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Efficacy of Reliable Milk and Blood Biomarkers for Diagnosing Clinical and Subclinical Bovine Mastitis

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Abstract | Mastitis diagnosis is of increasing importance and required appropriate detection methods since it is the most economically costly disease in dairy animals. The present study was conducted for detecting subclinical and clinical bovine mastitis using eclectic biochemical parameters of milk and serum including; aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), Calcium (Ca), Magnesium (Mg) and Phosphorus (P). A total of 111 milk and blood samples were collected from 111 Holstein Friesian cows in the mid-lactation period, from different reigns in Basrah city, Iraq. Therefore, the investigated cows were including 62 subclinical mastitic cows with positive to California mastitis test (CMT) and no signs of clinical mastitis, 27 clinical mastitic cows that showing various manifestations concerning milk and udder, and 22 control negative cows. Somatic cell count (SCC) and pH of milk were higher in subclinical and clinical mastitic cows than the control. Serum and milk levels of Ca, Mg, and P were significantly ($p < 0.05$) low in subclinical and clinical mastitic cows contrast to control. In addition, serum and milk levels of AST, ALP, and LDH were significantly high ($p < 0.05$) in subclinical and clinical mastitis compared to control. The findings of the present study indicate that the alteration in the levels of AST, ALP, LDH Ca, Mg, and P in serum and milk may serve as useful biomarkers of subclinical and clinical mastitis.

Keywords | Bovine, Biomarkers, Biochemical parameters, Clinical Mastitis, Subclinical Mastitis.

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INTRODUCTION

Cows are the most farm animals raised in Basrah governorate for milk production in addition to being an essential source of meat. Mastitis considered one of the most important economic factors in the field of dairy production, which affects the quantity and quality of milk, as well as its effect on the health of newborns and the general health of the animal (Halasa et al., 2007). Mastitis can be clinical or subclinical, subclinical mastitis (SCM) is difficult to be detected due to the absence of any visible signs, and it has major cost implications (Viguier et al., 2009). Clinical mastitis shows various manifestations like abnormal milk, mammary gland inflammation (redness, heat, swelling, pain and impaired function) with varying degrees according to the severity with or without systemic reaction. Thus, bacteria could be present in the milk and milk can vary in consistency and may become serum-like or watery with milk clots (Philpot and Nickerson, 2000). Different methods were used for detecting subclinical mastitis, including CMT, electrical conductivity, and estimation of milk SCC (Biggs, 2009). Recently, new techniques have been adopted for the early detection of mastitis, regardless to the type of the causative agent, by measuring the activities of selective biochemical parameters in the milk and blood that reflect the general health status of the animal and the udder condition (Pandey et al, 2012; Zeinhom et al., 2013; Ogurinade et al., 2015). However, little information of the host cell response of the clinical and subclinical mastitic cows in Iraq. In addition, the currently available

diagnostic tools used for diagnosing mastitis especially in the early stage of the disease is not clearly developed. Therefore, this study aimed to evaluate the changes of biochemical parameters in serum and milk as a result of clinical and subclinical mastitis and its potential use as an early indicator of inflammation in Holstein Friesian lactating cows that born in Iraq.

MATERIALS AND METHODS

Animals and Experimental Design

A total of 111 Holstein Friesian cows born in Iraq, aged between 3-6 years old at the mid-lactation stage, were selected from different reigns in Basrah / Iraq during the period from September 2018 to June 2019. All the cows were examined clinically for evidence of clinical mastitis and divided in to; 62 subclinical mastitic cows (CMT positive), 27 clinical mastitis cows, and 22 control healthy cows (CMT negative). Inspection and palpation of the udder were performed to detect abnormalities of bovine mammary glands, according to (Constable et al., 2016). Physical examination of milk includes color, odor, and, consistency was conducted, as mentioned in (Marth, 1978). California Mastitis Test was performed for each cow suspected with subclinical mastitis according to (Schalm et al., 1971).

Collection of Samples

Milk samples were collected, as described in (Biggs, 2009). The udder was cleaned thoroughly using cotton soaked in 70% ethyl alcohol; 40 ml of milk was collected in a sterile plastic container for the determination of milk pH, SCC, milk minerals and milk enzymes. Blood samples were collected according to (Jackson et al., 2017). 10 ml of blood were collected aseptically from jugular vein and divided into two tubes (9ml in a plan tube for serum biochemical analysis; and 1 ml in EDTA tube for estimation DLC). Then, samples were transported immediately in an icebox to Clinical Pathology Laboratory/ College of Veterinary Medicine/ University of Basrah, Iraq.

Determination of Milk pH and SCC

The pH of milk was determined with the aid of CT-6021A-type electronic pH meter (Kedida/ China). Direct microscopic SCC was determined, using Breed's smears stained with Levowitz-Weber modification of the Newman-Lampert as described in (Marth, 1978).

Enzymes Analysis

Milk and blood samples were prepared according to the instructions of the manufacturer of the diagnostic kits. Milk whey and serum were stored in Eppendorf tubes at -20°C for biochemical analysis. Milk enzymes analysis were conducted on 88 samples selected of total 111 milk samples, including 51 subclinical mastitis samples, 27 clinical mastitis and 10 control samples to be tested using 96-micro ELIZA plate provided with commercial enzymes kit. Milk enzymes AST and ALP were estimated using bovine Aspartate Aminotransferase ELIZA kit and bovine alkaline phosphatase ELIZA kit respectively (Wuhan Fine Biotech/ China). While LDH was estimated using bovine Lactate Dehydrogenase ELIZA kit (My BioSource/ USA). The optical density (O.D.) absorbance was read at 450 nm using a Microplate ELISA reader (Biotek Instrument/ USA). Levels of serum AST, ALP and, LDH were estimated using commercial enzymes kit (Cobas/ USA) according to the instruction manual of Chemistry auto-analyzer (Cobas Integra 400 plus/Germany). The absorbance of serum AST, ALP, and LDH was measured at 340nm, 480nm, and 340nm, respectively. Level of milk enzymes was expressed as ng/ml as instructed by the kit manufacturer.

Mineral Analysis

Serum and milk samples were prepared by acid digestion as mentioned in (Xueping et al., 2002; Rao et al., 2017), for estimation of Ca and Mg level**.** While the preparation of milk and serum for evaluation of phosphorus level was done according to (Galluci, 1973). The levels of Ca and Mg in milk and serum were estimated according to the instruction manual of Atomic Absorption Spectrophotometer AAS (Biotech Engineering Management CO., LTD. /UK). The concentration of phosphorus in milk and serum was determined by colorimetric method as phosphovanadomolybdate using Spectrophotometer (Labomed, INC. /USA) (Marczenko, 1986). The mineral analysis was conducted in the Central Research Laboratory of the Faculty of Agriculture/ University of Basrah, Iraq.

Differential Leucocyte Count

Differential Leucocyte Count was examined according to (Stockham et al., 2013).

Statistical Analysis

Data were analyzed using SPSS (version 24); one-way ANOVA test and student t-test were used to assess the significance between groups. P value < 0.05 considered statistically significant.

RESULTS

According to clinical examination and CMT on 111 cows, it was found that 27 (24.32%) cows infected with clinical mastitis, 62 (55.85%) were infected with subclinical mastitis (positive to CMT), and 22 (19.82%) were negative to CMT and considered as a control group. Clinical mastitic cows show various manifestations in relation to milk and udder (Table 1), the main clinical abnormalities of milk includes watery milk (51.85%), presence of blood (14.81%),

Table 1: Main clinical manifestation of milk and udder in clinical mastitis cows

Clinical manifestations (n=27)		No. of affected cows	Percentage
Abnormalities of milk	Watery milk	14	51.85%
	Blood	4	14.81%
	Pus	4	14.81%
	Clots	12	44.44%
Abnormalities of mammary gland	Swelling	21	77.77%
	Hotness	12	44.44%
	Pain	22	81.48%
	Dropped udder	1	3.70%
	Teat Hyper-keratosis	4	14.81%

Table 2: Milk pH, SCC and biochemical parameters in control, subclinical and clinical mastitis (Mean ± Standard Error)

Parameters	Control	Subclinical mastitis	Clinical mastitis
pH	6.54±0.03 b	6.96±0.02 a	7.12±0.03 a
SCC Cell/ml	16×10^4±665 c	16×10^5±257 b	12×10^6±204 a
Ca (mg/dl)	59.68±1.39 a	37.89±1.88 b	30.70±2.69 c
Mg (mg/dl)	9.83±0.15 a	9.33±0.04 b	9.11±0.06 c
P (mg/dl)	36.73±1.51 a	28.01±0.76 b	24.68±1.14 c
AST (ng\ml)	42.62±5.60 c	49.55±3.18 b	90.86±5.84 a
ALP (ng/ml)	10.76±1.58 c	29.02±1.44 b	40.91±5.12 a
LDH (ng/ml)	25.48±2.41 c	59.22±3.94 b	131.74±11.71 a

Values expressed in small letters mean significant differences at (P<0.05) level.

pus (14.81%) and, clots in the milk (44.44%) while clinical manifestation of udder includes swelling of udder (77.77%), hotness (44.44%), pain (81.48%), dropped udder (3.70%), and teat hyperkeratosis (14.81%). Milk pH and SCC show significant (P< 0.05) increase in subclinical and clinical mastitic cows compared to control (Table 2). However, significant (P< 0.05) decrease in the levels of milk Ca, Mg, and P were observed in subclinical and clinical mastitic cows compared to control (Table 2). In addition, milk ALP and LDH levels show significant (P< 0.05) increase in subclinical and clinical mastitic cows when compared to control. Milk AST levels were significant (P< 0.05) increase in clinical mastitis compared to control, although there is an increase in milk AST level in subclinical mastitis compared to control, the increase is not significant (Table 2).

Table 3: Serum biochemical parameters and differential leucocytes count in control, subclinical and clinical mastitis (Mean ± Standard Error)

Parameters	Control	Subclinical mastitis	Clinical mastitis
Ca (mg/dl)	8.65±0.28 a	6.28±0.19 b	4.66±0.14 c
Mg (mg/dl)	2.35±0.10 a	1.91±0.05 b	1.92±0.08 b
P (mg/dl)	6.21±0.27 a	4.12±0.09 b	3.26±0.09 b
AST (U/L)	76.72±4.94 c	82.09±5.43 b	99.22±6.20 a
ALP (U/L)	52.41±5.55 c	99.93±9.73 b	117.28±10.55 a
LDH (U/L)	504.59±55.93 c	967.72±51.63 b	1189.77±35.9 a
Neutrophil %	35.45±1.13 b	47.87±1.35 a	46.81±1.13 a
Lymphocyte %	51.54±1.56 a	41.59±1.37 b	45.03±1.11 b
Monocyte %	6.04±0.70 a	6.11±0.34 a	5.62±0.43 a
Eosinophil %	6.18±0.92 a	3.85±0.51 b	2.00±0.36 c
Basophil %	0.77±0.17 a	0.56±0.15 a	0.52±0.19 a

Values expressed in small letters mean significant differences at (P<0.05) level

Levels of serum Ca, Mg, and, P of subclinical, and clinical mastitic cows was significantly (P< 0.05) decrease contrast to the control group (Table 3). On the other hand, levels of AST, ALP and, LDH in serum of subclinical and clinical mastitic cows were significantly (P< 0.05) increase when compared to control group (Table 3). Results of differential leucocyte counts show significant (P< 0.05) increase in the mean of Neutrophils and Lymphocytes in both subclinical and clinical mastitic cows compared to control group. However, no significant (P> 0.05) differences in the mean Monocytes, Eosinophil, and Basophils in both subclinical and clinical mastitic cows compared to the control group (Table 3).

DISCUSSION

Mastitis diagnosis is of increasing importance and required appropriate detection methods since it is the most economically costly disease in dairy animals. Mastitis leads to induce physical and chemical changes in milk and pathological changes in the udder (Babaei et al., 2007). In the present study, physical examination of milk from cows infected with clinical mastitis show discolored milk and abnormalities in consistency which varies from watery to viscous secretion which may be attributed to the presence of blood, pus and milk clots respectively (Biggs, 2009; Srivastava et al., 2015). The main signs of clinical mastitis that related with udder (Table 1) were probably occurred as a result of the force handling on the teat end during milking process (Philpot et al., 2000; Constable et al., 2016).

The results of CMT in the present study displays that the occurrence of subclinical mastitis was (55.85%) which indicates a high prevalence rate. However, this finding is in corroborated with (Dasohari et al., 2018), who reported a high percentage (48.69%) of positive CMT cases.

The CMT test is widely used in dairy fields and recommended as a rapid indicator for mammary gland infection (Al-Anbari et al., 2006). It is useful method for monitoring the alteration of milk pH and SCC in the field for early detection of subclinical mastitis. However, the accurecy of CMT is still debatable.

In the present study, the pH value of milk samples from subclinical and clinical mastitis cows was markedly increased (Table 2), same finding were reported by (Koop et al., 2010; Riaz et al., 2012; Hassan, 2013). Clinical and subclinical mastitis may elicit higher milk pH as a result of increasing in the permeability of blood-milk barrier, which in turn affects the rate of leakage of blood components like sodium, chloride, and bicarbonate to milk based on the severity of the inflammation (Harmon, 1994).

Estimation of milk SCC was highlighted in several studies since it considered as an indicator of subclinical mastitis in which more than 200,000 cell/ ml considered positive (Atakisi et al., 2010; Riaz et al., 2012). Present study shows a significant increase in SCC in subclinical and clinical mastitis (Table 2) and these results were an agreement with previous related studies (Atakisi et al., 2010; Zeinhom et al., 2013). Increasing SCC in milk samples from mastitic cows could be explained as a result of a response to the inflammation of mammary gland (Dosogne et al., 2003) which in turn leads to increase of infiltration of leukocytes mainly polymorphonuclear cells.

Regarding the results of mineral analysis in milk samples (Table 3), it was found to be relevant to the findings of the other researchers (Batavani et al., 2007; Hussain et al., 2012), who reported that the concentrations of milk Ca, Mg and P were significantly lower in sub-clinical mastitis compared to control group. Although their results appear to be higher than the present study, it could be attributed to differences in the stage of lactation and the nature of the diet. In contrast to our study, Yildiz et al. (2005) did not find a correlation of Mg levels in milk between subclinical mastitic and healthy animals.

Decrease minerals levels in milk during clinical and subclinical mastitis may be explained as most of the milk minerals are exist in the colloidal form bounded to casein Casein concentrations reduced during mastitis because mastitis causes an overall reduction in milk yield due to the destruction of milk secretory cells (Ogola et al., 2007). Moreover, levels of serum minerals (Table 3) were lower in subclinical and clinical mastitis than the control, which is similar to (Al-autaish et al., 2018) who reported that there is a significant decrease in serum Ca, Mg and P in subclinical mastitic animals compared to control.

In the current study, there was an increase in milk AST, ALP and, LDH in mastitic cows compared to control (Table 2). The elevated activity of milk enzymes during both clinical and subclinical mastitis may be originated from destructed mammary epithelial cells, and damaged leukocyte since the intensity of increase in these enzymes is positively proportional to the severity of mastitis. (Ogurinade et al., 2015). Furthermore, the activity of enzymes in serum and milk has been studied extensively and included in the list of enzymes that considered as a biomarker for inflammation (Babaei et al., 2007; Katsoulos et al., 2009). Results of serum enzymes (Table 3) corroborated with observation of (Ogurinade et al., 2015) which reported significant increase in serum AST, ALP and, LDH in subclinical mastitis than in control group. However, Sarvesha et al. (2016) concluded that the changes in haemato-biochemical parameters can be used as important indicators of the physiological or pathological state in the animal. They have reported a significant increase in serum AST level in subclinical mastitis and clinical mastitis compared to control. Despite their findings appear to be higher than our results, but it can be attributed to the different sample size and different detection methods.

In contrast, our finding is incompatible with (Mohammadian, 2011) where finds no significant difference in serum LDH level in subclinical mastitis compared to control, which may indicate that the severity of inflammation was not sufficient to produce a significant increase in serum LDH. Thus, the increased levels of serum enzymes observed in the present study in mastitis may be a

consequence of tissue destruction, also due to increase in the permeability of microcirculatory vessels of the udder, which leads to an elevation in serum enzymes (Åkerstedt et al., 2011).

In the present study, differential leukocyte count was estimated (Table 3). Significant increase of Neutrophil and Lymphocytes was observed in subclinical and clinical mastitic cows compared to control. In line with the present study, (Singh et al., 2014) evaluated some hematological parameters and reported a significant increase in Neutrophil in subclinical and clinical mastitis compared to control group. On the other hand, he reported a decrease in Lymphocytes, Monocytes, and Eosinophil in subclinical and clinical mastitis than the control group.

The increase in Neutrophils and Lymphocytes in subclinical and clinical mastitis may be attributed to high production and release of inflammatory cells from bone marrow. The increase is occur as a result of increased demand for these cells in response to inflammation as they have an essential role in the eliminating the pathogenic microorganism and control the infection. These cells can migrate to the site of infection via a process termed diapedesis, which stimulated by the chemical mediators released during mastitis form the affected glandular tissue (Day et al., 2014).

CONCLUSION

This study concluded that both subclinical and clinical mastitis will induce a marked change in the level of milk and serum enzymes (AST, ALP, LDH) and minerals (Ca, Mg and, P) that can be linked to tissue damage of mammary parenchyma. It may serve as potential rapid and sensitive test in conjunction with other screening tests like CMT for early detection of abnormalities of mammary gland in cows regardless of the causative agent. The change in DLC especially increased Neutrophils and Lymphocytes, may be suggestive on an inflammatory process.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHORS' CONTRIBUTIONS

All researchers participate in performing the work equally.

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