RESEARCH ARTICLE

F.U. Vet. J. Health Sci.
2024; 38 (3): 234 - 239
http://www.fusabil.org

Investigation of Virulence Factors of *Staphylococcus aureus* Isolates Associated with Sheep Non-Gangrenous or Gangrenous Mastitis *

Canan KEBABÇIOĞLU [1, a]
Büşra ÇELİK [1, b]
Serhat AYAN [2, c]
Adam TAWOR [1, d]
Aslı BALEVİ [1, e]
Zafer SAYIN [1, f]
Osman ERGANİŞ [1, g]
Hacer MARANGOZ [1, h]

[1] Selcuk University, Faculty of Veterinary Medicine, Department of Microbiology, Konya, TÜRKİYE

[2] Selcuk University, Experimental Research and Application Centre, Konya, TÜRKİYE

[a] ORCİD: 0000-0001-7299-9923
[b] ORCİD: 0009-0003-4292-9600
[c] ORCİD: 0000-0001-6234-0703
[d] ORCİD: 0000-0001-6865-1801
[e] ORCİD: 0000-0002-7266-1049
[f] ORCİD: 0000-0001-6096-4440
[g] ORCİD: 0000-0002-9340-9360
[h] ORCİD: 0009-0006-4983-3443

Staphylococcus aureus is an important cause of gangrenous mastitis (GM) in dairy farming and causes significant economic losses due to toxins such as alpha toxin, beta toxin, fibronectin binding protein, and leucocidins. Alpha toxin is defined as the primary virulence factor that initiates necrosis in udders. In this study, the virulence gene profile of *S. aureus* strains isolated from milk with GM was compared with isolates obtained from non-gangrenous mastitis cases. According to the data obtained, virulence gene profiles of mastitis isolates were compared with GM isolates and virulence gene similarity between mastitis strains and GM strains was investigated. Nine *S. aureus* strains were isolated from 85 milk samples and analysed for virulence factors causing GM. By PCR genomic analyses, 9 (10.5%) *S. aureus* strains were isolated. The isolates were positive for alpha toxin (44.4%), beta toxin (44.4%), leukocidin F (55.5%), fibronectin binding protein A (55.5%) by PCR. The strains carrying virulence genes, especially alpha toxin, were found to be associated with GM. Antibiotic resistance profiles were determined and found to be significantly resistant to various antibiotics. In the medium test for Hla production, Brain Heart Broth and Tryptic Soy Broth were found to be suitable for Hla production. PCR analyses showed that strains carrying more than one virulence gene, especially Hla, were obtained from strains with GM and statistical analyses showed that the isolates with GM did not resemble strains with mastitis in terms of virulence gene profile.

***Key Words*:** *Alpha toxin, gangrenous mastitis, PCR, SDS-PAGE, Stapylococcus aureus*

Koyun Mastitisinden İzole Edilen *Staphylococcus aureus* İzolatlarının Gangrenöz Mastite Neden Olma Potansiyelinin Araştırılması

Staphylococcus aureus, süt hayvancılığında gangrenöz mastitisin (GM) önemli bir nedeni olup alfa toksin, beta toksin, fibronektin bağlayıcı protein ve lökosidinler gibi toksinler nedeniyle önemli ekonomik kayıplara neden olmaktadır. Alfa toksin (Hla) memelerde nekrozu başlatan birincil virülans faktörü olarak tanımlanmaktadır. Bu çalışmada, gangrenöz mastitisli sütlerden izole edilen *S. aureus* suşlarının virülans gen profili, normal mastitis vakalarından elde edilen izolatlarla karşılaştırıldı. Elde edilen verilere göre, mastitis izolatlarının virülans gen profilleri GM izolatları ile karşılaştırılarak mastitis suşları ile GM suşlarının virülans gen benzerliği araştırılıldı. Dokuz *S. aureus* suşu 85 süt örneğinden izole edildi ve GM'ye neden olan virülans faktörleri açısından incelendi. PCR ile yapılan genomik analizler ile, 9 adet (%10.5) *S. aureus* izole edildi. İzolatlarda alfa toksin (%44.4), beta toksin (%44.4), lökosidin F (%55.5), fibronektin bağlayıcı protein A (%55.5) yönünden PZR ile pozitif bulundu. Başta alfa toksin olmak üzere virülans genlerini taşıyan suşların gangrenöz mastitis ile ilişkili olduğu tespit edildi. Antibiyotik direnç profilleri belirlenmiş ve çeşitli antibiyotiklere karşı önemli oranda dirençli bulundu. Alfa toksin üretimi için yapılan besiyeri testinde, Beyin kalp infüzyon broth ve Triptik soy broth alfa toksin üretimi için uygun bulundu. PCR ile yapılan analizlerde, başta alfa toksin olmak üzere birden fazla virülans geni taşıyan suşların gangrenöz mastitisli suşlardan elde edildiğini ve yapılan istatistiksel analizlerde gangrenöz mastitisli izolatların mastitisli suşlarla virülens gen profili yönünden benzemediği tespit edildi.

Anahtar Kelimeler: *Alfa toksin, gangrenöz mastitis, PZR, SDS-PAGE, Stapylococcus aureus*

Received : 14.06.2024
Accepted : 28.08.2024

Correspondence
Yazışma Adresi

Canan KEBABÇIOĞLU
Selcuk University,
Faculty of Veterinary Medicine,
Department of Microbiology
Konya – TÜRKİYE

kebabciogluc@gmail.com

Introduction

Mastitis is a quickly spreading illness that produces mammary gland inflammation as well as change of the physical and chemical structure of milk (1). This illness also causes severe infections in herds (2, 3). Although antibiotics can be used to treat mastitis in sheep flocks, it is cost-effective and not practical (4). Mastitis is a condition that causes large economic losses in the dairy sector (5) Subclinical mastitis, for instance, generates 1 million $ losses per year (1).

Since mastitis is a complex infection, the effectiveness of treatment is determined by the experience of the physician, selection of effective antibiotics, timing of treatment initiation and herd health management (6). Sheep mastitis are commonly associated with *Staphylococcus* spp. *Staphylococcus aureus* (17-57%) and coagulase-negative *Staphylococcus aureus* (10-52.6%) are isolated from sheep mastitis. Also, according to the frequency of occurrence; *Streptococci, Pasteurella, Enterobacteria* and *Corynebacteria* are isolated. These agents cause clinical and subclinical mastitis. The

* This study was derived from Canan KEBABÇIOĞLU's doctoral thesis. It was supported by Selcuk University Scientific Research Projects Coordination Unit (SUBAP) (project number: 23212012).

prognosis of the disease worsens. Gangrene occurs when *Staphylococcus aureus* produces alpha (Hla) and beta toxins (Hlb) and other components contributing to its pathogenicity (7, 8). The most important predisposing factors of gangrenous mastitis (GM) are lack of milking hygiene and irregular vacuum settings of milking machines. In large enterprises, irritations occur in the udder tissue during the milking process and some S. aureus in the environment may cause gangrene in the udder tissue with their toxins (9). GM is a severe condition that frequently results in mortality (10). In bacterial mastitis, *S. aureus* is the most important pathogen in sheep and goat mammary glands. Mastitis caused by *S. aureus* can cause GM in its subclinical, chronic, acute forms (11). GM is a peracute condition causing purple/black discolouration with increasing redness and oedema in the distal region of the udder. It is thought that GM may be caused by the accumulation of leukocytes in the mammary glands and the secretion of exotoxins by bacteria, as well as significant inflammation. Rainard et al. (12) found substantial levels of Hla in the milk of goats with GM, indicating that toxin-producing strains of *S. aureus* are the most prevalent cause of GM in livestock and dairy ruminants. According to Anderson et al. (13), Hla is primarily responsible for the gangrenous type of mastitis, which is caused by vasoconstriction of the arteries and ischemic necrosis. GM is extremely difficult to treat; medicines are ineffective, and if not treated promptly and effectively, it results in udder loss and death of the animal, as well as udder tissue degradation. Although Hla is the primary cause of GM in animals, beta toxin plays a role in the disease's development. The Hla causes pore development in mammalian cells, stimulating inflammatory responses. It is the most powerful membrane-damaging protein. This toxin's hemolytic, neurotoxic, and dermonecrotic qualities all contribute to its pathogenicity (14, 15). Beta toxin, also known as sphingomyelinase has significant hemolytic activity against sheep erythrocytes. It also aids bacterial colonization of the skin by destroying keratinocytes (16). Beta toxin kills secretory epithelial cells in cattle mammary glands, increasing the action of Hla. It enhances the agent's adherence to udder epithelial cells and boosts its proliferation in the area (8). Panton-Valentine leukocidin (PVL) is the toxin that has the greatest effect on immune cells. PVL positive *S. aureus* isolates that generate leukocyte toxins are commonly found in deep skin and soft tissue infections such as skin abscesses and severe necrotizing pneumonia, indicating that PVL is a significant virulence factor (17). Fibronectin-binding proteins (FnBP) binding to fibronectin/fibrinogen host cells, allowing them to spread (18).

In this study, the components isolated from 85 CMT positive clinical mastitis milk samples from two different areas were investigated in terms of antibiotic susceptibility and *S. aureus* virulence genes (Hla, Hlb, FnbPA, LukMF) that might cause GM. Various media were employed to naturally produce the toxin, which could then be used to make toxoid vaccines and antiserums. The hemolytic activity of the generated toxin was evaluated utilizing blood agar.

Materials and Methods

Research and Publication Ethics: The ethics committee permission of the research was obtained with the decision numbered 2024/113 of Selçuk University Experimental Animals Production and Research Centre.

Material: In this study, a total of 85 milk samples, 79 with non gangrenous mastitis and 6 with GM, sent to Selcuk University Microbiology Department for diagnosis from Southeastern Anatolia and Central Anatolia regions were used.

Cultivation and Biochemical Identification: The samples were cultivated on 5% defibrinated sheep blood agar (Merck, Darmstadt, Germany) and Mac Conkey agar (Merck, Darmstadt, Germany) and incubated for 24-48 hours at 37 °C in both aerobic and microaerophilic conditions. Gram staining was used to identify the colonies. Catalase and coagulase tests were performed for gram-positive bacteria. Lactose, mannitol, motility and urease tests were applied to gram negatives. All *S.aureus* suspected strains were subjected to antibiogram test to determine resistance status (OB: Cloxaciline, N: Neomycin, APX: Ampicillin/cloxacillin, L: Lincomycine, CEQ: Cefquinome, P: Penicilline, FEP: Cefepime, SXT: Trimethoprim /sulphamethoxazole, CFP: Cefoperazone). The antibiogram test was performed using the Kirby Bauer technique. Following incubation, zone diameters were measured and analyzed using the CLSI (19). After biochemical testing, gram-positive bacteria with positive catalase and coagulase tests were selected for DNA isolation.

DNA extraction and PCR: Wizard® Genomic DNA Purification Kit was used for DNA isolation. The isolated DNAs were stored at -20°C to be used in the PCR process. The isolated DNAs were firstly screened for *S. aureus* spp. The DNA isolates were first confirmed for *S. aureus* spp. and then the positive isolates were analysed for other virulence genes. Primers and loops specific to the genes analysed were used (Table 1). For the LukF gene, the gene region with accession number S65052.1 was used and primer design was made via NCBI. PCR mix was 4 µL 5x master mix (SolisBiodyne, Estonia), 0.2 pmol of each primer was taken, 5 µL DNA was added and completed to 20 µL with ultrapure water. After PCR, they were visualised under UV light using a 100 bp marker (Solisbiodyne, Estonia) on an agarose gel containing 10 µg/mL GelRed.

Table 1. Primers and loops specific to the genes analysed

Gen Adı	Primer	Kaynak	Size
LukF	F- ATCATCCGTTGCTACATCTATGG R- GCGACTTAATGTTCTGTAACTTTCT	This study	517 bp
Staphylococcus 16S	F-GGACGACATTAGACGAATCA R- CGGGCACCTATTTTCTATCT	(20)	1318 bp
Hla	F- GCGAAGAAGGTGCTAACA R-CAATTGGTAGTCATCACGAAC	(21)	569 bp
Hlb	F-GTGCACTTACTGACAATAGTGC R-GTTGATGAGTAGCTACCTTCAGT	(22)	309 bp
FnbB- A	F-GATACAAACCCAGGTGGTGG R-TGTGCTTGACCATGCTCTTC	(23)	191 bp

Medium Trial for Natural Production of Toxin in Bacteria: A medium was assessed for the synthesis of Hla, the most significant toxin involved in the pathogenesis of GM. In the medium test, the strain determined to be Hla positive by PCR was inoculated in Luria Bertani (LB) peptone water, Tryptic Soy Broth (TSB), Nutrient broth, Todd Hewitt Broth (TH) and Brain Heart Broth (BHI). The strain was inoculate the medium and incubated at 37 °C for 24-48 hours. After incubation, the media were centrifuged. The supernatant of each medium was taken and subjected to the SDS-page process (24-26).

Detection of Toxin by SDS-PAGE: For the separating gel, all ingredients are added to the solution. After adding the ammonium persulphate (APS), the liquid was poured between the glass plates, leaving a 1.5 cm space at the top. To smooth the surface of the gel, 0.3 mL of n-butanol saturated with pure water was squeezed onto the surface of the gel from the edge of the cassette. After polymerization was completed, n-butanol on the surface was poured off and washed with distilled waterIn another flask, a 5% loading gel was prepared. Again, all materials except APS were mixed in a magnetic stirrer. APS was also added and poured into the polymerized separation gel, with a comb used to produce wells in the gel. After the polymerization was finished, the comb was removed and put in an electrophoresis tank (Thermo Scientific, EC120 Mini Vertical Gel System) filled with Tris-Glycine Buffer. Samples with 4× SDS Loading Buffer and 1 M DTT were mixed with sample preparation buffer for a total of 10 min. After incubation at 95 °C, 25 µL of each sample was loaded into the wells. Electricity current was adjusted to 90 volts as the proteins moved through the 5% stacking gel and 150 volts when they reached the 12% separation gel. The electrophoresis procedure was stopped 2.5 hours after the sample reached the separation gel line.

Hemolytic Activity of Strains: After it was determined that the toxin was produced in BHI and TSB, BHI was used to determine the haemolytic activity of the strains since the most prominent band was obtained from BHI. Four Hla-positive bacteria obtained from GM samples were grown in BHI and cultured at 37°C for 24-48 hours. After incubation, it was cultivated in 100 µl of blood agar and incubated at 37°C for 24-48 hours.

Statistical Analysis: Statistical analyses were carried out using the GraphPad Prism 9.5.0 version, c, test was performed to see wether or not there is significant diferernce betwwen virulence genes of GM and non gangrenous mastitis isolates, p values of <0.05 were considered significant.

Results

Bacteriological Findings: Growth was observed in 60 out of 85 cultivated samples. As a result of gram staining and biochemical tests performed on the growth colonies, 10.5% (9/85) of the colonies were diagnosed as *Staphylococcus aureus* and 89.5% were other bacteria. Other bacteria were not included because they were not focus of the study. Antibiotic resistance of the isolated strains was determined by antibiogram test (Table 2).

PCR Findings: By culture and biochemical tests, 9 strains were found to be suspected *S. aureus*. A total of 9 strains were confirmed to be *S. aureus* by 16S rRNA in PCR analyses. Five strains were obtained from milk with suspected non gangrenous mastitis, and four were isolated from milk with GM. The virulence genes of the isolates (Table 3) were examined, and the probability of generating GM was assessed using the virulence genes caused by mastitis *S. aureus* isolates. Two-tailed t-test results revealed that virulence genes of strains with non-gangrenous mastitis and virulence genes of gangrenous mastitis were significantly different. Since *p* value was recorded as <0.0001 in two-tailed t-test results.

According to Table 3, only 5 of the isolates isolated from non ganrenous mastitis milk contained Hlb, LukF, and FnBA. Non-gangrenous mastitis isolates were

negative for the virulence genes screened. In strains with GM, isolate 4 was found to be Hlb-negative. Figures 1 and 2 show the Hla PCR images of the isolates, which play the most critical role in developing GM.

SDS-PAGE Analysis Findings: By SDS-PAGE, BHI and TSB were found to be the best media for the production of Hla (Figure 3).

Hemolytic Activity Findings: After incubation in BHI, the strains were cultivated in 100 µL of sheep blood agar and incubated at 37 °C for 24-48 hours. After incubation, the capacity of all strains isolated from GM milk to produce active haemolysis on blood agar was evaluated. Phenotype was shown to represent the genotypic trait.

Table 2. Antimicrobial resistance of isolated strains

Antimicrobial	Resistance (%)	İntermediate Sensitive (%)	Sensitive (%)
Cloxaciline (OB)	77.7	(11.1	-
Neomycin (N)	66.6	22.2	-
Ampicillin/cloxacillin (APX)	88.8	-	-
Lincomycine (L)	55.5	55.5	-
Cefquinome (CEQ)	11.1	77.7	-
Penicilline (P)	44.4	77.7	22.2
Cefepime (FEP)	44.4	44.4	-
Trimethoprim/sulphametaxazole (SXT)	-	88.8	-
Cefoperazone (CFP)	22.2	33.3	33,3

Table 3. Virulence gene distribution of the strains

Suş adı	*S. aureus* spp.	Hla	Hlb	Luk F	FnBA
M 1	+	-	-	-	-
M 2	+	-	-	-	-
M 3	+	-	-	-	-
M 4	+	-	-	-	-
M 5	+	-	+	+	+
GM 1	+	+	+	+	+
GM 2	+	+	+	+	+
GM 3	+	+	+	+	+
GM 4	+	+	-	+	+

Figure 1. PCR image of Hla gene in GM isolates (1,2,3,4 numbered strains found positive)

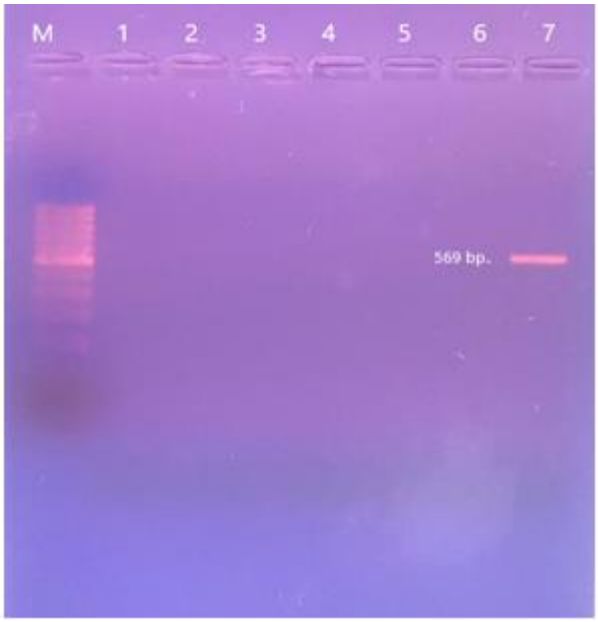

Figure 2. PCR image of Hla gene in non-GM isolates (1,2,3,4,5 numbered strains found negative) 6: Negative control, 7:Positive control M: marker

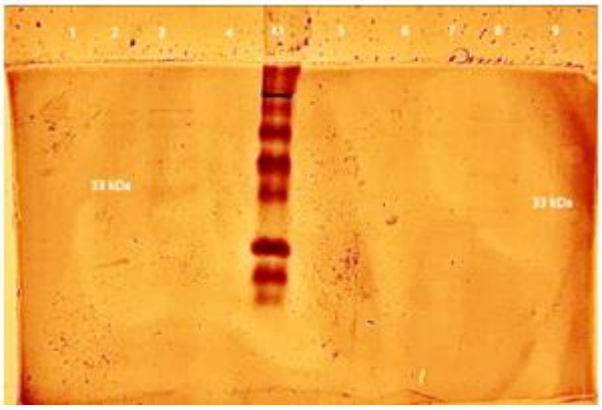

Figure 3. Hla production demonstration with SDS-PAGE. (Sample no. 3 is TSB, sample no. 8 is BH medium and these samples were found positive for Hla 33 kDa, other samples are negative)

Discussion

Staphylococcus spp. is the cause of a large number of animal diseases. It is most often found in non-ganrenous mastitis cases in dairy farms (27). Mastitis in sheep is a disease that causes significant economic losses. When an infection in the udder proceeds to gangrene, it results in udder loss and hence milk loss. *S. aureus* toxins also kill the animal (28). While most clinical and subclinical mastitis may be treated to some extent, GM cannot be treated with antibiotics and is irreversable. In addition, prevention is very important in this disease. Frequent monitoring of the agent and determination of antibiotic sensitivity and virulence factors are important in control and treatment, and are also necessary for vaccine studies.

In this study, virulence genes and antibiotic susceptibilities of the isolates obtained from mastitic milk were investigated. In addition, due to the importance of the losses caused by gangrenous mastitis in dairy sheep farms, virulence genes harboured by *S. aureus* isolated from mastitis strains and the potential of strains harbouring these genes to cause gangrenous mastitis were investigated. The predominance of Hla, the most significant toxin of *S. aureus*, which causes GM in mastitis strains, beta toxin, leucocidin F, and fibronectin-binding protein A were investigated. The virulence gene profile of gangrenous strains was compared with the virulence gene profile of non gangrenous strains.

According to the results obtained from the study; *Staphylococcus aureus* was detected in 15% (9/60) of 60 colonies (5 from non ganrenous mastitis and 4 from GM milk) and 85% (51/60) of other bacteria were isolated. In studies conducted with clinical mastitis milk, 13.95%, 20%, 7.6%, 9% isolated *S. aureus* (29-32). When similar studies were taken into consideration, it was determined that the findings obtained in this study were close.

Antibiogram results indicate that the strains we the most resistant to Cloxacillin. According to the antibiogram results of similar studies, tetracycline, penicillin and chloramphenicol were the most resistant antibiotics (31, 33, 34).

According to the PCR results from this investigation, only one of the mastitis-related isolates tested positive for beta toxin, luk F, and FnbP A. Hla, Hlb, luk F, and FnbP A were identified positively in isolates from GM. *S. aureus* 38% isolated from clinical and subclinical mastitis samples in sheep and goats tested positive for LukMF (35). Since there were not enough studies on gangrenous mastitis in sheep, studies on goats and cattle were taken into consideration. In a study on goats with subclinical mastitis, 7.5% of the isolated *S.aureus* were found to be FnBA positive (36). In a study conducted with milk of cattle with subclinical and clinical mastitis, Hla, Hlb, FnbA, LukF were found positive in 100% of the strains in the virulence genes sequence analysis. Better results were obtained in this study due to the difference in method and primers used (37). In another study examining the virulence genes of *S. aureus* isolated from bovine mastitis, 83.8% FnBA, 98.7% hla, 99.1% hlb were found positive (38).

The medium utilized in the toxin generation experiment included TSB, nutrient broth, TH, BHI, LB strain, and peptone water. According to the findings, experiments on manufacturing BHI and TSB had comparable outcomes. Similar to this investigation, Palmer et al. (25), Hildebrand et al. (24), Menzies and Kernodle (26) employed TSB to produce Hla and were satisfactory.

Consequently, PCR was used in this study to detect the presence of Hla and other virulence genes that are the primary cause of gangrenous mastitis. Ampicillin/cloxacillin resistance was found in 88.8% of *S. aureus* isolates obtained from mastitis and GM strains. When the virulence gene profiles of the isolated mastitis-related strains were investigated, the presence of the other virulence genes tested, particularly the Hla gene, was shown to be statistically insignificant. It has been established that GM strains' hemolytic characteristics are genotypically and phenotypically compatible. According to the data obtained with the strains employed in the study and other technique components, it is believed that strains with this strain profile are unlikely to induce gangrenous mastitis.

References

1. Ruegg PL. Investigation of mastitis problems on farms. Veterinary Clinics: Food Animal Practice 2003; 19: 47-73.
2. Smith MC, Roguinsky M. Mastitis and other diseases of the goat's udder. J Am Vet Med Assoc 1977; 171: 1241-1248.
3. White EC, Hinckley LS. Prevalence of mastitis pathogens in goat milk. Small Rumin Res 1999; 33: 117-121.
4. Yeşilmen S, Işık N, Özyurtlu N, et al. Koyun ve keçilerde *Staphylococcus aureus* kaynaklı gangrenli mastitis olgularında mastitis aşısının (Startvac®) etkinliğinin

araştırılması. Dicle Üniversitesi Veteriner Fakültesi Dergisi 2017; 10: 107-114.

5. Olechnowicz J, Jaśkowski JM. Mastitis in small ruminants. Med Weter 2014; 70: 67-72.
6. Paramasivam R, Gopal DR, Dhandapani R, et al. Is AMR in dairy products a threat to human health? An updated review on the origin, prevention, treatment, and economic impacts of subclinical mastitis. Infect Drug Resist 2023; 16: 155-178.
7. Bergonier D, Berthelot X. New advances in epizootiology and control of ewe mastitis. Livest Prod Sci 2003; 79: 1-16.
8. Vautor E, Cockfield J, Le Marechal C, et al. Difference in virulence between *Staphylococcus aureus* isolates causing gangrenous mastitis versus subclinical mastitis in a dairy sheep flock. Vet Res 2009; 40: 56.
9. Mein GA, Relnemann D, Ohnstad IC, O'Callaghan E. 100 years with liners and pulsators: Where the rubber meets the teat and what happens to milking characteristics. Bulletin International Dairy Federation 2003; 388: 28-34.
10. Machado G. Mastitis in small ruminants. Anim Husb Dairy Vet Sci 2018; 2: 1-9.
11. Kahinda CTM, Mastitis in small ruminants. In: Dego OK (Editor). Mastitis in Dairy Cattle, Sheep and Goats. 2nd Edition, United Kingdom: London, 2021: 87-111.
12. Rainard P, Gitton C, Chaumeil T, et al. Host factors determine the evolution of infection with *Staphylococcus aureus* to gangrenous mastitis in goats. Vet Res 2018; 49: 72.
13. Contreras A, Luengo C, Sánchez A, Corrales JC. The role of intramammary pathogens in dairy goats. Livestock Production Science 2003; 79: 273-283.
14. Abril GA, Villa TG, Barros-Velázquez J. et al. *Staphylococcus aureus* exotoxins and their detection in the dairy industry and mastitis. Toxins 2020; 12: 537.
15. Moreillon P, Que Y, Glauser M. Principles and Practice of Infectious Diseases In: Bennett JE, Dolin R, Blaser MJ (Editors). Staphylococcus aureus. 8th Edition, Philadelphia: Elsevier Churchill Livingstone, 2005: 2321-2351.
16. Oliveira D, Borges A, Simões M. *Staphylococcus aureus* toxins and their molecular activity in infectious diseases. Toxins 2018; 10: 252.
17. Friesen J, Neuber R, Fuhrmann J, et al. Panton-Valentine leukocidin–positive *Staphylococcus aureus* in skin and soft tissue infections from primary care patients. Clin Microbiol Infect 2020; 26: 1411-1414.
18. Costa DTM, Viljoen A, Towell AM, Dufrêne YF, Geoghegan JA. Fibronectin binding protein B binds to loricrin and promotes corneocyte adhesion by Staphylococcus aureus. Nat Commun 2022; 13: 2517.
19. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. In: Wayne PA (Editor). 30th Edition, CLSI supplement. Clinical and Laboratory Standards Institute; 2020
20. Riffon R, Sayasith K, Khalil H, et al. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. J Clin Microbiol 2001; 39: 2584-2589.
21. Reddy PK, Shekar A, Kingston JJ, Sripathy MH, Batra H. Evaluation of IgY capture ELISA for sensitive detection of alpha hemolysin of *Staphylococcus aureus* without staphylococcal protein A interference. J Immunol Methods 2013; 391: 31-38.
22. Jarraud S, Mougel C, Thioulouse J, et al. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun 2002; 70: 631-641.
23. Arciola CR, Campoccia D, Gamberini S, Baldassarri L, Montanaro L. Prevalence of cna fnbA and fnbB adhesin genes among *Staphylococcus aureus* isolates from orthopedic infections associated to different types of implant. FEMS Microbiology Letters 2005; 246: 81-86.
24. Hildebrand A, Pohl M, Bhakdi S. *Staphylococcus aureus* alpha-toxin. Dual mechanism of binding to target cells. JBC 1991; 266: 17195-17200.
25. Palmer M, Jursch R, Weller U, et al. *Staphylococcus aureus* alpha-toxin. Production of functionally intact, site-specifically modifiable protein by introduction of cysteine at positions 69, 130, and 186. JBC 1993; 268: 11959-11962.
26. Menzies BE, Kernodle DS. Site-directed mutagenesis of the alpha-toxin gene of *Staphylococcus aureus*: Role of histidines in toxin activity in vitro and in a murine model. Infection and Immunity 1994; 62: 1843-1847.
27. Friedman S, Shoshani E, Ezra E. Economical losses from clinical mastitis in four dairy herds in Israel. Isr J Vet Med 2004; 59: 16-19.
28. Jain NC. Common mammary pathogens and factors in infection and mastitis. J Dairy Sci 1979; 62: 128-134.
29. Stoimenov A, Popova T, Stoimenov G, Hristov K. Determination of causative agents of subclinical mastitis in sheep in Bulgaria. TMVM 2023; 8: 48-55.
30. Djerrou Z, Gabli Z, Gabli A. Study of bacteria associated with mastitis in dairy sheep farms in the highlands of eastern Algeria. Trop Agric 2023; 100: 301-307.
31. Michael CK, Lianou DT, Tsilipounidaki K, et al. Recovery of staphylococci from teatcups in milking parlours in goat herds in greece: Prevalence, identification, biofilm formation, patterns of antibiotic susceptibility, predictors for isolation. Antibiotics 2023; 12: 1428.
32. Danmallam FA, Pimenov NV. Study on prevalence, clinical presentation, and associated bacterial pathogens of goat mastitis in Bauchi, Plateau, and Edo states, Nigeria. Vet World 2019; 12: 638-645.
33. Othman N, Bahaman A. Prevalence of subclinical mastitis andantibiotic resistant bacteria in three selected cattle farms in serdang, selangor and kluang, johor. J Vet Malaysia 2005; 17: 27-31.
34. Dhital B, Chuang ST, Hsieh JC, et al. Prevalence, virulence and antimicrobial resistance of major mastitis pathogens ısolated from taiwanese dairy farms. Antibiotics 2024; 13: 36.
35. Hoekstra J, Rutten VPMG, Hout MVD, et al. Differences between *Staphylococcus aureus* lineages isolated from ovine and caprine mastitis but not between isolates from clinical or subclinical mastitis. J Dairy Sci 2019; 102: 5430-5437.
36. Salaberry SRS, Saidenberg ABS, Zuniga E, et al. Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. Microb Pathog 2015; 85: 35-39.
37. Rocha LS, Silva DM, Silva MP, et al. Comparative genomics of *Staphylococcus aureus* associated with subclinical and clinical bovine mastitis. PLoS One 2019; 14: 0220804.
38. Ote I, Taminiau B, Duprez JN, Dizier I, Mainil JG. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet Microbiol 2011; 153: 285-292.