Original Article

Cumulative Effect of Subclinical Mastitis on Immunological and Biochemical Parameters in Cow Milk

Saleem, H. D[1], Razooqi, M. A[2], Gharban, H. A. J[3] *

1. Department of Density, Al-Manara College of Medical Sciences, Al-Manara, Iraq
2. Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Iraq
3. Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, Iraq

Abstract

Mastitis is a complex and multifactorial disease that leads to chemical, physical, and bacteriological changes in milk, which is associated with great economic losses. This study was carried out on lactating cows to investigate the effect of subclinical mastitis (SCM) on milk production through the estimation of immunological and biochemical characteristics of milk. Therefore, a total of 200 apparently-healthy lactating cows were randomly selected from different areas in Baghdad and Maysan provinces in Iraq from April to July 2021, and 100 ml of fresh milk was directly collected from each cow. Milk samples were examined by the California mastitis test (CMT), and IgG concentration in milk was estimated by the ELISA method. Moreover, the changes in biochemical parameters and enzymatic parameters were analyzed to determine the prevalence of SCM. Based on the results of the CMT test, the prevalence of SCM was 41.5% with a significant increase in a mild degree of severity (61.45%). IgG antibodies increased significantly in positive cows (4.92± 0.21 µg/ml). Significant prevalence of infection was observed among cows ≥5 years old compared to those <5 years, crossbred ones compared to natives, and those with lower milk production without udder/milk abnormalities, compared to cows with normal milk production. However, a significant difference was observed between the two regions under investigation (i.e., Baghdad and Maysan provinces of Iraq). Results of biochemical and enzymatic parameters showed significant increases in the concentration of Cl, FFA, and Na, and a reduction in LAC and TP, though not in Ca and K. The pH of mastitis milk was significantly higher. Although no significant difference was observed in the values of ALT and LDH, AST was increased in mastitis milk. In conclusion, this study is one of the first reports on the estimated concentration of IgG in mastitis milk samples in Iraq. Increases in the concentration of SSCs and IgG in milk can apply to the detection of intra-mammary infections.

Keywords: Biochemical parameter; California mastitis test; ELISA; Iraq

1. Introduction

Subclinical mastitis (SCM) is one of the most common intra-mammary infections, which results in huge economic losses due to the reduction in the quality and production of milk, the costs of veterinary intervention, and mortalities (1, 2). Although many animals can become infected with SCM, lactating cows are more sensitive to significant changes in milk components (3, 4). Additionally, predisposing factors for the emergence of infection strongly depend on the awareness of dairy farmers, management practices, stage of lactation, and breed type (5). However, different infectious microorganisms (e.g., coagulase-negative *Staphylococcus* spp., *Corynebacterium bovis*, members of Enterobacteriaceae, *Pseudomonas*, *Serratia*, and bovine herpesvirus 4) are implicated in

bovine SCM (6, 7).

In some cases, SCMs occur due to microbial invasion through the mammary duct during lactation or opportunistic infections from the environment without local symptoms or systemic inflammation. Once established, several pathogens can exist during the cow's entire lactation period (8-10). Therefore, early detection of SCM is essential for minimizing losses and preventing zoonotic transmission. Although the culture of milk samples is the gold standard for detecting SCM, it remains highly expensive and impractical for routine application (11, 12). Throughout the years, much attention has been paid to developing indirect tests which are especially used in screening tests for predicting the existence of intra-mammary infections (13). Globally, the determination of milk somatic cell counts (SCCs) is considered to be one of the best-recommended methods to diagnose the prevalence of pathogenic SCM (14). The CMT and automated electronic counters are the most developed and available commercially-verified methods for determining the level of SCC. The CMT, first designed in 1957 as a qualitative assay, is widely used due to its low cost, ease of use, and rapid detection of infection status (15). Due to the multifactorial nature of intra-mammary gland infection, the inflammation of epithelial cells results in the release of various products and enzymes, which lower the milk quality. These products and enzymes can be regarded as great and valuable biomarkers of udder health. Furthermore, different biochemical changes in milk are useful to monitor the health status of the udder (16, 17).

In Iraq, there is a paucity of research on the prevalence of SCM, and therefore alterations in milk quality due to infection (18-20). Therefore, this study aimed to detect the prevalence of SCM in the milk samples of lactating cows based on CMT and investigate the negative effects of infection on milk quality through the estimation of the immunological and biochemical characteristics of milk.

2. Materials and Methods

2.1. Samples and Preparation of Collected Milk

Totally, 200 apparently healthy adult lactating cows were randomly selected from different areas in Baghdad and Maysan provinces of Iraq from April to July 2021. Afterward, 100 ml of fresh milk was directly collected from the active-quarter (s) of each cow under aseptic conditions into plastic-labeled containers. In the laboratory, milk samples were tested initially by the CMT, first centrifuged at 7000 rpm for 5 min, and then clear supernatant under the cream layer was pipetted into glass tubes and re-centrifuged at 5,000 rpm for 3 min. The obtained sera were kept frozen into 1.5 ml Eppendorf tubes to be later used for immunological and biochemical testing. Clinical data regarding the age and breed of cows, the level of milk production, and abnormality in udder/milk of each cow during the last 6 months prior to the current study were recorded based on the owners' information.

2.2. CMT Test

According to the manufacturer's instructions on the CMT reagent kit (Apee Eskay Enterprises, India), the collected milk samples were immediately tested, and the interpreted results are presented in table 1.

Table 1. Mixture and score classification of infection determined by CMT method in collected milk samples

Color	Score	Description of mixture	Result
Grey	0	Remained liquid, no slim or gel	No infection
Grey / light purple	1	Became slimy or gel-like	Mild infection
Purple	2	Distinctly formed a gel	Moderate infection
Dark purple	3	Thickened immediately, formed gel	Severe infection

2.3. Immunology Assay

Bovine Sandwich IgG ELISA kit (Sunlong Biotech, China) was used in this study to measure the concentration of IgG antibodies in milk samples. Following the manufacturer`s instructions of the kit, the

samples, standards, and reagents were prepared and subjected to a specific procedure. The Microplate Reader System (BioTek, USA) was used to detect absorbance optical density (OD) of the ELISA kit at 450 nm. Eventually, the concentration of IgG antibodies was calculated based on the concentration and OD of the standards as well as the ODs of the sera samples. The findings of IgG concentration were classified according to the results of the CMT test to estimate their relationships with the SCM.

2.4. Biochemical Parameters and Enzymatic Evaluation

The concentrations of total protein (TP), sodium (Na), Chloride (Cl), potassium (K), calcium (Ca), free fatty acid (FFA), and lactose (LAC) along with aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were measured in milk samples using Abbott Architect Plus analyzer (Germany) and Ultrasonic Milk Analyser (REIL, India). The pH values were also estimated using the pH meter.

2.5. Statistical Analysis

The results of CMT, immunology and biochemical parameters in addition to clinical data were documented and analyzed using Microsoft Office Excel (Version 2016; Microsoft, USA), and GraphPad Prism software (Version 6.0.1; GraphPad Software Inc., USA). Statistical analysis of all data was performed through the Chi-square (x^2), one-way analysis of variance (ANOVA), and odds ratio tests. Values (mean± SE) were considered significant at $P<0.05$ and $P<0.01$.

3. Results

The result of the CMT test revealed that 83 (41.5%) lactating cows were positive for SCM (Table 2). In addition, different scores of infection were obtained in positive samples; however, there was a significant difference was observed ($P≤0.028$) in mild positive cases (61.45%), compared to moderate (32.53%) and severe (6.02%) infection cases (Figure 1).

Table 2. Total results of CMT test on milk samples of lactating cows

Total No.	Positive		Negative	
	No.	%	No.	%
200	83	41.5	117	58.5
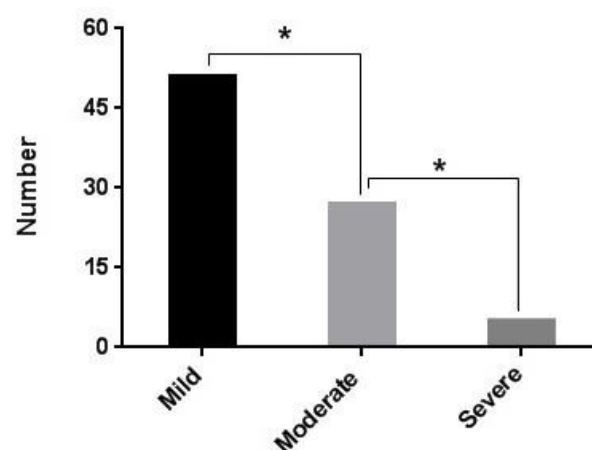
Figure 1. Score classification result of positive samples by CMT

Based on the results of clinical data, there was a significant difference ($P<0.05$) between evaluated factors in positive samples (table 3). Regarding the age factor, a significant increase was observed in the prevalence of SCM ($P≤0.026$) in the ≥5 years, compared to the <5 years age group (52.71% vs. 21.13%). Concerning the region variable, an insignificant difference ($P≤0.083$) was observed between the results of samples from Baghdad (42.5%) and Maysan (40.83%) provinces. However, it appeared that cows from Maysan province of Iraq were at higher risk (1.39) of developing SCM infection than cows from Baghdad province (0.72). Respecting the breeding factor, a statistically significant difference was reported ($P<0.05$) in the rate of infection ($P≤0.041$) and the prevalence of metastasis in crossbred cows (44.83%), compared to native cows (19.23%). In this study, lowered milk production (52.17%) was significantly more prevalent ($P≤0.032$) than the normal milk production (17.74%) in cows infected with SCM. In addition, a significant absence of udder/ milk

abnormalities (57.41%) was reported in cows that were almost SCM positive ($P \leq 0.037$).

Significantly higher values of IgG antibodies were detected in cows with positives SCM (4.92±0.21 µg/ml), compared to cows with negative SCM (3.74±0.19 µg/ml) (Figure 2). In addition, an insignificant difference was reported ($P \leq 0.064$ µg/ml) in the levels of IgG antibodies in mild (4.75±0.17 µg/ml), moderate (4.91 ± 0.24 µg/ml), and severe (5.02±0.21 µg/ml) degrees of SCM infection (Figure 3).

In the analysis of biochemical parameters of cows with positive SCM, significant increases ($P<0.05$) were reported in values of Cl (17.29±1.14), FFA (0.81±0.01), and Na (46.79±4.83), while a significant decrease ($P<0.05$) was observed in values of LAC (3.44±0.82) and TP (6.46 ±1.25). However, no significant differences ($P>0.05$) were detected in values

of Ca and K in cows with positive SCM (74.33±4.67 and 139.2±5.03, respectively), and negative SCM (78.64±3.23 and 141.15±3.48, respectively) (Table 4).

A significant elevation was observed in milk pH ($P<0.05$) in cows with positive (7.31±0.81), compared to negative SCM (6.73±0.64), (Figure 4). In addition, the milk pH in cows with positive SCM tended to increase with higher severity of SCM, as reported in mild (6.77±0.64), moderate (7.21±0.61), and severe (7.94±0.85) infections (Figure 5).

Insignificant elevation in ALT and reduction in LDH contents were observed and in cows with positive SCM (42.53±5.38 U/L and 33.47±4.11 U/L, respectively), compared to cows with negatives SCM (39.67±3.98 U/L and 36.41±3.78 U/L, respectively). Inversely, the level of AST was significantly higher in cows with positive (108.29±8.52 U/L) than negative SCM (61.39±5.66 U/L), (Figure 6).

Table 3. Analysis of risk factors associated with SCM in cows

| Factor	Group	Positive / tested	Prevalence	Odds ratio	Risk
Age (Year)	< 5	15 / 71	21.13	0.24	0.39
	≥ 5	68 / 129	52.71 *	4.11	2.52
Region	Baghdad	34 / 80	42.5	1.07	0.72
	Maysan	49 / 120	40.83	0.93	1.39
Breed	Crossbred	78 / 174	44.83	3.42	2.33
	Native	5 / 26	19.23	0.29	0.43
Milk production	Normal	11 / 62	17.74	0.39	0.35
	Lowered	72 / 138	52.17 *	2.53	2.89
Udder / Milk abnormality	Exist	21 / 92	22.83	0.22	0.4
	Absent	62 / 108	57.41 *	4.55	2.52

* ($P<0.05$)
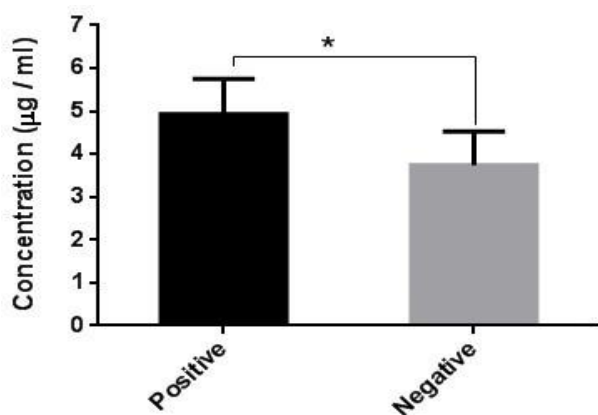
**Figure 2.** Concentration of IgG antibodies among cows with positive and negative SCM
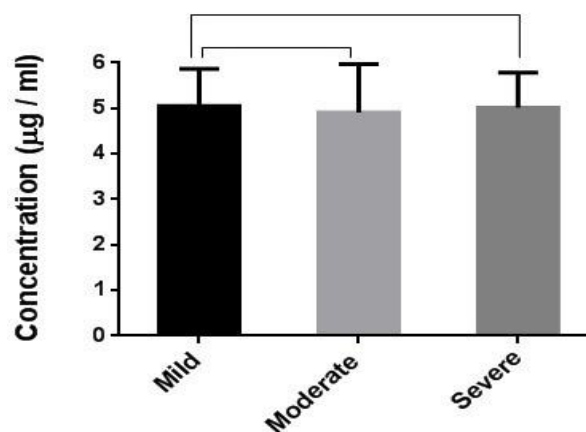
Figure 3. Concentration of IgG antibodies in SCM positive cows based on degrees of SCM severity

Table 4. Total results of biochemical parameters for cows with positive and negative SCM* ($P<0.05$)

Parameter	Unit	Positive	Negative	x^2	Statistics
Ca	mg/ml	74.33±4.67	78.64±3.23	1.923	NS
Cl	%	17.29±2.24 *	11.73±2.21	4.518	S
FFA	μeqv/ml	0.81±0.01 *	0.66±0.01	5.007	S
K	mg/ml	139.2±5.03	141.15±3.48	2.164	NS
LAC	%	3.44±0.82	4.45±0.79 *	3.97	S
Na	mg/ml	46.79±4.83 *	34.15±3.72	4.251	S
TP	mg/ml	6.46±1.25	7.87±1.09 *	5.189	S
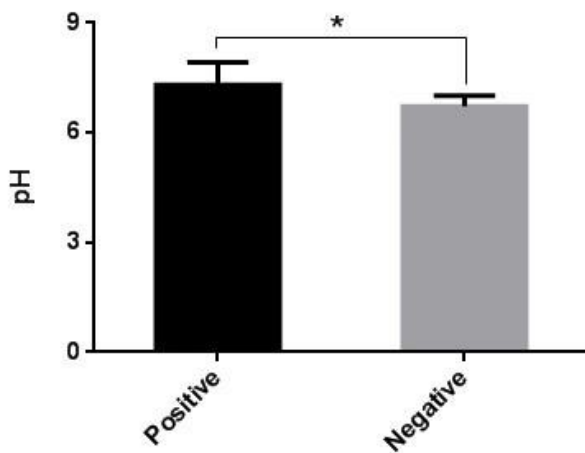
Figure 4. Total pH results for cows with positive and negative SCM
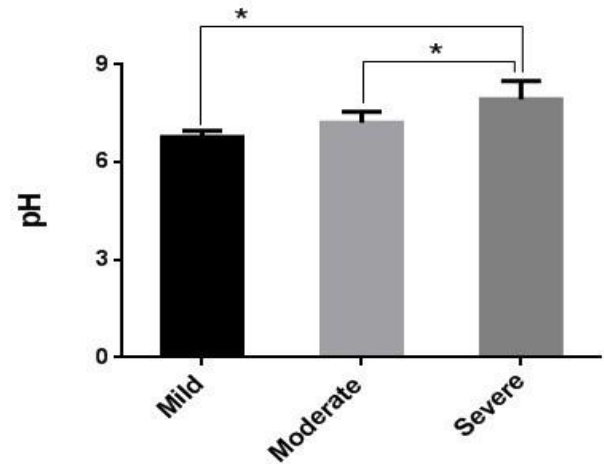
Figure 5. pH results in cows with positive SCM based on degrees of SCM severity
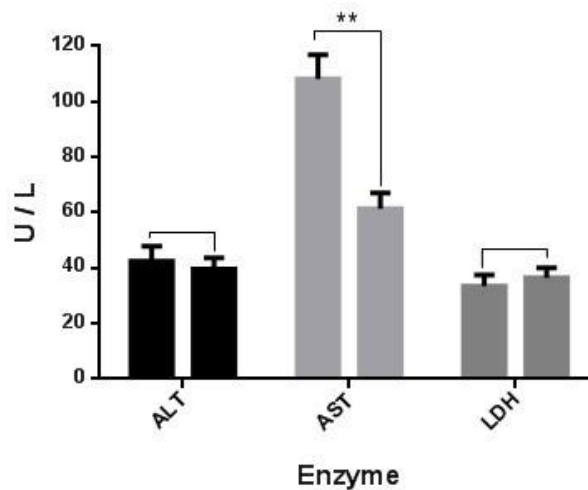
Figure 6. Total results of enzyme analysis in cows with positive and negative SCM cow

4. Discussion

Results of studies on the prevalence of SCM are useful for monitoring the health status of the udder in dairy animals, as well as the impacts of structural alterations in dairy industries (21). The obtained revealed that the prevalence of SCM was 41.5%, with a significant increase in a mild degree of SCM in positive samples (61.45%). This might be attributed to the fact that most farmers in the regions under study did not practice proper farming management and SCM screening at earlier stages. In addition, different pathogens elicit different immune responses in the mammary gland, and depending on the etiology, differences have been observed in somatic cell count (SSC) trends and milk quality (22). In comparison to other studies, the prevalence of SCM in Iraq was 38.89% (18) and 68% (19), while it was 51.6% in Tanzania (23), 21.6% in Iran (24), 85.3% in Nigeria (1), 45% in India (25), 88.6% in Vietnam (26), 86.2% in Uganda (27), 46.4% in Brazil (28), 15-47.8% in Finland (21), 55.2% in Colombia (29), 67.9% in Bangladesh (30), and 74.83% in Paraguay (31). These variations may be attributed to differences in the causative agent-related factors, animal risk factors, production system, environmental conditions, and management and husbandry-related factors.

According to the clinical data, instances of SCM increased with age in lactating cows, which was in line with the findings of the previous studies (32-34). Other studies reported that SCM was more prevalent in the age groups of 4-9 years (35), 7-10 years (36), and >9 years (37). The association of SCM with age may imply that older cows have been more exposed to causative factors, including increased milk production, decreased immunity, and resistance of bacteria to antibiotics that were used indiscriminately to treat mastitis during previous infections (34, 38).

In this study, clinical data of cows obtained from their owners revealed that there were significant decreases in milk production in the absence of udder/milk abnormalities. Very limited published data are available to quantify milk production losses associated with SCM worldwide. Based on de Graaf and Dwinger (39) and Mungube et al. (2005), the average losses of potential milk production from each cow infected with SCM have been 17.6% and 17.2%, respectively. Klaas (40) reported that higher SSCs were related to more severe tissue alterations due to long-lasting infections with pathogens. It is worth mentioning that SCM causes more than 3-4 times losses, compared to CM or milk production decrease with no clinical signs (41). The Food and Agriculture Organization of the United Nations states that SCM needs to be recognized early since its effective management depends on multiple recommendations, based on a better understanding of the disease (29).

The results of this study showed that the level of IgG increased more significantly in mastitis cows than the healthy ones; however, insignificant variation was observed among the mild, moderate, and severe cases of positive SCM. In cows, IgG is the main isotype in ruminants' milk, and IgG2 is considered to be the main opsonin supporting neutrophil phagocytosis in the milk of infected mammary glands due to the presence of specific Fc receptors in bovine neutrophils and macrophages which bind to IgG2 (42-44). It is suggested that the high level of IgG may explain its important role in udder resistance against infection. Kociņa, Antāne (45) mentioned that in the case of SCM, IgG immigration from blood circulation into the udder tissue is intensified to protect the udder from the manifestation of infection. Galfi, Radinović (46) showed a high concentration of IgG in the milk of SCM cows, and the highest values of IgG were found in milk samples obtained from quarters infected with minor mastitis pathogens.

The effect of udder health on milk quantity, quality, and milk derivatives has been widely studied (22, 47, 48). The findings of the present study revealed that the values of Cl, FFA, LAC, Na, and TP differed significantly between the mastitis and healthy milk samples. Fetherston, Lai (49) found that LAC concentration was conversely related to the concentrations of Na and Cl. This suggests that in cases

of SCM, a lower concentration of LAC affects the osmolality of milk which results in higher-than-normal concentrations of Na and Cl. In addition, preterm birth, teat trauma, self-perceived oversupply, low supply, and increase in acute phase response due to systemic reaction can elevate the Na/K ratio in milk. According to Jones and Bailey (50), mastitis plays a crucial role in increasing milk conductivity, which in turn results in elevated concentrations of Na and Cl. As observed in this study, Hunt, Williams (51) also confirmed high levels of FFAs in mastitis milk samples. Nonetheless, elevation in the levels of FFAs is interesting since trace amounts of FFAs have exhibited antibacterial activity as well. The effect of FFAs on mastitis pathogens may be very much dependent upon the activity of bacterial strain associated with infection (52-54).

The milk pH in the mastitis group was higher than the healthy group, which confirmed the results reported by previous studies (55-57). They found that the milk pH increased positively in association with the severity of the inflammatory process. In a study conducted by Kandeel, Megahed (58), it was initially hypothesized that milk pH testing could provide an accurate, low-cost, and practical on-farm method for diagnosing SCM; however, it was concluded that milk pH was not a clinically useful method for diagnosing SCM in dairy cattle. Other previous reports suggested that the level of milk pH was primarily influenced by the concentration of milk Na, citrate, and bicarbonate during udder inflammation (59-61).

Therefore, the results of this study revealed a high concentration of AST in the milk of sub-clinically mastitis cows, compared to the healthy ones, which is similar to those reported by other researchers (62-64). Pandey, Pratiksha (65) mentioned that the increase in the level of AST indicated the damage to the secretory epithelium of the udder.

The CMT is still the superior screening diagnostic aid for the detection of SCM in the field, since it is performed easily and rapidly at a low cost. Even if lactating animals appear to be without clinical abnormalities and give normal milk, their routine and regular testing can provide important data on the status of mammary glands and milk production. The high prevalence of SCM in dairy animals affects milk production and quality, and the coexistence of pathogenic bacteria is alarming since it threatens human health and has a public health significance. Therefore, herd health improvement interventions are required to protect human and society's health. Economic effects of SCM vary and must be calculated at the farm or herd level based on local, regional, epidemiological, managerial, and economic conditions.

Authors' Contribution

H. A. J. G. was responsible for the collection and testing of milk samples by C. M. T. and ELISA, and H. D. S. and M. A. R. were responsible for biochemical and enzymatic testing of milk samples.

Ethics

The current study was licensed and approved by the Scientific Committees of Al-Manara College of Medical Sciences, in Maysan, Iraq, and the Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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