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Determination of Antimicrobial Susceptibility and Some Virulence Genes of *Staphylococococcus* spp. Strains Isolated from Keratoconjunctivitis Cases in Sheep and Goats

Özgül GÜLAYDIN[1,*] Muazzez YEŞİLYURT[1] Ali GÜLAYDIN[2]

[1] *Siirt University, Faculty of Veterinary Medicine, Department of Microbiology, 56100, Siirt, Türkiye*
[2] *Siirt University, Faculty of Veterinary Medicine, Department of Surgery, 56100, Siirt, Türkiye*

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ABSTRACT This study aimed to isolate *Staphylococcus* spp. from sheep and goats clinically diagnosed with keratoconjunctivitis, and to determine their antimicrobial susceptibility. Phenotypic and genotypic characterisation of biofilm forming ability of the isolates obtained was carried out. *Staphylococcus* spp. strains isolated from 288 ocular swab samples were identified by MALDI-TOF MS. Antimicrobial susceptibilities of the isolates were determined by the using disk diffusion method. The Congo-red agar method was used to determine the ability to form biofilms. The presence of genes associated to formation of biofilm and toxin synthesis was investigated by PCR. While *Staphylococcus aureus* was identified in 2 of the 35 strains identified in the research, the remaining isolates were found to be coagulase-negative *Staphylococcus* spp. The most frequently isolated coagulase-negative strain was identified to be *Staphylococcus equorum*. The strains were susceptible to enrofloxacin, gentamicin, and tobramycin. While 68.57% of the isolates phenotypically formed biofilms, the *AtlE* gene had a high positivity rate. Furthermore, the presence of genes responsible for toxin synthesis was not identified in the strains analysed. Based on the findings of the study, it was determined that *Staphylococcus* spp. isolates should be considered for small ruminant keratoconjunctivitis cases. It was concluded that antimicrobial agents such as enrofloxacin, gentamicin, and tobramycin would achieve success in the treatment of the disease caused by the causative agents.

Keywords: *Biofilm, Goat, Keratoconjunctivitis, Sheep, Staphylococcus* spp.

ÖZ

Koyun ve Keçilerde Keratokonjunktivitis Olgularından İzole Edilen *Staphylococococcus* Spp. Suşlarının Antimikrobiyal Duyarlılığının ve Bazı Virülens Genlerinin Belirlenmesi

Bu çalışmada koyun ve keçilerde klinik olarak tespit edilen keratokonjunktivitis olgularından *Staphylococcus* spp. izolasyonu ve antimikrobiyal duyarlılığının belirlenmesi amaçlandı. Elde edilen izolatlarda biyofilm oluşturma yeteneğinin fenotipik ve genotipik karakterizasyonu gerçekleştirildi. Koyun ve keçilerden alınan 288 adet göz svabı örneğinden izole edilen *Staphylococcus* spp. suşları MALDI-TOF MS yöntemiyle identifiye edildi. İzolatların antimikrobiyal duyarlılıkları disk difüzyon testi ile belirlendi. Biyofilm oluşturma yeteneğinin belirlenmesinde Kongo-red agar yöntemi kullanıldı. Biyofilm oluşumundan ve toksin sentezinden sorumlu gen varlığı ise PCR ile araştırıldı. Araştırmada izole edilen 35 suşun 2'si *Staphylococcus aureus* olarak identifiye edilirken, geri kalan izolatların koagulaz negatif *Staphylococcus* spp. olduğu belirlendi. Koagulaz negatif türler arasında çoğunlukla izole edilen türün *Staphylococcus equorum* olduğu tespit edildi. Suşlar, enrofloksasin, gentamisin ve tobramisine duyarlı bulundu. İzolatların %68.57'sinin fenotipik olarak biyofilm oluşturduğu belirlenirken, *AtlE* gen pozitiflik oranının da yüksek olduğu görüldü. Ancak, çalışmada biyofilm oluşturma yeteneği ile antimikrobiyal direnç profili arasında herhangi bir ilişki saptanmadı. Bununla birlikte incelenen suşlarda toksin sentezinden sorumlu gen varlığı da tespit edilmedi. Çalışmadan elde edilen bulgular doğrultusunda küçük ruminant keratokonjunktivitis olgularında *Staphylococcus* spp. izolatlarının göz önünde bulundurulması gerektiği belirlendi. Etkenlerin neden olduğu enfeksiyonların tedavisinde enrofloksasin, gentamisin ve tobramisin gibi antimikrobiyal maddelerin başarı sağlayacağı kanısına varıldı.

Anahtar Kelimeler: *Biofilm, Keçi, Keratokonjuktivitis, Koyun, Staphylooccus* spp.

✉ *Corresponding author: ozgul.gulaydin@siirt.edu.tr

INTRODUCTION

Infectious keratoconjunctivitis (IKC), which negatively affects animal well-being in small ruminants, is a contagious disease characterised by clinical findings such as redness on conjunctiva, purulent or serous discharge, and blepharospasm. The disease not only causes a drop in yield in sheep and goats but also leads to serious conditions such as pregnancy toxaemia as a result of lack of feed conversion (Işik et al. 2018). It has been reported that bacterial agents such as *Moraxella ovis*, *Chlamydia* spp., *Mycoplasma* spp. *Staphylococcus aureus* and *Listeria monocytogenes* are frequently isolated and identified from disease cases (Fernandez-Aguilar et al. 2017). Since *Staphylococcus* spp. isolates are opportunistic pathogens and are available in the natural flora of mucous membranes, they are considered to be less important than other bacterial agents in determining the a etiology of keratoconjunctivitis cases diagnosed in small ruminants. However, the strains that develop resistance to topical and/or systematic antibiotics used especially in the treatment of keratoconjunctivitis cases negatively affect the prognosis of the disease and animal well-being (Udegbunam et al. 2014). *Staphylococcus* spp. strains have various virulence factors that allow them to evade the host immune system and play a role in the occurrence of infection. These virulence factors are synthesised in bacteria under the control of some enzymes (Karahan et al. 2009). The formation of biofilm in staphylococcal isolates is among the important virulence factors. The mechanism of biofilm formation plays a role in the development of antimicrobial resistance of the agents against both antibiotics and phagocytosis mediated by macrophages (Watkins and Unnikrishnan 2020; Gurler et al. 2022). The biofilm formation in *Staphylococcus* spp. isolates is induced by the icaABCD operon (Arciola et al. 2015). However, it has been known that various adhesin proteins (AtlE, aap) are involved in biofilm formation in isolates. These proteins mediate the accumulation and adhesion stages of biofilm (Soumya et al. 2017). It has been reported that panton-valentine leukocidin and exfoliative toxin synthesised by the agent play a role, especially in soft tissue infections, and these toxins are induced by *luk-PV*, as well as *eta*, *etb* and *etd* genes, respectively (Boyle-Vavra and Daum 2007). In numerous studies, different bacterial agents were isolated and identified from clinically diagnosed conjunctivitis cases in sheep and goats, and different treatment procedures were applied (Angelos and Rowe 2014; Biswas and Saifuddin 2017; Athira et al. 2018). Previous studies showed that antimicrobial agents such as chloramphenicol, tetracycline, enrofloxacin, ceftiofur, gentamicin, and tobramycin could be used topically and/or systemically in small ruminant conjunctivitis cases (Angelos and Rowe 2014; Jesse et al. 2017; Athira et al. 2018). This study was aimed to determine the antimicrobial susceptibility of *Staphylococcus* spp., which are frequently isolated from conjunctivitis cases in sheep and goats to antimicrobial agents frequently used in the treatment of conjunctivitis cases with current data. Furthermore, it is also aimed to investigate the virulence associated genes that play a role in the occurring of the disease.

MATERIAL AND METHODS

This study was approved by the Local Ethics Committee for Animal Experiments at Siirt University with decision number 2024/04/22 on 30/04/2024.

Ocular swab samples collected from 188 Hamdani sheep and 100 hair goats clinically diagnosed with keratoconjunctivitis between 2019 and 2023 were used. The ages of the sheep and goats were found to range from 1 to 3 years. 134 (72.27%) of the sheep were ewe and 54 (28.72%) were rams, while 71 (71.00%) of the goats were does and 29 (29.00%) were billy.

Collection of Swab Samples

The swabs were collected from the affected eye and/or eyes of animals diagnosed with keratoconjunctivitis based on clinical examination by following the rules of asepsis and antisepsis. The lower eyelid was gently turned upside down, and a cotton-tipped swab was rubbed on the conjunctiva, and samples were collected. If both eyes were found to be infected in the same animal, the same swab was used for sampling from both eyes.

Isolation and Identification of *Staphylococcus* spp.

The swabs were inoculated on Columbia blood agar (Oxoid, CM 03331, UK) and Mannitol Salt Agar (Oxoid, CM85, UK) media containing 5-7% defibrinated sheep blood. The inoculated media were allowed to incubate at 37 °C in an aerobic environment for 24-48 hours. Pure cultures were obtained from the formed colonies. The obtained isolates were analysed according to Gram staining, microscopic morphology, catalase reaction, ability to grow on mannitol salt agar medium, and tube coagulase test results (Quinn et al. 2011).

The isolates were identified by using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Microorganism Identification device. Ethanol formic acid extraction method was used to obtain the protein profiles. The identification process was completed by comparing the spectra obtained with the flex control software (Biotyper 3.0; Microflex LT; Bruker Daltonics GmbH, Bremen, Germany) with the Maldi Biotyper Real-Time Classification (RTC) software (version.9) (Uysal et al. 2019).

Determination of Antimicrobial Susceptibility

Antimicrobial susceptibility of the isolates was determined by the disk diffusion method using chloramphenicol (30 µg), tetracycline (30 µg), enrofloxacin (5 µg), ceftiofur (30 µg), gentamicin (10 µg), and tobramycin (10 µg) disks. The criteria reported in CLSI (2018) were taken into consideration in the performance and analysis of the test. The isolates were categorized as susceptible (S), intermediate (I), or resistant (R).

Determination of the Biofilm-Forming Ability by Congo-Red agar Method

Congo-Red Agar method was used to determine the ability of biofilm-forming in the strains. To prepare Congo red agar, 0.8 g of Congo red and 36 g of sucrose were added to 1 liter of brain heart infusion agar medium (Merck 1.10493, Germany). The prepared media was inoculated and then, incubated at 37 °C for 24 hours. After that, the plates were kept at room temperature for 48 hours. Isolates forming black colour were considered as biofilm-forming, and strains forming burgundy or red colour were considered as non-biofilm-forming strains (Gurler et al. 2022).

Identification of Genes Responsible for Biofilm and Toxin Synthesis

The genes responsible for toxin synthesis and biofilm formation in the isolates were determined by PCR using specific primers. Genomic DNA used in the PCR method was extracted by the boiling method. PCR mixture was

Table 1: Primer sequences for investigating the presence of genes associated with toxin synthesis and biofilm formation in isolates by PCR.

Gene	Oligonucleotide (5'-3')	Amplicon size (bp)	Denaturation - annealing - extension (35 cycles)	References
Biofilm and adhesion factors to hydrophobic surfaces				
AtlE	F: CAACTGCTCAACCGAGAACA R: TTTGTAGATGTTGTGCCCCA	682	94°C / 1 min. - 51°C / 1 min. - 72°C / 1 min.	Frebourg et al., 2000
icaAB	F: TTATCAATGCCGCAGTTGTC R: GTTTAACGCGAGTGCGCTAT	546	94°C / 1 min. - 51°C / 1 min. - 72°C / 1 min.	Frebourg et al., 2000
aap	F: ATACAACTGGTGCAGATGGTTG R: GTAGCCGTCCAAGTTTTACCAG	399	94°C / 1 min. - 54°C / 1 min. - 72°C / 1 min.	Vandecasteele et al., 2003
icaA	F: CCTAACTAACGAAAGGTAG R: AAGATATAGCGATAAGTGC	1315	94°C / 1 min. - 51°C / 1 min. - 72°C / 1 min.	Vandecasteele et al., 2003
icaD	F: AAACGTAAGAGAGGTGG R: GGCAATATGATCAAGATAC	381	94°C / 1 min. - 50°C / 1 min. - 72°C / 1 min.	Vandecasteele et al., 2003
Toxin genes				
Exfoliative toxin				
eta	F: CTATTTACTGTAGGAGCTAG R: ATTTATTTGATGCTCTCTAT	741	94°C / 1 min. - 45°C / 1 min. - 72°C / 1 min.	Yamaguchi et al., 2002
etb	F: ATACACACATTACGGATAAT R: CAAAGTGTCCAAAAGTAT	629	94°C / 1 min. - 45°C / 1 min. - 72°C / 1 min.	Yamaguchi et al., 2002
etd	F: AACTATCATGTATCAAGG R: CAGAATTTCCCGACTCAG	376	94°C / 1 min. - 45°C / 1 min. - 72°C / 1 min.	Yamaguchi et al., 2002
Panton-Valentine leukocidin toxin				
luk-PV	F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAASTGTATTGGATAGCAAAAGC	433	94°C / 1 min. - 57°C / 1 min. - 72°C / 1 min.	Lina et al., 1999

prepared using a commercial mastermix (2X PCR Mastermix, ABT®, Ankara, Turkey). The mix was consisted of 5 µl of genomic DNA, 1.5 µl of each of primers (10 µM) and 12.5 µl of mastermix. The total volume of PCR mix was completed up to 25 µl with PCR water. Table 1 shown primer sequences and PCR process. DNA-free PCR water was used as a negative control in the assay. The amplicons were electrophoresed at 80 V for 1.5 h in agarose gel. Amplicons were compared with DNA markers and analysed in the gel imaging device (Gen-Box ImagER, Ankara, Türkiye).

RESULTS

Staphylococcus spp. was isolated in 35 (12.15%) swab samples. *S. arlettoe* was identified in 1 (2.85%) of the isolates, *S. cohnii* was identified in 1 (2.85%) isolate, *S. lentus* was identified in 1 (2.85%) isolate, *S. sciuri* was identified in 1 (2.85%) isolate, *S. aureus* was identified in 2 (5.71%) isolates, *S. haemolyticus* was identified in 2 (5.71%) isolates, *S. simulans* was identified in 2 (5.71%) isolates, *S. xylosus* was identified in 2 (5.71%) isolates, *S. chromogenes* was identified in 4 (11.42%) isolates, *S. vitulinus* was identified in 7 (20.00%) isolates, and *S. equorum* was identified in 12 (34.28%) isolates. All *Staphylococcus* spp. isolates were susceptible to enrofloxacin, gentamicin, and tobramycin. While 2.85% of the isolates were resistant to tetracycline and ceftiofur, 11.42% and 2.85% of the isolates were found to be moderately susceptible to tetracycline and chloramphenicol, respectively. Moreover, 24 (68.57%) of *Staphylococcus* spp. isolates phenotypically formed biofilm. The *aap* gene was identified in 6 (25.0%) of the strains that were able to form biofilms, *AtlE* gene in 9 (37.5%), and *icaA* gene in 2 (8.33%). The *icaD* and *icaAB* genes responsible for biofilm formation were not identified in the isolates. One of each of the strains resistant to tetracycline and ceftiofur formed biofilm. Table 2 shows the distribution of the presence of genes responsible for biofilm formation in strains found to phenotypically form biofilm. No *eta*, *etb*, *etd*, or *luk-PV* genes responsible for toxin production were identified in the strains analysed in the study.

Table 2: Distribution of the gene profile associated with biofilm formation in strains determined to form biofilms phenotypically (n=24).

Gene profile	Number of the isolates	%
aap+AtlE+icaA+icaB+icaAB	11	45.83
aap	4	16.66
aap+AtlE	2	8.33
AtlE	5*	20.83
AtlE+icaA	2**	8.33

*: 1 isolate resistant to ceftiofur; **: 1 isolate resistant to tetracycline

DISCUSSION AND CONCLUSION

Staphylococci are agents that are available in the natural flora of mucous membranes; therefore, *Mycoplasma* spp., *Moraxella* spp., and *Chlamydia* spp. strains are considered to play a role in the aetiology of keratoconjunctivitis cases in sheep and goats (Fernandez-Aguilar et al. 2017; Gulaydin et al. 2024). However, the transfer of antibiotic resistance developed, especially in staphylococcal strains, to other bacterial agents in the flora jeopardises both animal and human health and brings challenges in the treatment of the disease (Udegbunam et al. 2014). Therefore, it is critical to continuously monitor antimicrobial resistance in bacteria using up-to-date data. Various studies have analysed the presence of *Staphylococcus* spp. isolates in ocular swab samples collected from small ruminants. The study conducted by Hammadi (2015) in Iraq reported that they isolated *Staphylococcus* spp. in 30.5% of 200 ocular swab samples collected from sheep. In another study conducted in the

same country, *S. aureus* was isolated in 6.8% of ocular swab samples collected from goats and *S. epidermidis* and *S. saprophyticus* were isolated in 4.5%. On the other hand, it was reported that the isolation rates of the agents were 25.9%, 11.1%, and 3.7%, respectively, in samples collected from sheep (Rhaymah et al. 2013). A study conducted in Nigeria reported that *S. aureus* was identified in 49.2% of ocular swab samples collected from ruminants (Udegbunam et al. 2014). In a study conducted in the United States of America, *Staphylococcus* spp. strains were identified in most of the samples collected from goats (Meekins et al. 2017). The related studies have revealed that the isolation rate of *Staphylococcus* spp. varies in ocular swab samples collected from small ruminants. In this study, *Staphylococcus* spp. was isolated in 12.15% of the samples collected from clinically diagnosed keratoconjunctivitis cases in small ruminants raised in the Siirt region, and most of the isolates were identified as coagulase-negative species. Although the isolation rate was generally similar to other studies, it was lower than the data obtained by Udegbunam et al. (2014) and Meekins et al. (2017). Regional differences, breed, age, and disease conditions of the animals examined in the study, the sampling process, and the applied laboratory test methods were considered to cause differences in the isolation rates achieved in the studies. It has been reported that the use of antimicrobial agents such as oxytetracycline, chloramphenicol, ceftiofur, and gatifloxacin as eye drops locally or systemically in the treatment of keratoconjunctivitis cases in small ruminants yielded successful outcomes in the treatment of the disease (Biswas and Saifuddin 2017; Athira et al. 2018). Although chloramphenicol resistance in *Staphylococcus* spp. strains isolated from ocular swab samples of sheep and goat were reported to be low by Hammadi (2015) and Rhayman et al. (2013), Udegbunam et al. (2014) reported that 71% of the isolates were resistant to chloramphenicol. On the other hand, no resistance to chloramphenicol was found in the isolates obtained in this study. Likewise, the study revealed that all of the isolates were susceptible to gentamicin. However, this result was different from the data reported by Hammadi (2015) and Rhayman et al. (2013). While only one of the *Staphylococcus* spp. isolates isolated from the ocular swab samples was resistant to tetracycline, this result is compatible with the data obtained by Rhayman et al. (2013). On the other hand, another study reported that the rate of resistance to tetracycline group antimicrobial agents was quite high in *Staphylococcus* spp. isolates (Udegbunam et al. 2014). It was thought that the differences in antibiotic use habits in livestock breeding in different geographical regions may have caused differences between the results obtained in the studies. It was also concluded that the nomadic breeding in the Siirt region posed a problem with access to antibiotics, and consequently, antibiotic resistance rates in bacterial agents may have been limited. The biofilm-forming ability has been one of the important virulence factors in *Staphylococcus* spp. isolates. The biofilm formation has been known to play a role in the evasion of the agent from the host defence system and in the development of resistance to antimicrobials (Andrade et al. 2021). The biofilm-forming ability in staphylococcal strains isolated from sheep and goats has been mostly investigated in isolates obtained from milk samples. No studies have been found to investigate the biofilm formation and the presence of genes related to biofilm formation in *Staphylococcus* spp. isolates obtained from ocular samples. In their study, Andrade et al. (2021) reported that 75% of 137 *Staphylococcus* spp. isolates formed biofilm. They reported that the *icaA* gene was identified in 15.90% of 44 isolates and the *icaD* gene was identified in 43.18% (Andrade et al. 2021). Lira et al. (2016) determined that 28% (n=17) of the strains isolated from milk samples formed biofilm, while 82% of these isolates tested positive for the *icaD* gene. Lianou et al. (2023) showed that the majority (71.8%) of *Staphylococcus* spp. strains that they isolated from tank milk in sheep and goat herds were able to form biofilm. The present study revealed biofilm formation in 68.57% of the isolates obtained, as in other studies. Unlike the strains isolated from milk samples, the *icaD* gene was not identified in the isolates that this study examined. However, biofilm formation was not significantly correlated with antibiotic resistance in this study. This result was compatible with the data obtained by Lianou et al. (2021). Conclusively, this study revealed that it is necessary to consider especially coagulase-negative *Staphylococcus* spp. isolates in the cases of keratoconjunctivitis leading to well-being problems in small ruminants. It was demonstrated that antimicrobial agents such as enrofloxacin, gentamicin, and tobramycin can be used for the effective treatment of cases induced by the causative agents. While biofilm formation was found in most of the isolates, it was observed that this was ineffective in the formation of an antimicrobial resistance profile. It was considered that the gathered data would contribute to the studies on the subject.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: ÖG
Supervision / Consultancy: ÖG
Data Collection and / or Processing: AG
Analysis and / or Interpretation: MY
Writing the Article: ÖG
Critical Review: ÖG

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