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Mastitis in Sheep - Procalcitonin Level as a Biomarker for Early Diagnosis

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ABSTRACT

***Background*:** Mastitis in sheep is significant due to its ability to cause both clinical and subclinical infections, being one of the most important diseases affecting dairy sheep worldwide. Infection caused by *Staphylococcus aureus* (*S. aureus*) is significant due to its ability to cause both clinical and subclinical mastitis. Procalcitonin (PCT) is a prohormone that increases in cases of bacterial inflammation, sepsis, and multiple organ failure. This study was conducted to determine changes in procalcitonin levels in subclinical and clinical mastitis in sheep.

***Materials, Methods & Results*:** The study used 60 Ivesi breed sheep that were within the 1st 45 days of lactation and had not received any medication in the past 20 days. The sheep were divided into 3 groups based on clinical examination, somatic cell count (SCC), California mastitis test (CMT), and results from bacteriological isolation and identification. Group 1 (n = 20) consisted of healthy sheep; Group 2 (n = 20) consisted of sheep with subclinical mastitis, and Group 3 (n = 20) consisted of sheep with clinical mastitis. Blood samples were collected from the jugular vein and then centrifuged. Serum Amyloid A (SAA), Haptoglobin (Hp), and PCT levels were assessed using commercial kits. The obtained data were analyzed using one-way ANOVA. Body temperature and leukocyte count were found to be higher in the clinical mastitis group compared to the other groups ($P < 0.001$). SCC, SAA, Hp, and PCT levels were lowest in the healthy group and highest in the clinical mastitis group ($P < 0.001$). Significant positive correlations were found between procalcitonin and body temperature, leukocyte count, SCC, SAA, and Hp ($r = 0.897$, $P < 0.01$; $r = 0.940$, $P < 0.01$; $r = 0.985$, $P < 0.01$; $r = 0.928$, $P < 0.01$; $r = 0.956$, $P < 0.01$, respectively).

***Discussion*:** In our study, higher PCT levels were detected in sheep with clinical mastitis. This finding supports the theory that PCT increases in bacterial infections. The presence of infection triggers an inflammatory response, which increases PCT production. The increase in leukocyte count and PCT levels reflects the severity of the infection and the immune system's response. This parallel increase explains the positive correlation between PCT and leukocyte count observed in our study. SCC is particularly indicative of mammary gland infections. During mastitis, somatic cells, especially leukocytes, increase in milk. This increase leads to elevated PCT levels as part of the inflammatory response. The high correlation between SCC and PCT indicates that both parameters increase in response to the presence and severity of infection. This parallel increase explains the positive correlation between SCC and PCT observed in our study. SAA is another indicator of the inflammatory response and is classified as an acute phase protein. Both SAA and PCT reflect the severity and extent of the inflammatory response. This parallel increase explains the positive correlation between SAA and PCT observed in our study. Hp is another acute phase protein produced by the liver as part of the inflammatory response. Hp levels increase during infection or tissue damage. Similarly, PCT levels also rise in response to inflammatory cytokines. In our study, the positive correlation between Hp and PCT indicates that both biomarkers increase in response to the severity of inflammatory processes. In conclusion, procalcitonin may be a novel biomarker for diagnosing both subclinical and clinical mastitis in sheep.

Keywords: sheep, haptoglobin, procalcitonin, biomarker, diagnosis, clinical mastitis, subclinical mastitis, bacterial infection.

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INTRODUCTION

Mastitis is considered one of the most important diseases affecting dairy sheep worldwide [3,15,17]. Bacterial infection caused by *Staphylococcus aureus* (*S. aureus*) is significant due to its ability to cause both clinical and subclinical mastitis [3,10,20].

The determination of plasma and serum levels of acute phase proteins (APPs) is used to assess the degree of inflammation and diagnose diseases [7,9,24]. APPs are induced by proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), and they help restore disrupted physiological processes. The response of APPs varies among pets, livestock, and humans [24]. In particular, haptoglobin and SAA are useful for determining the severity and prognosis of bovine mastitis [5,13].

PCT is a prohormone of calcitonin and is produced in the C cells of the thyroid in healthy organisms. During infection, PCT is produced through 2 main mechanisms: indirect and direct. Due to these mechanisms, the serum concentration of PCT rises rapidly during bacterial infections and returns quickly to normal levels once the infection resolves. Numerous studies have proven that PCT is an effective marker for disease and sepsis. In veterinary medicine, PCT is considered an important biomarker for determining the severity of bacterial infections. PCT has been studied in various animal species, including horses, cattle, and dogs [2].

This study aims to examine PCT levels in sheep with clinical and subclinical mastitis and evaluate PCT as a biomarker for early diagnosis of mastitis caused by bacteria.

MATERIALS AND METHODS

Animal selection and experimental protocol

The study material consisted of 60 Ivesi breed sheep brought to the Obstetrics and Gynecology Clinic of the Veterinary Faculty Animal Hospital at Harran University. The sheep used in the study were selected based on the following criteria: they were in the 1st 45 days of lactation, had not received any medication in the past 20 days, and had not undergone any treatment for mastitis. The study was conducted between February and May. Group 1 (n = 20, CMT and microbiological culture negative) consisted of healthy sheep; Group 2 (n = 20, *S. aureus* identified by MALDI-TOF MS) comprised sheep with subclinical mastitis, and Group 3 (n = 20, *S. aureus* identified by MALDI-TOF MS) included sheep with clinical mastitis. The subclinical mastitis group comprised clinically healthy sheep showing no signs of udder infection, with no changes in the morphological characteristics of the milk, but with a positive somatic cell count (SCC > 250 × 10^3 cells/mL). The clinical mastitis group included sheep with acute or hyperacute clinical mastitis affecting at least 1 lobe of the udder, a body temperature above 39.6°C, disease symptoms starting no more than 12 h prior, and signs of reduced appetite and/or other general illness symptoms.

Somatic Cell Count (SCC) and California Mastitis Test (CMT)

The CMT was performed on all milk samples. For the CMT, the collected milk samples were mixed in equal amounts with 2 mL of milk and 2 mL of test solution (containing bromocresol purple and anionic detergent). The test plate was slowly rotated to observe changes such as color alterations or gel formation, and the results were interpreted based on previous study [16]. The somatic cell count was determined using an automatic somatic cell counter (Lactoscan SCC, Milkotronic)[1] at a specialized dairy farm.

Culture and bacterial identification

Milk samples collected from healthy, subclinical, and clinical mastitis study groups were transferred to the laboratory under the cold chain. Subsequently, 100 µL of milk were inoculated on 5% blood agar[2] and plates were incubated at 37°C for 24-48 h. Plates revealing the growth of 3 or more different types of colonies were not subjected to further investigation due to contamination. Additionally, plates that had greater than 25 colonies per 100 µL were considered indicative of a mastitis infection. A representative colony selected from plates indicating the growth of 1 or 2 distinct colony types was subcultured on 5% sheep blood agar and incubated at 37°C for 24 h [19]. Colonies grown on blood agar were evaluated based on Gram staining, hemolysis characteristics,

pigment production, oxidase and catalase activity, and coagulase production. Gram-positive cocci in clusters, hemolytic, oxidase-negative, catalase-positive, and coagulase-positive isolates were identified as presumptive *Staphylococcus* strains. The species delineation of all fresh and pure isolates was established by MALDI-TOF MS [33]. Briefly, a single colony was transferred to a stainless steel plate via a toothpick. Following colony smearing, the direct transfer-formic acid approach was employed to extract bacterial proteins on-plate [28]. The MALDI-TOF MS results were evaluated in accordance with the manufacturer's guidelines based on the biotyper databases supplied by Bruker Daltonik. When applying a 2.0 cutoff, scores ≥ 2.0 indicated species-level identification, scores < 2.0 and ≥ 1.7 demonstrated genus-level identification, and scores < 1.7 suggested unreliable identification.

Blood sampling and measurement of serum PCT and acute phase proteins

To obtain serum from the sheep in the study groups, blood samples were collected from the jugular vein into 5 mL tubes containing a coagulation activator, following aseptic and antiseptic protocols. The collected blood samples were transported to the laboratory under cold chain conditions and centrifuged at 1207 *g*. The resulting serum samples were transferred to 2 mL microcentrifuge tubes and stored at -80°C until analysis.

Serum levels of PCT (Sheep Procalcitonin ELISA Kit)[3], Hp (Sheep Haptoglobin ELISA Kit)[3], and SAA (Serum Amyloid A ELISA Kit)[3] were evaluated using the ELISA method at the MEGATIP Laboratory (Gaziantep). Measurements were conducted according to the manufacturer's instructions with the Molecular Devices[4].

Statistical analysis

Statistical analysis were performed using SPSS version 26 software. The normality of variable distributions was assessed using visual methods (histograms and probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Descriptive statistics for normally distributed variables were reported as mean and standard deviation. Serum PCT values were compared between groups using a one-way ANOVA test. The homogeneity of variances was assessed using the Levene test. Correlation analyses were performed using the Pearson test to determine the relationships between the data. Results with a *P*-value below 0.05 were considered statistically significant. In cases where significant differences were found between groups, pairwise post-hoc comparisons were conducted using Tukey's tests.

RESULTS

The mean body temperature, leukocyte count, SCC, acute phase proteins, and PCT levels for the study groups are presented in Table 1. Significant differences in body temperature, leukocyte count, SCC, acute phase proteins, and PCT levels were found between the groups ($P < 0.001$). In the clinical mastitis group, body temperature and leukocyte count were observed to be higher compared to the other groups ($P < 0.001$). However, there were no significant differences in body temperature and leukocyte count between the healthy and subclinical mastitis groups ($P > 0.05$). The SCC, SAA, Hp, and PCT levels were found to be lowest in the healthy group and highest in the clinical mastitis group ($P < 0.001$).

The correlation analysis results between serum PCT levels and body temperature, leukocyte count, SCC, SAA, and Hp levels are presented in Table 2. A strong correlation was found between procalcitonin levels and body temperature, leukocyte count, SCC, SAA, and Hp levels ($P < 0.001$). Significant positive correlations were observed between procalcitonin and body temperature (r = 0.897, $P < 0.01$), leukocyte count (r = 0.940, $P < 0.01$), SCC (r= 0.985, $P < 0.01$), SAA (r= 0.928, $P < 0.01$), and Hp (r= 0.956, $P < 0.01$). Additionally, significant positive correlations were determined between body temperature and leukocyte count (r = 0.875, $P < 0.01$), SCC and body temperature (r= 0.908, $P < 0.01$) and leukocyte count (r = 0.967, $P < 0.01$), and between SAA and body temperature (r= 0.859, $P < 0.01$), leukocyte count (r= 0.938, $P < 0.01$), and SCC (r= 0.954, $P < 0.001$). Significant positive correlations were also found between haptoglobin and body temperature (r = 0.892, $P < 0.01$), leukocyte count (r = 0.945, $P < 0.01$), SCC (r = 0.978, $P < 0.01$), and SAA (r = 0.918, $P < 0.01$).

Table 1. Mean body temperature, leukocyte count, somatic cell count, acute phase proteins, and procalcitonin levels for the study groups.

Groups	n	Temperature (ºC)	Leukocytes (*10^3/µL)	Somatic cells*10^3/mL (n = measurable samples)	SAA (µg/mL)	Hp (µg/mL)	PCT (pg/mL)
		$\bar{X}$ ± SEM	$\bar{X}$ ± SEM	$\bar{X}$ ± SEM	$\bar{X}$ ± SEM	$\bar{X}$ ± SEM	$\bar{X}$ ± SEM
Healthy	20	38.78 ± 0.22[a]	4.83 ± 0.30[a]	200.70 ± 22.48[a]	8.44 ± 1.89[a]	39.66 ± 0.99[a]	508.51 ± 4.58[a]
SCM	20	38.93 ± 0.39[a]	5.01 ± 0.36[a]	447.25 ± 59.39[b]	19.57 ± 2.13[b]	51.58 ± 0.78[b]	879.47 ± 5.76[b]
CM	20	40.22 ± 0.30[b]	8.24 ± 0.41[b]	1820.90 ± 68.90[c]	95.73 ± 10.97[c]	162,45 ± 4.95[c]	1209.66 ± 33.33[c]
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a,b,c]Different letters in the same column indicate a statistically significant difference. SCM: Subclinical mastitis; CM: Clinical mastitis; SAA: Serum amyloid A; Hp: Haptoglobin; PCT: Procalcitonin; SEM: Standard error of the mean.

Table 2. Correlation analysis results between procalcitonin and body temperature, leukocyte count, somatic cell count, SAA, and Hp levels.

Correlation		Temperature	Leukocytes	SCC	SAA	Hp	PCT
Temperature	r	1	0.875**	0.908**	0.859**	0.892**	0.897**
Leukocytes	r	0.875**	1	0.967**	0.938**	0.945**	0.940**
SCC	r	0.908**	0.967**	1	0.954**	0.978**	0.985**
SAA	r	0.859**	0.938**	0.954**	1	0.918**	0.928**
Hp	r	0.892**	0.945**	0.978**	0.918**	1	0.956**
PCT	r	0.897**	0.940**	0.985**	0.928**	0.956**	1

**$P < 0.01$. SCC: Somatic cell count; SAA: Serum amyloid A; Hp: Haptoglobin; PCT: Procalcitonin.

DISCUSSION

Mastitis in dairy sheep flocks leads to significant economic losses due to reduced milk yield, decreased milk quality, and milk losses resulting from antibiotic use [29]. Laboratory diagnosis plays an important role in improving production efficiency and controlling diseases, including mastitis [34]. Procalcitonin has been studied as a potential marker of systemic inflammatory response to infections. Numerous studies have demonstrated its efficacy as a marker in critical illness and sepsis [31]. Our study provides significant data as the 1st to determine serum PCT levels in sheep with clinical and subclinical mastitis and to elucidate the relationship of these levels with other clinical and laboratory parameters.

Studies on the use of PCT in veterinary medicine are limited [2]. Considering the significant economic losses caused by mastitis in sheep, we are investigating the role of PCT in this disease for the 1st time. Previous studies have shown that PCT levels are significantly higher in cows with clinical mastitis compared to healthy cows [6]. Similarly, in our study, higher PCT levels were detected in sheep with clinical mastitis. This finding supports the theory that PCT increases in bacterial infections [1]. Since samples were taken from sheep with clinical mastitis immediately after the onset of symptoms, it was observed that PCT levels were already high at this stage, confirming that PCT is an early marker of infection [27], on the other hand serum PCT levels were significantly higher in cows with mastitis due to *S. aureus* infection compared to healthy controls [6]. PCT rapidly increases in the blood in response to *S. aureus* infection and the subsequent synthesis of pro-inflammatory cytokines [25]. Neumann *et al*. [22] found that serum PCT concentrations were significantly higher in cows with subclinical mastitis compared to healthy controls. However, they noted that serum PCT levels were significantly lower in the subclinical mastitis group compared to the clinical mastitis group, and these levels fell into an intermedi-

ate range between those of cows with clinical mastitis and healthy cows. Consistent with the literature, our study observed a directly proportional increase in PCT levels with the clinical severity in sheep with S. aureus mastitis. The production of pro-inflammatory cytokines, such as TNF-α, which exacerbate inflammatory processes and stimulate neutrophil phagocytic activity, varies depending on the clinical severity of the disease. Therefore, it is concluded that PCT levels are lower in the subclinical mastitis group [14].

Hp is an important acute phase protein in sheep, as it is in cattle [21]. It is known to be a marker of acute inflammatory processes and their aftermath. The main biological function of haptoglobin is to bind hemoglobin with high affinity, thereby preventing renal parenchymal damage caused by hemoglobin and iron loss following intravascular hemolysis [21]. Significant increases in Hp and SAA levels were found in cows with mastitis due to *S. aureus* infection, highlighting a significant acute phase response in intramammary *S. aureus* infections [6]. Consistent with the literature, our study found that SAA and Hp levels were higher in sheep with *S. aureus* mastitis compared to healthy sheep. High Hp levels may be associated with pro-inflammatory cytokines released from damaged mammary tissue in sheep with infection-related mastitis. Similarly, the SAA response in sheep with *S. aureus* mastitis can be linked to the important role of the immune system in modulating tissue damage. These effects primarily occur through the induction of leukocyte migration, neutrophil differentiation, activation of default receptors on neutrophils, and increased secretion of IL-8 [11,23]. Additionally, SAA initiates the synthesis of tissue-degrading enzymes and pro-inflammatory cytokines [8].

Neumann *et al*. [22] examined PCT levels in cows with acute clinical and subclinical mastitis and compared them with other clinical and laboratory parameters. In their study, they did not find a significant correlation between PCT concentrations and somatic cell count, body temperature, and leukocyte count. In contrast to the literature, our study found a significant correlation between PCT concentration and somatic cell count, body temperature, and leukocyte count. This difference may be due to the homogeneity of the parameters obtained from evaluating only *S. aureus*-infected mastitis in our study, rather than assessing multiple microorganisms causing mastitis simultaneously. The evaluation of PCT levels in cows with various diseases and noted that there was no correlation between PCT and leukocyte count, which could be attributed to differences in the timing of the response to infection [4]. To minimize timing-related differences, samples in our study were collected within 12 h of the onset of infection, and animals exceeding this timeframe were excluded from the study. The current standard for detecting and assessing mastitis is the measurement of somatic cell count. However, due to various factors such as changes in pH values or protein precipitations, somatic cell counting may not always provide accurate results. Some authours were unable to differentiate between clinical and subclinical mastitis using somatic cell count but achieved this differentiation with PCT [22]. In our study, somatic cell count and the CMT test were used as study parameters and were also effective in group classification. Therefore, differences in somatic cell count were observed across all groups, and a positive correlation with PCT was detected.

Infections cause an increase in body temperature, and during this process, PCT levels also rise. During infection and inflammation, the body's immune system releases various cytokines (e.g., IL-6, TNF-α) that stimulate the hypothalamus, leading to fever. These same cytokines also increase procalcitonin production [18]. Therefore, as the severity of infection and inflammation increases, both body temperature and PCT levels rise. This explains the strong positive correlation between these 2 parameters observed in our study.

Leukocytes are the body's 1st line of defense against infections, and their numbers increase in response to infection. The presence of infection triggers an inflammatory response, which increases PCT production. The increase in leukocyte count and PCT levels reflects the severity of the infection and the immune system's response [30]. This parallel increase explains the positive correlation between PCT and leukocyte count observed in our study.

SCC is particularly indicative of mammary gland infections. During mastitis, somatic cells, especially leukocytes, increase in milk. This increase leads to elevated procalcitonin (PCT) levels as part of the inflammatory response. The high correlation between SCC and PCT indicates that both parameters increase in response to the presence and severity of infection

[26]. This parallel increase explains the positive correlation between SCC and PCT observed in our study.

SAA is another indicator of the inflammatory response and is classified as an acute phase protein. During infection or inflammation, the liver increases SAA production. Similarly, PCT levels also rise as part of the inflammatory response. Both SAA and PCT reflect the severity and extent of the inflammatory response [32]. This parallel increase explains the positive correlation between SAA and PCT observed in our study.

Hp is another acute phase protein produced by the liver as part of the inflammatory response. Hp levels increase during infection or tissue damage [12]. Similarly, PCT levels also rise in response to inflammatory cytokines [25]. In our study, the positive correlation between Hp and PCT indicates that both biomarkers increase in response to the severity of inflammatory processes.

CONCLUSION

In conclusion, this study demonstrates that procalcitonin can be used as a novel biomarker for diagnosing both subclinical and clinical forms of mastitis in sheep. Procalcitonin provides valuable information, particularly in assessing the severity of infection and the systemic response. Future studies should evaluate the effectiveness and reliability of procalcitonin in different animal species, larger populations, and with other microorganisms causing mastitis. Additionally, monitoring procalcitonin levels could serve as a practical tool for the early detection and management of mastitis, contributing to efforts to protect animal health and optimize milk production.

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***Ethical approval*.** This study was conducted with permission from the Harran University Local Ethics Committee for Animal Experiments (HRU-HADYEK) on March 28, 2024 (Protocol Number: 2024/002).

***Declaration of interest*.** The authors declare no conflicts of interest. The authors are solely responsible for the content and writing of the article.

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