incubated aerobically at 37ºC for 24 hrs. then centrifuged at 3000 rpm for 20minutes. The supernatant fluid was discarded and a sterile loopful from the sediment was cultured onto the surface of Mannitol salt agar, blood agar and MacConkey agar plates. The plates were incubated aerobically at 37ºC for 24 - 48 hrs., then examined for bacterial growth, the growing surface colonies were purified, picked up and identified biochemically using catalase test**,** Coagulase test **(Taylor and Achanzer, 1972),** Mannitol test **(Cowan and Steel, 1974),** Urease test **(APHA, 2004),** TSI **(Macfaddin, 2000),** Indol test **(Kovacs, 1928),** Citrate utilization test **(Baiely and Scott, 1998)**, Methyl red test **(Cowan and Steel, 1974),** Voges - Proskauer test **(Cowan and Steel, 1974).**

Assessment of LDH:

LDH Activity was measured by the spectrophotometer using Lactate dehydrogenase (LDH)-Liquizyme (4+1) kits (spectrum diagnostics) as described by **(Mohammadian, 2011)**.

Assessment of ALP:

ALP analysis was measured by spectrophotometer using (ALP) liquizyme (9+1) (spectrum diagnostics) as described by **(Batavani *et al*., 2003)**.

Statistical analysis:

All statistical analyses were performed using pro-stat programme as described by **(Petrie and Watson, 1999)**, the mean and median values of each parameter were compared between the healthy cows and cows with subclinical mastitis. The difference was considered statistically significant at P-value of < 0.05.

RESULTS

Bacteriological examination of milk samples:

In the current study, it was found that 50 milk samples out of 108 samples (46%) were diagnosed using conventional techniques as subclinical mastitis. It was positive for California mastitis test and somatic cell count test (SCC was higher than 200 × 1000 cells/ml).

As shown in (Table. 1) and Fig. (1), Bacteriological examination of milk samples from these animals was positive and the following bacterial isolates were recovered; *Staphylococcus aureus* (18%), *Staphylococcus epidermidis* (14%), *E. coli*(14%), *Klebsiella* spp(36%), *proteus* spp (18%).

Table (1): Incidence of bacterial pathogens isolated from milk samples of cattle diagnosed positive for subclinical mastitis using SSC and CMT.

Isolated Bacterial species	No.	Incidence % *
Staphylococcus aureus	**9**	**18**
Staphylococcus epidermidis	**7**	**14**
E. coli	**7**	**14**
***Klebsiella* spp**	**18**	**36**
***Proteus* pp**	**9**	**18**
Total	**50**	**46**

***Percentages were calculated according to total positive samples for CMT and SCC.**

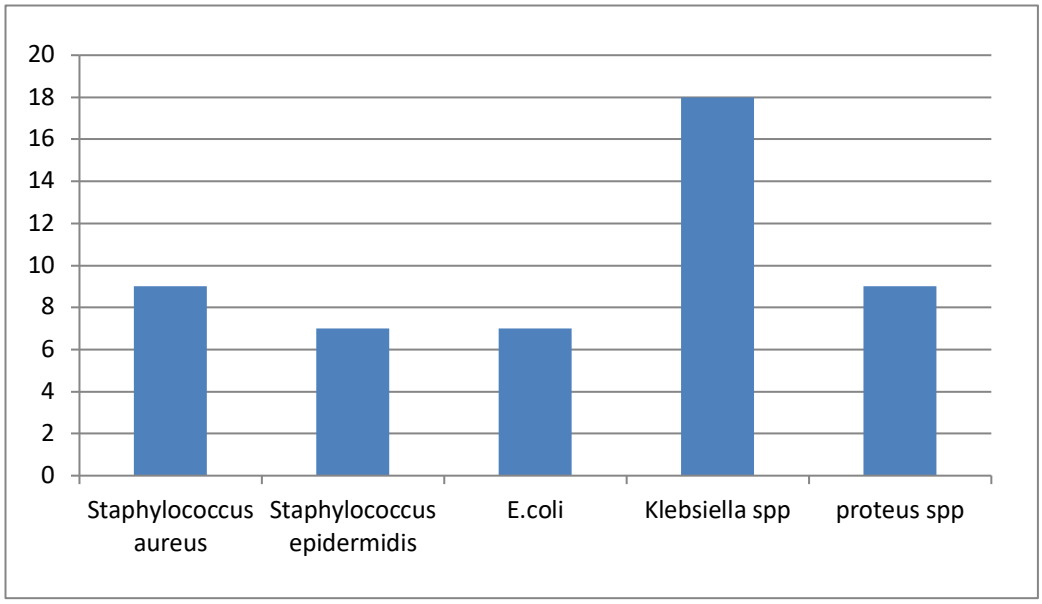

Fig. (1): Incidence of bacterial pathogens isolated from bovine milk samples collected from cattle diagnosed positive for subclinical mastitis using SCC and CMT.

Result of assessment of LDH and ALP enzymatic activity in bovine milk samples:

Data presented in Table (2), Fig. (2) showed the activity of LDH and ALP enzyme in milk samples from cattle diagnosed suffering from subclinical mastitic using the conventional diagnostic tests, namely SSC, CMT and bacteriological examination. The LDH and ALP enzyme activities were significantly high in milk samples from cows with subclinical mastitic and reached to 818 ± 38.2 and 123.4 ± 3.2 µ/L, respectively, as compared to 103^{A} ± 2.5 and 30.3^{A} ± 1.3 µ/L with milk from apparently normal cows.

Table (2): The enzymatic activities of LDH and ALP in milk samples from normal cows and cows with subclinical mastitis.

Parameters	Normal cows	Subclinical mastitic cows
Somatic cell count × 10^3	**89^{A}± 1.8**	**683^{B*} ± 11.5**
LDH (µ/L)	**103^{A} ± 2.5**	**818^{B*}± 38.2**
ALP (µ/L)	**30.3^{A} ± 1.3**	**123.4^{B*} ± 3.2**

*** Significantly different from control at P<0.05.**

Means that have different subscripts in rows were significantly different at P<0.05.

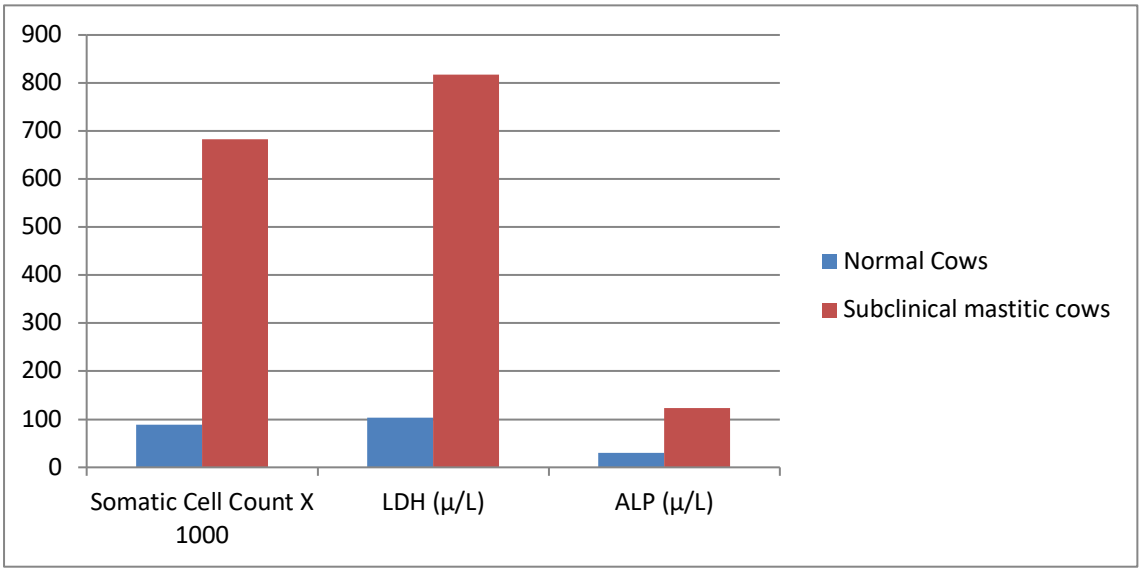

Fig. (2): The enzymatic activities of LDH and ALP in milk samples from mastitic and healthy cows

DISCUSSION

Subclinical mastitis is one of the most health problems affecting dairy industry where the infected animal shows no obvious clinical symptoms and secretes apparently normal milk for a long time, during which the causative pathogens spread the infection in the herd. This represents an important feature of the epidemiology of many forms of bovine mastitis. Early diagnosis of mastitis is essential for reduction of production losses and for enhancing the prospects of recovery **(Bakken and Gudding, 1982)**. The most common conventional test for detection of bovine subclinical mastitis depends upon the detection of the cellular players of the inflammatory process i.e., the SCC. More recently the attention of the researchers is directed to look for the initiators of the inflammatory process (e.g., the proinflammatory cytokines) or the products of the inflammatory cells like the secreted cellular enzymes rather than the cells itself. Therefore, in the present study we tried to correlate the conventional diagnostic tools used for detection of subclinical mastitis with the enzymatic activities of LDH and ALP in milk samples in order to evaluate its sensitivity as a diagnostic tool. Using the conventional diagnostic tests an incidence of 46% subclinical mastitis cases out of was 108 samples was identified. Bacteriological examination of these positive samples revealed the recovery of the following bacterial spp. *Klebsiella* spp (36%), *Staphylococcus aureus* (18%), *Staphylococcus epidermidis* (14%), *E.coli* (14%), *proteus*spp (18%) (Table 1), Fig. (1). Similar results were recorded by (**El-Khodery and Osman, 2008**). Although CMT and SCC

are field, easy, rapid and cheap tools helping as screening tests for detection of subclinical forms of bovine mastitis and it directs attention to individual mammary quarter that is secreting milk of high **(Abdel-Rady and Sayed, 2009**) but among the drawbacks of CMT it is un suitable in early lactation or in dry period. Therefore, the detection of milk enzymatic activities of LDH and ALP might represent a reliable diagnostic method for identifying subclinical mastitis in early lactation or in dry period **(Babaei *et al*., 2007)**. Data presented in (Table 2), Fig. (2) Confirmed the sensitivity of using LDH and ALP enzymatic activity as biomarker for diagnosis of subclinical mastitis. The mean LDH and ALP activities in milks cows with subclinical mastitis were significantly ($P < 0.05$) higher than those from healthy normal cows. Our finding is consistent with the results recorded by **(Batvani *et al*., 2007), (Yang *et al.,* 2011) and (Kalantari *et al*., 2013)**. 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 **(Akerstedt, 2008)**. Intramammary infection can increase the permeability of small vessels through secretion of chemical mediators such as histamine, prostaglandins, quinine, and oxygen free radicals from inflammatory cells**.** The origin of increased LDH is the leukocytes in the milk from affected quarters **(Hiss *et al*., 2007)** or the damaged epithelial mammary and interstitial cells during inflammatory processes **(Babaei *et al.,* 2007).** The increased ALP in the milk of cows with mastitis originates also from mammary leukocytes and epithelial cells and from damaged interstitial cells during inflammation, especially from damaged leukocytes **(Anirban *et al.,* 2012)**. The present research showed that there is a significant positive correlation between LDH and ALP activity and somatic cells and also proved that measuring LDH and ALP activities in milk is both easy and low cost compared to the other diagnostic tools. Transformation of the enzyme activities detection in milk sample into a field test necessitates further investigations.

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