

Investigation of cathelicidin, amyloid A, and haptoglobin levels in milk from ewe with subclinical mastitis caused by *Mycoplasma agalactiae* and coagulase-negative *Staphylococcus* spp.

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Abstract

Subclinical mastitis, a significant disease in the dairy industry, also poses an important risk to small ruminants in terms of both animal health and economic loss. The aim of this study was to evaluate the status of acute phase proteins (APP) (cathelicidin [CATH], serum amyloid A [SAA], Haptoglobin [Hp]) in mastitis caused by *Mycoplasma agalactiae* and coagulase-negative staphylococci (CoNS), which are important causative agents of mastitis in ewes. The study material consisted of milk from 90 ewes divided into *M. agalactiae* (n = 30), CoNS (n = 30), and control (n = 30). After performing the California Mastitis Test (CMT) on the ewe, milk samples were collected with CMT scoring (–, +, ++, +++). Subsequently, microbiological analyses were performed to isolate the causative agent. After the groups were completed according to the pathogen isolation, ELISA tests were performed on milk samples. Normality analysis was performed after ELISA tests. ANOVA was applied to parametric cases, and Kruskal-Wallis test was applied to non-parametric cases. The SAA and CATH concentration in the milk of ewes with subclinical mastitis varied depending on the pathogens, but was found to be statistically significant ($P < 0.001$). Haptoglobin concentration was found to be significantly higher in the CoNS group compared to the other two groups ($P < 0.01$). In conclusion, it was determined that depending on the causative agent, APP can be considered as biomarkers, and studies targeting mycoplasma-induced mastitis as a common problem in small ruminants can be increased.

Ovine, acute phase proteins, Mycoplasma agalactiae, mammary gland

Mastitis, inflammation of the mammary gland, is a widespread disease affecting ruminants in the dairy industry, including ewes (Safak and Risvanli 2021; Jesse et al. 2023; Kiouvrekis et al. 2024). Subclinical mastitis (SCM) is characterized by changes in the chemical composition of milk without any physical changes in the mammary gland. Subclinical mastitis causes major financial losses for dairy farms due to decreased milk production and reduced milk quality. It is also an important disease from an animal welfare perspective (Safak and Risvanli 2022; Michael et al. 2023; Iancu et al. 2025).

Somatic cell count (SCC) is a commonly used marker in the diagnosis of subclinical mastitis. The California mastitis test (CMT) is frequently used to evaluate SCC in milk. Because it is a semi-quantitative test, interpretation is subjective and may lead to inaccurate results. Furthermore, SCC often remains elevated for some time after successful treatment of an intramammary infection (Dingwell et al. 2003; Viguier et al. 2009;

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Wollovski et al. 2021). Many factors influence SCC, including age, breed, level of genetic selection, lactation period, number of births, milking technique, feeding, grazing style, udder morphology, seasonal variations, other environmental stressors, and vaccination status (Bergonier et al. 2003; Souza et al. 2012; Williamson et al. 2022). On the other hand, considering the time, labour, and cost requirements for bacteriological culture, it does not appear to be feasible as a mastitis screening tool (Giagu et al. 2022).

Another method for diagnosing subclinical mastitis is through changes in milk conductivity. However, this method also does not meet the 80% sensitivity requirement (ISO 2007) necessary for diagnostic tests in automated milking systems (Hovinen et al. 2006; Wollovski et al. 2021).

Recent advances in proteomic analysis have enabled the identification of neutrophil-derived proteins involved in the immune response to mastitis. These proteins appear to have the potential to be used as biomarkers in the diagnosis of mastitis (Wollovski et al. 2021). Acute phase proteins (APP) such as serum amyloid A (SAA) or haptoglobin (Hp) are part of the inflammatory process in bacterial infections and appear to be important markers for the diagnosis of mastitis in ruminant animals (Eckersall et al. 2007; Tothova et al. 2014; Wollovski et al. 2021).

The aim of this study was to investigate the contents of cathelicidin (CATH), AA, and Hp in milk from ewes with mastitis, according to bacterial agents. We aimed to compare APP values in *Mycoplasma agalactiae* and coagulase-negative staphylococci (CoNS), which are frequently encountered in ewes.

Materials and Methods

Animals

Ethical approval for this study was obtained from the Siirt University Local Animal Experiments Ethics Committee (Decision date: 29.11.2024 and decision number: 2024/09/62). In this study, milk samples collected from ewe farms in Siirt province were subjected to bacteriological analysis. The study used three groups: *M. agalactiae*, CoNS, and control. A total of 90 milk samples were used, 30 in each group. The animals were hand-milked during a lactation period ranging from 50 to 80 days.

California mastitis test

California mastitis test was applied to milk samples taken from the ewes. Before this test, pre-milking was performed from each udder lobe. A milk sample was taken from each mammary lobe and placed in a separate compartment of the CMT container for testing. Next, equal amounts of CMT solution and milk were added to each section of the container. To allow the solution to interact with the milk, circular motions were performed horizontally for 10–15 s. Then, the same person rated CMT based on the gel's consistency. Results were divided into CMT negative (–) and positive (+) (within the degrees of +, ++ and +++). If at least one teat quadrant received one of the values +, ++, and +++, the ewe was considered positive for CMT. If both mammary lobes were negative, CMT(–) was considered (Safak et al. 2023).

Milk sample collection, bacterial isolation, and identification

After thoroughly cleaning the ewe's udders and disinfecting them with cotton soaked in 70% alcohol, milk samples (approximately 2 ml) were aseptically collected into sterile plastic tubes for microbiological analysis (Safak and Risvanli 2021). The samples were transported under cold chain conditions to the Microbiology Laboratory of the Faculty of Veterinary Medicine, Siirt University, and delivered within a maximum of 2 h (Plate IX, Fig. 1)

Isolation of coagulase-negative *Staphylococcus* spp.

Mastitis milk samples will be homogenized by vortexing, then inoculated onto blood agar medium (Oxoid, Basingstoke, United Kingdom) and mannitol salt agar (Oxoid) containing 5% defibrinated ewe blood, using a loop. Culture media were incubated at 37 °C in an aerobic environment for 24–48 h. Pure cultures were obtained from the colonies formed on the culture medium at the end of the incubation period. Isolates obtained from pure cultures were evaluated according to Gram staining, macroscopic and microscopic morphological characteristics, catalase, oxidase, and tube coagulase reactions (Quinn et al. 2011). Isolates that were Gram-positive, had cocci morphology, were haemolytic and/or non-haemolytic, had a positive catalase reaction, and were negative for oxidase and tube coagulase tests, were considered CoNS.

Isolation and identification of *M. agalactiae*

Milk samples brought to the laboratory were homogenized by vortexing and then inoculated into Mycoplasma Broth Base (Oxoid) for the isolation of *Mycoplasma* spp. They were then incubated for 7 days at 37 °C in a medium containing 5–10% CO₂. After the incubation period, the liquid culture media showing growth were inoculated onto Mycoplasma Agar Base (Oxoid) and incubated for 7–10 days at 37 °C in a medium containing 5–10% CO₂. After incubation, the presence of growth in the culture medium was examined under a stereomicroscope at ×40 magnification, and colonies resembling fried eggs were considered suspected *Mycoplasma* spp. (Samiullah 2013; Farooq et al. 2017; Abd-Elrahman et al. 2020). Suspected isolates were stored in Mycoplasma Broth Base supplemented with 50% horse serum at –20 °C until species-level identification was performed by PCR.

For PCR analysis, genomic DNA from suspect isolates was extracted using a commercial kit (Exgene™ Cell SV 106-152, GeneAll, Korea) in accordance with the manufacturer's protocol. To identify *M. agalactiae* isolates, specific primers targeting the *p80* gene region as reported by Tola et al. (1996) were used. The PCR assay was carried out using a commercial master mix (2X PCR Mastermix, ABT®, Ankara, Türkiye). For reaction optimization, the 25 µl total volume contained 12.5 µl of 2X PCR Mastermix, 5 µl of template genomic DNA, 1.5 µl of each primer (10 µM), and nuclease-free water. Thermal cycling conditions were optimized based on the instructions for both the master mix and the primers. The thermal profile consisted of an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 15 min. Nuclease-free PCR water served as a negative control, whereas genomic DNA from *M. agalactiae* ATCC® 35890 was used as a positive control. Amplified products were resolved by agarose gel electrophoresis (80 V, 1.5 h) stained with GelRed®. Bands were visualized under a gel documentation system (Gen-Box ImagER, Ankara, Türkiye) and evaluated alongside a molecular weight marker (100 bp Plus Opti-DNA Marker G193, ABM, Canada). Isolates yielding a distinct 375 bp amplicon were confirmed as *M. agalactiae*.

After microbiological analyses

Milk samples that were CMT-negative and showed no bacterial growth were considered as the control group (n:30). The study groups consisted of milk samples in which *M. agalactiae* (n:30) and CoNS spp (n:30) species grew in CMT (+) milk samples. Milk samples showing the growth of two or more bacteria were excluded from the study.

Measurement of acute phase proteins in milk samples

The concentrations of CATH, SAA, and Hp in the collected milk samples were determined using species-specific ELISA kits. Cathelicidin (Cathelicidin ELISA Kit, YLA0158SH), SAA (Serum Amyloid A/SAA ELISA Kit, YLA0009SH), and Hp (Haptoglobin/Hp ELISA Kit, YLA0004SH) were analysed according to the manufacturer's instructions. Results obtained from the assays were measured using an ELISA microplate reader (BioTek Instruments, USA). All laboratory analyses were performed at the Laboratory of the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Siirt University.

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). The distributional characteristics of the APPs, including CATH, Hp, and SAA, were assessed prior to statistical comparisons. Normality was evaluated both visually, using histograms and normal probability (Q-Q) plots, and analytically using the Kolmogorov-Smirnov test. Based on the distributional characteristics of the data, appropriate parametric or non-parametric statistical tests were selected. For variables showing a normal distribution, differences among the three groups were evaluated using one-way analysis of variance (ANOVA). When the ANOVA indicated a statistically significant overall difference, Tukey's test was performed for *post hoc* pairwise comparisons to identify which groups differed from each other. For variables that did not meet the assumptions of parametric analysis, the Kruskal-Wallis H test was used to assess differences among the three groups. When a significant difference was detected by the Kruskal-Wallis test, pairwise comparisons between groups were subsequently performed using the Mann-Whitney U test.

Results

Serum amyloid A, Hp, and CATH concentrations in ewes with subclinical mastitis are given in Table 1. Serum amyloid A concentration was highest in the CoNS group (2.76 ± 0.12 µg/ml), while it was lowest in the *M. agalactiae* group (1.98 ± 0.15 µg/ml). The SAA content in the milk of ewes with subclinical mastitis varied depending on the pathogens, but was found to be significant ($P < 0.001$). When we looked at the CATH concentration, the highest concentration was observed in the CoNS group (46.27 ± 1.12 pg/ml) and the difference was significant compared to the other two groups ($P < 0.001$). No significant

difference was observed between the control and mycoplasma groups ($P > 0.05$). However, a significant difference was found in Hp concentrations in the CoNS group compared to the other two groups ($P < 0.01$). No significant difference was found between the control and mycoplasma groups ($P > 0.05$). The highest Hp concentration was observed in the CoNS group ($60.46 \pm 3.20 \mu\text{g/ml}$), while the lowest was observed in the mycoplasma group ($45.32 \pm 2.90 \mu\text{g/ml}$) (Fig. 2).

Table 1. Comparison of acute phase protein concentrations among groups (values are presented as means $\pm$ sample standard deviation).

	Control (n = 30)		<i>Mycoplasma agalactiae</i> (n = 30)		CoNS (n = 30)		<i>P</i>
	$\bar{x} \pm \text{sx}$	Median	$\bar{x} \pm \text{sx}$	Median	$\bar{x} \pm \text{sx}$	Median	
Hp ($\mu\text{g/ml}$)	48.20 ± 4.49^a	39.97	45.32 ± 2.90^a	42.45	60.46 ± 3.20^b	54.40	**
SAA ($\mu\text{g/ml}$)	2.04 ± 0.18^a	1.61	1.98 ± 0.15^a	1.88	2.76 ± 0.12^b	2.67	***
CATH (pg/ml)	37.87 ± 1.61^a	36.26	37.70 ± 1.01^a	37.77	46.27 ± 1.12^b	46.25	***

CoNS: coagulase-negative staphylococci; Hp: haptoglobin; SAA: serum amyloid A; CATH: cathelicidin

^{a,b} Different superscripts in the same row denote a significant difference between the groups ($P < 0.01$);

** $P < 0.01$; *** $P < 0.001$

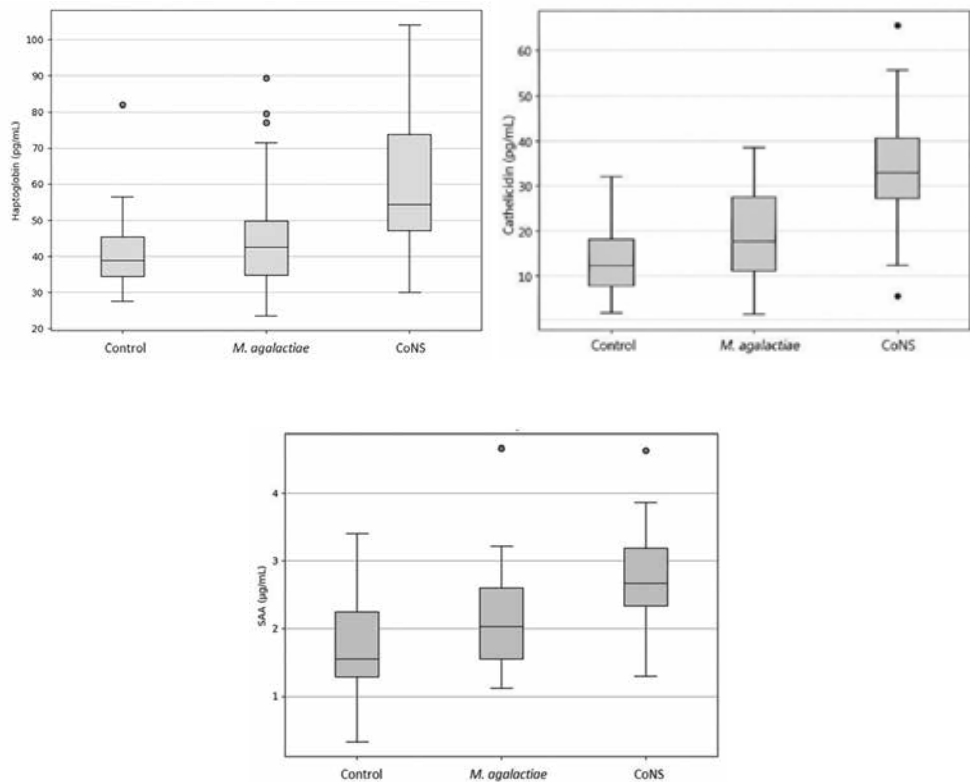


Fig. 2. Haptoglobin (Hp), cathelicidin (CATH), and serum amyloid A (SAA) concentrations among control, *M. agalactiae*, and CoNS groups.

Discussion

Cathelicidin is a biomolecule synthesized in the epithelial cells of the mammary gland and released when these cells in the mammary gland are exposed to an invading pathogen. It is known that in cases of infection, CATHs cross into the mammary gland even before leukocytes do, and play a role in the early stages of mammary defense against pathogens. Their role in the early stages of infection also allows the use of the CATH biomolecule as an early biomarker in mastitis (Cubeddu et al. 2017; Katsafadou et al. 2019; Bourganou et al. 2025). Studies in goats have revealed that CATH 1, CATH 2, CATH 3, CATH 6, and CATH 7 are present in milk during mastitis. These studies have reported a positive correlation between the presence of CATH in milk and SCC (Tedde et al. 2019; Bourganou et al. 2025).

Cathelicidin is almost undetectable in the milk of healthy animals, but its concentrations are known to increase upon exposure to an inflammatory stimulus. Cathelicidin contents decrease when there is no infection or in late and resolving infections (Smolenski et al. 2011; Addis et al. 2016). In their experimental study of *M. agalacticae*, Addis et al. (2011) observed a significant increase in milk production and mammary tissue. Similarly, Cubeddu et al. (2017) also found an increase in CATH concentrations in mastitis caused by *M. agalactiae* in their study. Studies involving different bacterial agents (*Staphylococcus aureus*, *Streptococcus uberis*, *S. chromogenes*, *Mannheimia haemolytica*) have also shown an increase in CATH contents (Addis et al. 2013; Katsafadou et al. 2019).

In our study, a statistically significant difference was observed in the CoNS group compared to the control and *M. agalactiae* groups. This may be due to the fact that CATHs appear in the early stages of infection. In ewes with mycoplasma infections, even if no clinical symptoms are seen in the milk, there may be conditions affecting the general condition, such as lameness or conjunctivitis. Therefore, the absence of early-stage infection can also differentiate between CoNS infections and *M. agalactiae* infections. In animals with mastitis, APP levels increase, indicating a defensive response to the infection, and it is predicted that it could be used as a biomarker (Ceciliani et al. 2012; Thulasiraman et al. 2013). In experimentally induced or spontaneously occurring mastitis of the mammary gland, a correlation has been found between SCC and bacteriological test findings and APP (Kleczkowski et al. 2017). Many APP are produced locally in the mammary gland and increase during infection, which strengthens their use as biomarkers in subclinical mastitis (Safi et al. 2009; Whelehan et al. 2011; Ceciliani et al. 2012; Kumar et al. 2014; Saleh et al. 2022). In a study conducted by Domanska et al. (2025) on mares with subclinical and clinical mastitis, significantly higher SAA and Hp concentrations were found compared to healthy mares.

Sadek et al. (2017) found an increase in SAA and Hp levels in cows with subclinical and clinical mastitis in their study. In the study conducted by Szczubial et al. (2012), while the highest increase in SAA was observed in animals with subclinical mastitis, an increase in ceruloplasmin and Hp contents was also observed. Saleh et al. (2022) reported an increase in SAA and Hp levels, in line with these two studies. Apart from these, many studies on animals with subclinical mastitis show an increase in SAA and Hp values (Gronlund et al. 2003; Hiss et al. 2004; Eckersall et al. 2007; Colla et al. 2011; Kováč et al. 2011; Nazifi et al. 2011; Çenesiz et al. 2018; Hussein et al. 2018; Aydın and Emre 2021; Wollowski et al. 2021). Winter et al. (2006) showed in their study that SAA contents in ewe milk can be used as a biomarker in subclinical mastitis.

In contrast to all these studies, there are also studies emphasizing that the increase in Hp and SAA in animals with subclinical mastitis was not significant (Safi et al. 2009; Gerardi et al. 2009; Pyorala et al. 2011). The findings in their studies were based on the detection of bacterial agents. In their study, Pyorala et al. (2011) reported

an increase in APP concentration in the *S. aureus* group, while its concentration was low in mastitis caused by CoNS and streptococci. In contrast, the study by Zalewska et al. (2020) showed an increase in animals with CoNS mastitis. In a study conducted by Winter et al. (2003), an increase in SAA was detected in mastitis caused by *Staphylococcus epidermidis* in ewe. Hiss et al. (2004) found that major mastitis pathogens such as *Escherichia coli* and *S. aureus* were more prevalent than minor mastitis pathogens. While Szczubiał et al. (2012) found low SAA contents in CoNS-related mastitis, there are also studies supporting a slight increase in SAA levels (Simojoki et al. 2009; Pyorala et al. 2011). In a study by Zalewska et al. (2020) comparing healthy animals with CoNS and coagulase-positive staphylococci (CoPS), an increase in Hp and SAA levels was reported in the CoNS and CoPS groups. In the study conducted by Ariffin et al. (2022), experimental *S. aureus* was produced and APP concentration was examined as a function of time. As a result, they reported that in the infected group, SAA and Hp concentrations showed a time-dependent change compared to the control group. In our study, Hp and SAA concentrations were significantly increased in the CoNS group compared to the other two groups. However, although an increase was observed in the *Mycoplasma* group, no significant difference was observed compared to healthy animals. This can be explained by the fact that the body's response to bacterial agents varies. Furthermore, Ariffin et al. (2022) reported time-dependent changes in the concentration of *M. agalactiae*. Although the relatively high *M. agalactiae* burden observed in the present study may be associated with the recent emergence of the infection in the study population, the absence of a significant difference may also reflect temporal variation in the host response and/or the stage of infection.

In conclusion, this study has shown that APP can be used as a biomarker in animals with subclinical mastitis. Many bacteriological agents can cause subclinical mastitis, and studies evaluating APP based on these agents are generally limited to cows. Many of the studies involve microorganisms such as *S. aureus*, CoNS, and *E. coli*. This study in ewes suggested that, based on the evaluation of bacteriological agents, APP could be a potential biomarker for diagnosis. Studies on bacteriological agents in ewe are limited, and this study is thought to serve as a reference for future research.

Conflict of interest

The authors declare no conflicts of interest. The authors have no relevant financial or non-financial interests to disclose.

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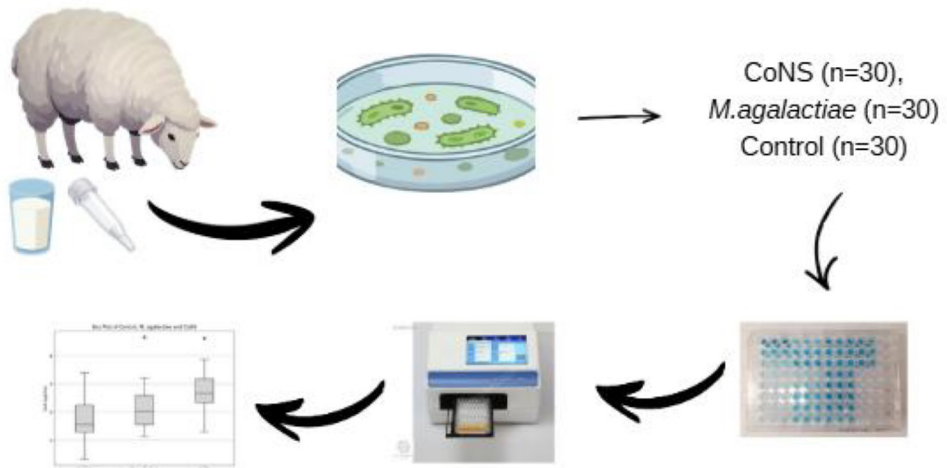


Fig. 1. Schematic representation of sample collection and laboratory procedures.