Original Article

Inflamatory Markers in Cows Affected by Subclinical Mastitis Due To *Escherichia Coli* and *Staphylococcus* Infections

Ghasemian, SO[1*], Asri-Rezaei, S[2]

1. *Department of Veterinary, Behbahan Branch, Islamic Azad University, Behbahan, Iran.*
2. *Internal Medicine and Clinical Pathology Department, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.*

How to cite this article: **Ghasemian SO, Asri-Rezaei S. Inflamatory Markers in Cows Affected by Subclinical Mastitis Due To *Escherichia Coli* and *Staphylococcus* Infections. Archives of Razi Institute. 2024;79(6):1319-1328.** DOI: 10.32592/ARI.2024.79.6.1319

ABSTRACT

The investigation of serum amyloid A (SAA), IL-6, and IL-8 concentrations in serum during episodes of clinical and subclinical mastitis is of significant value. The objective of this study was to assess the diagnostic value of Serum Amyloid A (SAA), IL-6, and IL-8 in the early detection of subclinical mastitis in cows infected with Escherichia coli (E. coli) and staphylococcus infections. This cross-sectional analytical study, conducted in 2023 at the Veterinary Laboratory in Urmia, Iran, evaluated inflammatory markers in 79 dairy cows with clinical and subclinical mastitis. The cows were divided into three groups: healthy cows, cows with subclinical mastitis, and cows with clinical mastitis. Each of these groups was then evaluated for Serum Amyloid A (SAA), IL-6, and IL-8. The diagnostic value of the inflammatory markers was determined by calculating the areas under the curves (AUCs) of the receiver operating characteristic (ROC) curves. In general, among patients with a positive culture test result (57%), 19% were found to be infected with E. coli, 22.8% with Streptococcus uberis, and 15.2% (12 cases) with Staphylococcus aureus. A strong correlation was observed between the mean SCC and the values of IL-6 ($P<0.005$), IL-8 ($P<0.005$), and SAA ($P<0.005$). Furthermore, a strong correlation was observed between SAA and IL-8 ($P<0.005$). The value of IL-6 exhibited a moderate correlation with both IL-8 ($P<0.005$) and SAA ($P<0.005$). The sensitivity and specificity of SCC (0.98), SAA (0.90), IL-6 (0.95), and IL-8 (0.87) were high for the diagnosis of mastitis in cows. The present study demonstrated that mastitis in dairy cows is associated with an increase in inflammatory cytokines, including amyloid A, IL-6, and IL-8. The findings of this study indicate that fluctuations in these biomarkers may serve as a potential indicator for disease diagnosis.

Keywords: *Escherichia Coli*, Inflamatory Markers, Mastitis, *Staphylococcus Aureus.*

Article Info:
Received: 13 November 2023
Accepted: 24 April 2024
Published: 31 December 2024

Corresponding Author's E-Mail:
Ghasemian1249@yahoo.com

1. Introduction

Mastitis in cows is a widely recognized disease with significant economic implications for dairy farms globally (1). The financial impact of the disease is typically manifested in reduced milk yield, elevated veterinary care expenses, and the premature removal of affected animals from the herd. The annual incidence rate of mastitis has been documented to be 37% (2). The primary cause of mastitis in cattle is bacterial infection, although other infectious agents, including mycoplasma and fungi, can also be involved. It is widely accepted that Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) are the primary causes of bovine mastitis. Once an infection with S. aureus has been established, eradication becomes exceedingly difficult. Furthermore, the infected cattle may act as a vector for transmission. Bovine mastitis, which can be transmitted to consumers either through milk or by workers, represents a significant one-health issue and poses a zoonotic risk to humans (3). Although the use of antimicrobial agents for the eradication of S. aureus and E. coli on cattle farms can frequently be advantageous, the emergence of antimicrobial resistance may be an unintended consequence of the excessive utilization of these agents in the management or prevention of mastitis (2). Furthermore, the presence of antimicrobial residues has been demonstrated to result in a reduction in milk quality (4). Bovine mastitis can be classified into two distinct categories: clinical and subclinical. Clinical mastitis is characterized by the presence of visible signs of udder inflammation and concomitant disturbances in the animal's general health status. In contrast, subclinical mastitis, which is more prevalent than clinical mastitis, is more challenging to detect promptly due to the absence of overt symptoms (5). In a healthy udder, macrophages are the most common cells. However, in the initial phases of mastitis, neutrophils become the predominant cell type. The presence of somatic cells, including macrophages, neutrophils, lymphocytes, and a few mammary epithelial cells, in milk can serve as an indicator for diagnosing subclinical mastitis (6). The interactions between the host and the pathogen elicit the innate immune response, which either identifies the infected microorganisms or modulates the components of the immune system. This results in an increase in the number of macrophages and the release of cytokines. These cytokines attract leukocytes to the site of infection and initiate both local and systemic acute-phase responses (7). It has been observed that during the course of an infection, there is an increase in the presence of acute phase proteins, including serum amyloid A (SAA). This protein plays a pivotal role in the immune response, with its expression markedly elevated during inflammatory conditions. Furthermore, there is an increased expression of cytokines, including interleukin-1 beta (IL-1β), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α), which are essential mediators of the body's immune response to infection (8). To effectively identify the early signs of udder bacterial infections in bovines, it is essential to have a comprehensive understanding of both the pathogenesis and the bovine immune response. This understanding allows for the identification of reliable biomarkers that can be detected at the earliest stages of infection. The examination of serum amyloid A (SAA), IL-6, and IL-8 concentrations in serum during episodes of clinical and subclinical mastitis is of considerable significance. These biomarkers serve as vital indicators of inflammatory and immune responses. Fluctuations in their concentrations can provide invaluable insights into the pathogenesis and progression of mastitis. In this study, we employed precise methodologies to quantify inflammatory markers, thereby identifying alterations associated with bacterial invasion. The objective of this study is to assess whether the concentrations of SAA, IL-6, and IL-8 in the serum increase during instances of clinical and subclinical mastitis in cows infected with E. coli and S. aureus.

2. Materials and Methods

2.1. Study Design and Research Area

The present study employed a cross-sectional analytical approach to investigate the inflammatory markers in dairy cows with clinical and subclinical mastitis. The study was conducted in Isfahan, Iran, in 2023.

2.2. Sample size calculation method

In this study, Cochran's B formula was employed for the calculation of the requisite sample size. A sample size of 79 cows was determined, considering a 5% error level and a z-value of 1.96 and a Z2 value of 3.8416. The calculation is as follows:

$$n = \frac{\frac{z^2 pq}{d^2}}{1 + \frac{1}{N}\left[\frac{z^2 pq}{d^2} - 1\right]}$$

2.3. Animal Population and Study Design

Samples were obtained from an industrial farm animal production facility situated in Isfahan, Iran. The study population consisted of 79 dairy cows, including 30 healthy cows, 30 cows with subclinical mastitis, and 19 cows with clinical mastitis. The cows were all of the Holstein breed, aged over three years, and at least three months had elapsed since the commencement of lactation. Cows with mastitis that had received either systemic or intramammary antibiotics at least two months prior were excluded from the study. The farm implemented cutting-edge management strategies and maintained rigorous hygiene protocols, exclusively utilizing automatic milking machines. The results of the biochemical parameter measurements were also documented in the pertinent sections of the questionnaire forms. A comprehensive clinical examination was conducted on all the cows under investigation. Cows exhibiting one or more of the following signs were identified as having clinical mastitis: systemic reaction symptoms, unusual physical characteristics of milk, as well as the presence of inflammation indicators in one or more quarters of the udder. The seventy-nine dairy cows were

separated into three distinct groups. The initial group, designated as the control group, comprised 30 cows exhibiting no indications of illness. These included the absence of fever, hyperemia, anemia of the mucous membranes, and depression. The udders of these cows were also observed to be healthy, exhibiting no signs of pain, swelling, abscesses, warmth, or redness. Furthermore, the California Mastitis Test (CMT) was conducted on the milk samples from all four quarters of the cows in the control group, and the results were negative. The second group, designated as the subclinical mastitis group, comprised 30 cows that appeared to be in good health and did not exhibit any overt signs of illness. Additionally, the udders of these cows were observed to be free of any observable abnormalities. Cows that tested positive for CMT but did not exhibit clinical symptoms were considered to be afflicted with subclinical mastitis. The third group, designated as the clinical mastitis group, comprised 19 cows exhibiting at least one symptom of mastitis, including pain, swelling, abscess, warmth, and redness, along with the presence of deposits or clots in their milk. Furthermore, the cows in this group tested positive for CMT.

2.4. Blood Sampling

Blood samples were collected from each cow in accordance with aseptic techniques using a Venoject apparatus. All samples were duly labeled in accordance with the relevant specifications and were promptly transferred to the Arin Dam Laboratory in Isfahan for the requisite testing. One blood sample was collected with an anticoagulant for the purpose of performing a complete blood count. The blood sample was collected in a heparinized tube and rapidly centrifuged at 1500× g for five minutes to separate the blood plasma. Subsequently, the serum and plasma were dispatched to the Clinical Pathology Laboratory of the Faculty of Veterinary Medicine at Urmia University for the requisite determinations of blood biochemical parameters.

2.5. California Mastitis Test (CMT)

The CMT was conducted in the following manner: A four-well plastic vessel was utilized to collect approximately 2 mL of milk from each quarter of the udder, which was subsequently combined with an equal volume of alkali reagent. A somatic cell count (SCC) test was performed on each milk sample using the Nucleo Counter® SCC-100™.

2.6. Milk Sample Collection

To obtain milk samples, visits were conducted at the dairy farms during the designated milking period. The samples were collected in the milking parlor, prior to the commencement of milking. Prior to the sampling process, the udders of the cows were meticulously sterilized using alcohol and subsequently dried. All specimens were collected in vials that had undergone a thorough sterilization process. Following the disinfection of the teats, the initial milk produced was discarded. Subsequently, 10 milliliters of milk from each quarter was collected and transferred into sterile tubes. Subsequently, the samples were dispatched to the laboratory in Isfahan, where they were maintained within the cold chain.

2.7. Bacterial Identification

The milk samples were subjected to a 10-minute centrifugation process. Subsequently, a droplet from the resulting sediment was cultivated on agar of varying types. The culturing process was conducted at a temperature of 37°C, with the incubation period lasting between 24 and 48 hours. The bacterial colonies that formed were identified based on their phenotypic traits, as observed on the culture media. The bacteria were tentatively identified based on their Gram staining properties and biochemical characteristics.

2.8. Molecular Confirmation of S. *Aureus*, *S. Uberis*, and *E. Coli* Isolates

DNA Extraction: The boiling method was employed for the extraction of DNA from all potential isolates. A number of groups from the isolates suspected to be Staphylococcus aureus, Staphylococcus uberis, and Escherichia coli were combined with 200 µL of deionized water and subsequently heated for 10 minutes. Subsequently, a 10-minute centrifugation was conducted. The supernatants were stored at -20 °C as DNA specimens.

2.9. Polymerase Chain Reaction (PCR)

All isolates that were biochemically typed and suspected to be Staphylococcus were verified by amplifying the species-specific nuc gene. This was achieved through the use of primers nuc-F and nuc-R, employing the polymerase chain reaction (PCR) amplification method. A 25 µL PCR reaction mix was prepared, containing 5 µL of DNA template, 12.5 µL of 2X PCR master mix, 6.5 µL of deionized nuclease-free water, and 1 µL of each primer. The polymerase chain reaction (PCR) conditions were carried out in five stages, commencing with an initial denaturation at 94 °C for two minutes and concluding with a final extension step at 68 °C for seven minutes. The series of reactions was conducted using a 96-well Applied Biosystem 2720 thermal cycler. Furthermore, all E. coli isolates, identified through biochemical methods, underwent PCR amplification to encode the genus-specific 16S-rRNA gene primers
(16S-rRNA-F-GCGGACGGGTGAGTAATGT and
16S-rRNA-R-TCATCCTCTCAGACCAGCTA).
The reaction was set up in a total volume of 25 µL, comprising 5 µL of DNA template, 12.5 µL of 2X PCR master mix, 6.5 µL of deionized nuclease-free water, and 1 µL of each primer. The PCR amplification process commenced with an initial denaturation phase at 94 °C for five minutes and concluded with a final extension phase at 72 °C for seven minutes. The PCR products for three isolates, namely S. aureus, S. uberis, and E. coli, were visualized on a 1% agarose gel using a UV transilluminator and a gel documentation system.

2.10. Inflammatory Cytokines Analysis

The concentrations of serum amyloid A and interleukins (IL-8 and IL-6) were quantified using commercially available ELISA kits designed for bovine samples (Cat. No. The kits used were MBS2024318, MBS739067, and MBS733925, which were sourced from MyBiosource in

the USA. These kits are sandwich enzyme immunoassays for the quantitative measurement of SAA, IL-8, and IL-6 in bovine serum. In summary, the principle of the assays is as follows: the microplate provided in this kit has been pre-coated with an antibody specific to cytokines. Subsequently, the standards or samples are introduced to the designated microplate wells, where they are bound by a biotin-conjugated antibody that is specific to cytokines. Subsequently, avidin-horseradish peroxidase (HRP) is added to each microplate well and incubated. Following the addition of TMB substrate solution, only those wells containing cytokines, biotin-conjugated antibody, and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution, and the resulting color change is then measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. Subsequently, the concentration of cytokines in the samples is determined by comparing the optical density (O.D.) of the samples to the standard curve. All measurements were conducted using a microplate reader (Labomed EMR-500, USA).

2.11. Statistical Analysis

The data were analyzed using the statistical software package SPSS. The centrality and dispersion indices were employed for the purpose of describing the results. The Chi-square test was employed for the purpose of comparing qualitative data, while the T-test facilitated comparisons of quantitative data before and after. Group comparisons were conducted using analysis of variance (ANOVA), and tests such as the area under the receiver operating characteristic curve were employed to ascertain sensitivity and specificity. The diagnostic value of the inflammatory markers was determined by calculating the areas under the receiver operating characteristic curves (AUCs).

2.12. Ethical Considerations

This study was conducted in accordance with the ethical guidelines set forth by the Helsinki Declaration. It is important to note that throughout the sampling process, the utmost care was taken to ensure the well-being and safety of the animals involved. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine at Behbahan University (IR.IAU.BEHBAHAN.REC.1401.817).

3. Results

In general, the CMT was negative in 38% (30 cases) and positive in 62% (49 cases) of cows. Among the patients with a positive culture test (57%: 45 cases), 19% (15 cases) were found to have E. coli, 22.8% (18 cases) had S. uberis, and 15.2% (12 cases) were infected with S. aureus. In a substantial majority of the mastitic milk samples, specifically 92% (45/49), bacterial agents were identified. The present study compares SCC, SAA, IL-6, and IL-8 in cows that tested either negative or positive in the CMT (Table 1). The results demonstrated a statistically significant difference in SCC, SAA, IL-6, and IL-8 between cows that tested negative and those that tested positive in the CMT ($P<0.005$). A comparative analysis of the mean levels of SCC, SAA, IL-6, and IL-8 in three distinct groups: healthy controls, subclinical mastitis, and clinical mastitis, is presented in Table 2. The results demonstrate a statistically significant difference in the levels of SCC, SAA, IL-6, and IL-8 among the three groups ($P<0.005$). The Post Hoc Tests reveal that all groups significantly differ from each other in terms of SCC, SAA, IL-6, and IL-8 ($P<0.005$) (Table 3). A comparison of the mean SCC, SAA, IL-6, and IL-8 in cows infected with different types of bacteria revealed a significant difference in these variables among cows infected with different types of bacteria ($P<0.005$) (Table 4). A pairwise comparison of cows infected with different types of bacteria in terms of somatic cell count (SCC), serum amyloid A (SAA), interleukin-6 (IL-6), and interleukin-8 (IL-8) revealed significant differences in SCC, SAA, IL-6, and IL-8 levels between cows with a negative culture test and those infected with Escherichia coli, Staphylococcus aureus, and Streptococcus uberis ($P<0.005$). Moreover, cows infected with S. aureus exhibited notable disparities in SCC, SAA, IL-6, and IL-8 levels when compared to those infected with E. coli and S. uberis ($P<0.005$). Nevertheless, no statistically significant difference was identified between E. coli and S. uberis in terms of SCC ($P=0.68$), SAA ($P<0.01$), IL-6 ($P=0.99$), and IL-8 ($P=0.09$) (Table 5). Further analysis revealed a strong correlation between the mean SCC and the values of IL-6 ($r=0.709$, $P < 0.005$), IL-8 ($r = 0.94$, $P<0.005$), and SAA ($r=0.84$, $P<0.005$). Furthermore, there was a strong correlation between SAA and IL-8 ($r=0.91$, $P<0.005$) (Table 5). The value of IL-6 was found to be moderately correlated with both IL-8 ($r=0.73$, $P<0.005$) and SAA ($r = 0.69$, $P<0.005$) (Table 5). The present study sought to evaluate the diagnostic accuracy of SCC, SAA, IL-6, and IL-8 in diagnosing mastitis in cows. The analysis revealed that SCC exhibited a sensitivity of 0.98 and a specificity of 0.98, while SAA demonstrated a sensitivity of 0.90 and a specificity of 0.90. Similarly, IL-6 and IL-8 exhibited sensitivity values of 0.95 and 0.87, respectively (Figure 1).

4. Discussion

The objective of this study was to assess the diagnostic utility of inflammatory markers in subclinical mastitic cows. In conclusion, the results demonstrated that E. coli and S. aureus were the predominant pathogens responsible for the development of mastitis. Serum IL-6 and IL-8 concentrations, along with SAA values, were found to be significantly correlated with subclinical mastitis caused by E. coli and Staphylococcus. The specificity and sensitivity of IL6, IL8, and SAA were high for the diagnosis of mastitis caused by E. coli and Staphylococcus. Mastitis is a complex disease with a multitude of potential causes, including contagious bacteria, environmental factors, and opportunistic organisms (9).

Table 1. Comparison of Cows with Positive and Negative California Mastitis Test Results in Terms of Somatic Cell Count, Serum Amyloid A, Interleukin-6, and Interleukin-8

Variables	CMT	Mean	SD	t-test	P-value
Somatic cell count (x1000/mL)	Negative	42.79	18.164	-7.244	<0.005
	Positive	3041.57	2261.63		
Interleukin 6 (pg/mL)	Negative	24.67	10.49	-5.411	<0.005
	Positive	2407.33	2405.93		
Interleukin 8 (pg/mL)	Negative	19.95	8.37	-10.211	<0.005
	Positive	420.408	214.15		
Serum amyloid A (mg/L)	Negative	12.98	5.73	-14.112	<0.005
	Positive	167.706	59.73		

Table 2. Comparison of the Mean Values of Somatic Cell Count, Serum Amyloid A, Interleukin-6, and Interleukin-8 across Three Groups.

Variables	Groups	Mean	SD	95% Confidence Interval for Mean		F*	P-value
				Lower Bound	Upper Bound		
Somatic cell count (x1000/mL)	Healthy Cattle	42.79	18.16	36.008	49.57	130.69	<0.005
	Subclinical Mastitis	1637.48	1158.69	1204.82	2070.14		
	Clinical Mastitis	5258.56	1732.94	4423.308	6093.81		
Serum amyloid A (mg/L)	Healthy Cattle	12.98	5.736	10.84	15.13	19.204	<0.005
	Subclinical Mastitis	140.62	41.11	125.26	155.97		
	Clinical Mastitis	210.4	60.31	181.39	239.54		
Interleukin 6 (pg/mL)	Healthy Cattle	24.67	10.49	20.75	28.59	135.25	<0.005
	Subclinical Mastitis	1860.87	2554.30	907.08	2814.67		
	Clinical Mastitis	3270.14	1908.11	2350.46	4189.82		
Interleukin 8 (pg/mL)	Healthy Cattle	19.95	8.37	16.82	23.08	165.27	<0.005
	Subclinical Mastitis	303.04	98.209	266.3	339.71		
	Clinical Mastitis	605.72	218.07	500.61	710.83		

* One-way ANOVA

Table 3. Pairwise Comparison of Groups in Terms of Somatic Cell Count, Serum Amyloid A, Interleukin-6, and Interleukin-8.

Dependent Variable	Groups		Mean Difference	Std. Error	P-value	95% Confidence Interval (Lower Bound)
Somatic cell count (x1000/mL)	Healthy Cattle	Subclinical Mastitis	-1594.69	285.62	<0.005	-2277.46
		Clinical Mastitis	-5215.77	324.33	<0.005	-5991.09
	Subclinical Mastitis	Clinical Mastitis	-3621.08	324.33	<0.005	-4396.39
Interleukin 6 (pg/mL)	Healthy Cattle	Subclinical Mastitis	-1836.208	472.71	0.001	-2966.23
		Clinical Mastitis	-3245.47	536.79	<0.005	-4528.67
	Subclinical Mastitis	Clinical Mastitis	-1409.26	536.79	0.02	-2692.46
Interleukin 8 (pg/mL)	Healthy Cattle	Subclinical Mastitis	-283.08	31.59	<0.005	-358.607
		Clinical Mastitis	-585.76	35.87	<0.005	-671.52
	Subclinical Mastitis	Clinical Mastitis	-302.68	35.87	<0.005	-388.43
Serum amyloid A (mg/L)	Healthy Cattle	Subclinical Mastitis	-127.63	10.06	<0.005	-151.68
		Clinical Mastitis	-197.48	11.42	<0.005	-224.8
	Subclinical Mastitis	Clinical Mastitis	-69.85	11.42	<0.005	-97.16

Table 4. Comparison of the Mean Values of Somatic Cell Count, Serum Amyloid A, Interleukin-6, and Interleukin-8 in Cows Infected with Different Types of Bacteria

Variables	Groups	Mean	SD	95% Confidence Interval for Mean		F	P-value
				Lower Bound	Upper Bound		
Somatic cell count (x1000/mL)	Negative	67.303	60.83	47.01	87.58	70.95	<0.005
	E. coli	2508.68	1534.66	1658.82	3358.55		
	S. uberis	2987.86	1535.21	2169.80	3805.92		
	S.aureus	5672.22	1977.62	4343.64	7000.81		
Serum amyloid A (mg/L)	Negative	25.309	29.92	15.33	35.28	55.16	<0.005
	E. coli	162.37	45.045	137.42	187.31		
	S. uberis	163.59	14.22	156.01	171.17		
	S.aureus	237.97	46.26	206.88	269.05		
Interleukin 6 (pg/mL)	Negative	99.42	254.19	14.67	184.17	124.28	<0.005
	E. coli	1633.32	1088.61	1030.47	2236.18		
	S. uberis	1769.86	443.61	1533.47	2006.24		
	S.aureus	5654.81	3126.01	3554.72	7754.904		
Interleukin 8 (pg/mL)	Negative	49.52	64.06	28.16	70.88	156.47	<0.005
	E. coli	325.72	69.63	287.16	364.28		
	S. uberis	417.001	126.35	349.67	484.33		
	S.aureus	709.84	203.37	573.21	846.47		

Table 5. Pairwise Comparison of Cows Infected with Different Types of Bacteria in Terms of Somatic Cell Count, Serum Amyloid A, Interleukin-6, and Interleukin-8.

Dependent Variable	(I) Culture	(J) Culture	Mean Difference (I-J)	SE	P-value	95% Confidence Interval	
						Lower Bound	Upper Bound
Somatic cell count (x1000/mL)	Negative	*E. coli*	-2441.38	366.57	<0.005	-3404.6	-1478.16
		S. uberis	-2920.56	358.33	<0.005	-3862.11	-1979.006
		S.aureus	-5604.92	411.27	<0.005	-6685.59	-4524.25
	E.coli	*S. uberis*	-479.17	430.41	0.68	-1610.12	651.77
		S.aureus	-3163.53	475.39	<0.005	-4412.68	-1914.39
	S.uberis	*S.aureus*	-2684.36	469.07	<0.005	-3916.88	-1451.83
Interleukin 6 (pg/mL)	Negative	*E.coli*	-1533.9	386.51	0.001	-2549.51	-518.29
		S. uberis	-1670.44	377.82	<0.005	-2663.21	-677.67
		S.aureus	-5555.39	433.64	<0.005	-6694.84	-4415.94
	E.coli	*S.uberis*	-136.53	453.82	0.99	-1329.0	1055.93
		S.aureus	-4021.48	501.25	<0.005	-5338.57	-2704.39
	S. uberis	*S.aureus*	-3884.95	494.58	<0.005	-5184.51	-2585.39
Interleukin 8 (pg/mL)	Negative	*E. coli*	-276.202	32.94	<0.005	-362.761	-189.64
		S.uberis	-367.47	32.201	<0.005	-452.08	-282.86
		S.aureus	-660.32	36.95	<0.005	-757.43	-563.207
	E.coli	*S. uberis*	-91.27	38.67	0.09	-192.907	10.35
		S.aureus	-384.119	42.72	<0.005	-496.37	-271.86
	S.uberis	*S.aureus*	-292.84	42.15	<0.005	-403.605	-182.08
Serum amyloid A (mg/L)	Negative	*E. coli*	-137.06	10.309	<0.005	-164.15	-109.97
		S. uberis	-138.28	10.07	<0.005	-164.77	-111.808
		S.aureus	-212.66	11.56	<0.005	-243.05	-182.26
	E. coli	*S. uberis*	-1.22	12.105	>0.99	-33.03	30.57
		S.aureus	-75.6	13.37	<0.005	-110.73	-40.46
	S.uberis	*S.aureus*	-74.37	13.19	<0.005	-109.03	-39.707

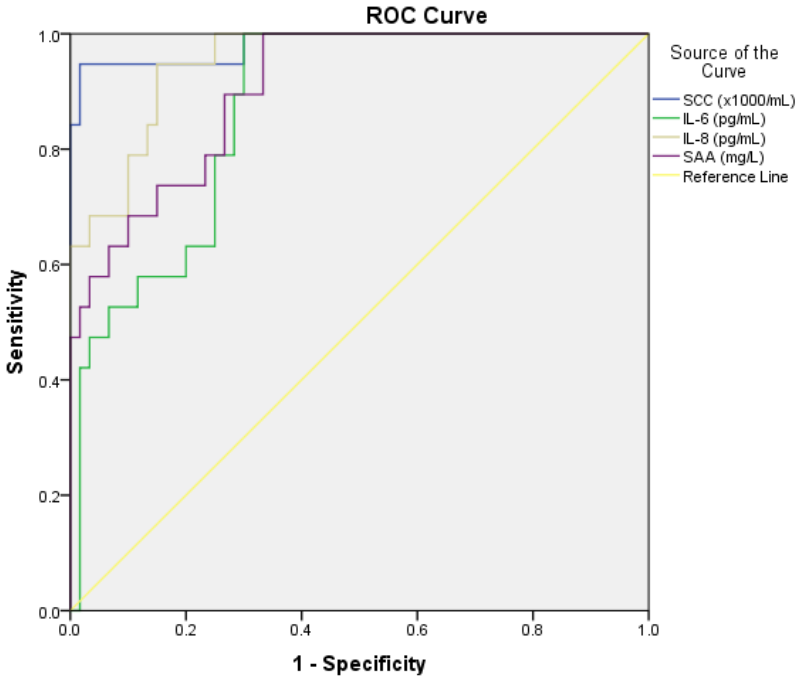

Figure 1. Sensitivity and Specificity of SCC, SAA, IL-6, and IL-8 in the Diagnosis of Mastitis in Cows.

In the present study, bacterial agents were identified in a substantial majority (92%) of all mastitic milk samples. In a separate study by Sadat et al., this figure was estimated to be 73.75%. This observation is consistent with previous studies that have identified bacterial mastitis as the most common cause of mastitis (6). Furthermore, our findings corroborate those of earlier studies which identified E. coli and Staphylococcus as the primary pathogens causing mastitis (6). In the present study, a slightly lower incidence (approximately 33.3%) of clinical mastitis cases was observed in which E. coli was the pathogen. Conversely, Staphylococcus aureus and Streptococcus uberis were responsible for clinical and subclinical mastitis in 66.7% of cases. This finding is consistent with that of a previous study, which reported a slightly lower incidence (approximately 31%) of clinical mastitis cases where E. coli was the pathogen (10). Nevertheless, a study conducted by Sadat et al. revealed that S. aureus was isolated in 76% of subclinical cases, a finding that is consistent with other studies conducted in Egypt (6, 11). The findings of the current study did not align with those of previous studies, but the rate was estimated to be significantly lower in our study. This discrepancy may be attributed to variations in bacterial resistance across different geographical locations.

Laboratory diagnostics are of paramount importance in the detection of cows with subclinical mastitis. The management of mastitis is primarily dependent on the somatic cell count (SCC) and the cumulative milk test (CMT), both of which quantify the cell count in milk samples. However, each of these diagnostic methods has its inherent limitations, underscoring the necessity for the development of novel biomarkers for subclinical mastitis. Sensitive markers, such as cytokines and acute-phase proteins, have the potential to serve as early diagnostic tools for mastitis and also aid in preventative measures. Previous examinations have demonstrated that quantifying acute-phase proteins (APPs), such as amyloid A, in blood plasma or serum can facilitate the diagnosis of disease, prognosis, and the detection of inflammation (12). The results of this study indicated that nIL6, IL8, and SAA exhibited high specificity and sensitivity for the diagnosis of mastitis caused by E. coli and Staphylococcus. Similarly, Sadat et al. (6) observed that cows suffering from illnesses exhibited significantly elevated levels of amyloid A in both subclinical and clinical mastitic animals compared to their healthy counterparts. A number of studies have demonstrated that the concentration of amyloid A is elevated in the milk of cows with mastitis in comparison to healthy cows (13, 14). In this context, the study by Bochniarz et al. revealed that the amyloid A value was markedly elevated in the milk of cows with health issues compared to those without the disease. Nevertheless, no distinction was discerned in the amyloid A concentrations in the serum between the two groups (15). Further research is required to elucidate these discrepancies. However, the lack of significant correlations between serum amyloid A concentration and milk amyloid A during subclinical mastitis indicates that amyloid A may be produced locally in the udder as a response to infection. The concentration of amyloid A in milk typically increases in conjunction with an elevation in the somatic cell count (SCC) of milk (15). It has been demonstrated that in certain instances, a considerable quantity of milk amyloid A was present in the milk of cows that exhibited no indications of mastitis and

had a somatic cell count of less than 200,000 cells per milliliter of milk. It is possible that these quarters have previously undergone an inflammatory process, as indicated by the elevated levels of amyloid A in the milk prior to the appearance of inflammatory symptoms and an increase in serum amyloid A in the milk (16). Therefore, SAA may serve as a more sensitive and early indicator of inflammation in the mammary gland. The ability to identify a subclinical form of inflammation is of paramount importance, as cows exhibiting no overt symptoms frequently remain untreated (17). Further analysis of the data yielded elevated levels of IL-6 and IL-8 in cows with mastitis and subclinical mastitis in comparison to healthy controls. These findings are consistent with other evidence presented in the literature (15, 18). Similarly, Sadat et al. (6) demonstrated that cows with mastitis and subclinical mastitis exhibited higher levels of TNF-α, IL-1β, and IL-6 compared to those without the disease. A study revealed that on the initial day of the disease, the mean concentration of IL-6 was 25 times greater in milk samples and 5 times greater in serum samples from cows afflicted with acute mastitis, in comparison to those from healthy cows (18). Other findings have corroborated the presence of elevated levels of IL-6 in the initial stage of infection. This finding is consistent with previous research indicating that pro-inflammatory cytokines are instrumental in combating the initial infection. The elevated levels of TNF-α and IL-1β observed in subclinical mastitis indicate that these factors may play a role in the initial stages of mastitis development (19). This elevation in pro-inflammatory concentrations appears to occur concurrently with the onset of disease symptoms (9). The concentration of IL-6 in the milk and serum of cows is influenced not only by the stage of infection but also by the specific microorganisms responsible for mastitis. The findings of Hagiwara and colleagues indicate that cows with mastitis induced by Escherichia coli and Staphylococcus aureus exhibited markedly elevated levels of IL-6 in both milk and serum. Nevertheless, the levels in question were found to be markedly lower in instances of mastitis caused by coagulase-negative staphylococci (18). Moreover, the researchers demonstrated that, in contrast to S. aureus, E. coli was capable of triggering the activation of NF-κB in bovine mammary epithelial cells. It is currently understood that the NF-κB subunit p65 undergoes a process of detachment from its inhibitory protein IκB, subsequently migrating from the cytoplasm to the nucleus. In the nucleus, it initiates the transcription of specific target genes, including TNF-, IL-1B, and IL-6. The generation of pro-inflammatory cytokines and chemokines is of paramount importance for the host's defense mechanisms and survival. The generation of these cytokines may result in the manifestation of systemic clinical symptoms. IL-6 plays a pivotal role in the acute phase of inflammation, while IL-8 is a crucial chemokine that attracts neutrophils to the site of infection (20). A higher level of cytokine expression has been observed in bovine mammary epithelial cells in response to E. coli compared to S. aureus. This variation in cytokine production may be a contributing factor to the differing outcomes observed with E. coli and S. aureus infections. E. coli is often associated with clinical mastitis, while S. aureus is more frequently linked to subclinical mastitis that presents as a chronic and persistent condition (20). The research conducted by Safak and his team (2022) revealed that the levels of TNF-α and interferon-gamma were notably elevated in the group associated with *E. coli* (21). Similarly, a study by Yunhea et al. observed that the expression levels of TNF, IL-6, and IL-8 were significantly higher and increased more quickly in bovine mammary epithelial cells when exposed to heat-inactivated *E. coli*, compared to when these cells were exposed to heat-inactivated *S. aureus* (20). Karthikeyan et al. demonstrated that the expression levels of toll-like receptor 2 (TLR-2) and interleukin-8 (IL-8) in the milk somatic cells of cows affected by subclinical mastitis were significantly elevated in comparison to those observed in healthy cows (22). Upon binding with its ligand, the IL-8 receptor initiates an intracellular signaling cascade. This activation enables neutrophils to sustain prolonged activation, survival, and migration. An increase in the expression of the IL-8 receptor in milk somatic cells was observed in cases of experimental infection with Staphylococcus chromogenes (23). The study by Karthikeyan observed a significant increase in the expression of the IL-8 receptor in milk somatic cells of cows affected by subclinical mastitis, with up to 12.94-fold changes, compared to healthy cows (22). The cytokines released by immune cells in response to infection may result in elevated expression of the IL-8 receptor. This plays a pivotal role in guiding neutrophils to the site of inflammation, which results in an increase in the somatic cell count during subclinical mastitis. Moreover, the IL-8 receptor has been associated with the survival and migration of alveolar epithelial cells, indicating a potentially beneficial role for IL-8 R expression during subclinical mastitis (24). This study offers significant insights into the diagnostic value of inflammatory markers in detecting subclinical mastitis in dairy cows. However, it is imperative to acknowledge the study's limitations and consider potential avenues for further exploration. For instance, the presence of inflammation due to mastitis may not exclusively be attributed to bacterial infection. For instance, the presence of bacterial infection could potentially weaken the immune system, thereby allowing secondary invaders to enter, or it could reduce the cow's food intake, leading to signs of nutritional deficiency. Consequently, further research in this domain is warranted. These prospective studies should entail the examination of a substantial number of cows afflicted with mastitis to discern the presence of various microbiological, environmental, and nutritional disorders. Furthermore, the research underscores the necessity for expanded examination of cytokines beyond those that have been previously studied. The present study reveals that mastitis in dairy cows is associated with an increase in inflammatory cytokines such

as amyloid A, IL-6, and IL-8. The study suggests that variations in these biomarkers could be utilized for disease diagnosis. The differential expression patterns of these inflammatory markers in dairy cows with subclinical and clinical mastitis have the potential to serve as indicators of the bovine immune status. This contributes to the prediction of vulnerable periods for disease onset and facilitates the establishment of effective management protocols, aimed at enhancing health through the implementation of appropriate breeding and vaccination schedules.

Acknowledgment
We would like to express our profound gratitude to all individuals who have participated in this study.

Authors' Contribution
Designed object, writing – original draft preparation and data collection: S. O. G .
Editing article, data collection and analyzed data: S. A.

Ethics
This study was conducted in accordance with the ethical guidelines established by the Helsinki Declaration. It is imperative to emphasize that meticulous care was exercised at every stage of the sampling process to ensure that no harm was inflicted on any of the animals involved.

Conflict of Interest
The authors have no conflict of interest.

Grant Support
There is no financial resource for this study.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

References

1. Maity S, Ambatipudi K. Quantitative proteomics of milk whey reveals breed and season specific variation in protein abundance in Holstein Friesian cow and Murrah buffalo. Research in veterinary science. 2019;125:244-52.
2. Cvetnić L, Samardžija M, Habrun B, Kompes G, Benić M. Microbiological monitoring of mastitis pathogens in the control of udder health in dairy cows. Slovenian Veterinary Research. 2016;53(3):131-40.
3. Petersson-Wolfe CS, Mullarky IK, Jones GM. Staphylococcus aureus mastitis: cause, detection, and control. 2010.
4. Yang M, Shi J, Tian J, Tao J, Chai M, Wang J, et al. Exogenous melatonin reduces somatic cell count of milk in Holstein cows. Scientific reports. 2017;7(1):43280.
5. Abdelmegid S, Murugaiyan J, Abo-Ismail M, Caswell JL, Kelton D, Kirby GM. Identification of host defense-related proteins using label-free quantitative proteomic analysis of milk whey from cows with Staphylococcus aureus subclinical mastitis. International journal of molecular sciences. 2017;19(1):78.
6. Sadat A, Farag AM, Elhanafi D, Awad A, Elmahallawy EK, Alsowayeh N, et al. Immunological and Oxidative Biomarkers in Bovine Serum from Healthy, Clinical, and Sub-Clinical Mastitis Caused by Escherichia coli and Staphylococcus aureus Infection. Animals. 2023;13(5):892.
7. Roncada P, Piras C, Soggiu A, Turk R, Urbani A, Bonizzi L. Farm animal milk proteomics. Journal of proteomics. 2012;75(14):4259-74.
8. Mudaliar M, Tassi R, Thomas FC, McNeilly TN, Weidt SK, McLaughlin M, et al. Mastitomics, the integrated omics of bovine milk in an experimental model of Streptococcus uberis mastitis: 2. Label-free relative quantitative proteomics. Molecular Biosystems. 2016;12(9):2748-61.
9. Shaheen M, Tantary H, Nabi S. A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. J Adv Dairy Res. 2016;4(1):1-10.
10. Ibrahim ESF, Khalil SA, Torky HA. Prevalence of Esbl Producing Enterobacteriacae Isolated from Bovine Mastitis Milk. Alexandria Journal for Veterinary Sciences. 2018;58(1).
11. Algammal AM, Enany ME, El-Tarabili RM, Ghobashy MO, Helmy YA. Prevalence, antimicrobial resistance profiles, virulence and enterotoxins-determinant genes of MRSA isolated from subclinical bovine mastitis in Egypt. Pathogens. 2020;9(5):362.
12. González MD, Arnold CF, Rodríguez MF, Checa R, Montoya A, Fuentes MP, et al. Effect of two treatments on changes in serum acute phase protein concentrations in dogs with clinical leishmaniosis. The Veterinary Journal. 2019;245:22-8.
13. Kováč G, Tóthová C, Nagy O, Seidel H. Milk amyloid A and selected serum proteins in cows suffering from mastitis. Acta veterinaria brno. 2011;80(1):3-9.
14. HR G. Evaluation of serum and milk amyloid A in some inflammatory diseases of cattle. 2008.
15. Bochniarz M, Zdzisińska B, Wawron W, Szczubiał M, Dąbrowski R. Milk and serum IL-4, IL-6, IL-10, and amyloid A concentrations in cows with subclinical mastitis caused by coagulase-negative staphylococci. Journal of dairy science. 2017;100(12):9674-80.
16. Kovačević-Filipović M, Ilić V, Vujčić Z, Dojnov B, Stevanov-Pavlović M, Mijačević Z, Božić T. Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis. Veterinary immunology and immunopathology. 2012;145(1-2):120-8.
17. Miglio A, Moscati L, Fruganti G, Pela M, Scoccia E, Valiani A, Maresca C. Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes. Journal of dairy research. 2013;80(4):496-502.
18. Hagiwara K, Yamanaka H, Hisaeda K, Taharaguchi S, Kirisawa R, Iwai H. Concentrations of IL-6 in serum and whey from healthy and mastitic cows. Veterinary research communications. 2001;25:99-108.

19. Johnzon C-F, Dahlberg J, Gustafson A-M, Waern I, Moazzami AA, Östensson K, Pejler G. The effect of lipopolysaccharide-induced experimental bovine mastitis on clinical parameters, inflammatory markers, and the metabolome: a kinetic approach. Frontiers in Immunology. 2018;9:1487.
20. Fu Y, Zhou E, Liu Z, Li F, Liang D, Liu B, et al. Staphylococcus aureus and Escherichia coli elicit different innate immune responses from bovine mammary epithelial cells. Veterinary immunology and immunopathology. 2013;155(4):245-52.
21. Safak T, Rısvanli A, Asci-Toraman Z. Th1/Th2 cytokine polarization in milk according to different pathogens causing subclinical mastitis in cows. Mljekarstvo: časopis za unaprjeđenje proizvodnje i prerade mlijeka. 2022;72(2):105-13.
22. Karthikeyan A, Radhika G, Aravindhakshan T, Anilkumar K. Expression profiling of innate immune genes in milk somatic cells during subclinical mastitis in crossbred dairy cows. Animal Biotechnology. 2016;27(4):303-9.
23. Verbeke J, Van Poucke M, Peelman L, De Vliegher S. Differential expression of CXCR1 and commonly used reference genes in bovine milk somatic cells following experimental intramammary challenge. BMC genetics. 2015;16:1-8.
24. Nannuru KC, Sharma B, Varney ML, Singh RK. Role of chemokine receptor CXCR2 expression in mammary tumor growth, angiogenesis and metastasis. Journal of carcinogenesis. 2011;10.