

Antimicrobial resistance patterns of *Streptococcus uberis* isolates recovered from bovine mastitis

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Abstract: *Streptococcus uberis*, is a major cause of bovine mastitis in the dairy industry, is commonly treated with antimicrobials. The objective of this study was to investigate the antimicrobial resistance (AMR) patterns of *S. uberis* isolates recovered from bovine mastitis cases. Totally 120 milk samples were collected in 2025 from cows with mastitis on a farm. *S. uberis* isolates were identified using standard bacteriological analyses and confirmed by sequence analysis of the 16S *rRNA* gene. Six different antimicrobials (gentamicin, streptomycin, penicillin, vancomycin, erythromycin, and tetracycline) were used for AMR test by the Kirby-Bauer disc diffusion method. Eighteen *S. uberis* isolates (15.0%, 18/120) were recovered, none of which were susceptible to all the tested antimicrobials. All isolates (100.0%, 18/18) were resistant to aminoglycosides, gentamicin, and streptomycin. In contrast, all isolates (100.0%, 18/18) were susceptible to penicillin and vancomycin. This finding supports the use of beta-lactams as first-line antimicrobials to treat *S. uberis* infections. Additionally, 27.8% (5/18) and 61.1% (11/18) of the isolates were resistant to erythromycin and tetracycline, respectively. The high resistance rate to tetracycline indicates that it is not an effective treatment option for *S. uberis* infections. Four different AMR patterns were determined. Isolates with the "gentamicin, streptomycin, erythromycin, tetracycline" (CN-S-E-TE) pattern were defined as multidrug-resistant (MDR) (16.7%, 3/18). The identification of MDR isolates emphasizes the necessity of conducting AMR testing prior to initiating antimicrobial treatment for bovine mastitis. In conclusion, these findings align with existing literature and demonstrate the significance of regular surveillance to guide evidence-based and sustainable approaches in bovine mastitis management.

Keywords: Bovine mastitis, Monitoring antimicrobial resistance, Penicillin, *Streptococcus uberis*, Tetracycline

Sığır mastitinden izole edilen *Streptococcus uberis* suşlarının antimikrobiyal direnç profilleri

Özet: Süt sığırı yetiştiriciliğinde sığır mastitisinin başlıca etkenlerinden biri olan *Streptococcus uberis*, genellikle antimikrobiyal ajanlarla tedavi edilmektedir. Bu çalışmanın amacı, mastitisli sığırlardan izole edilen *S. uberis* suşlarının antimikrobiyal direnç (AMR) profillerini araştırmaktır. Bu amaçla, 2025 yılında bir çiftlikteki mastitisli ineklerden toplam 120 adet süt numunesi toplandı. *S. uberis* izolatları, standart bakteriyolojik yöntemler ile tanımlandı ve 16S *rRNA* geni dizi analizi ile doğrulandı. Kirby-Bauer disk difüzyon yöntemi ile gerçekleştirilen AMR testi için altı farklı antimikrobiyal ajan (gentamisin, streptomisin, penisilin, vankomisin, eritromisin ve tetrasiklin) kullanıldı. Toplam 18 adet *S. uberis* izolatı (%15,0, 18/120) elde edildi ve test edilen antimikrobiyallerin tamamına duyarlı izolat bulunmadı. Tüm izolatlar (%100,0, 18/18), aminoglikozitler olan gentamisin ve streptomisine dirençli bulundu. Buna karşılık, tüm izolatlar (%100,0, 18/18) penisilin ve vankomisine duyarlı bulundu. Bu bulgu, *S. uberis* enfeksiyonlarının tedavisinde beta-laktamların birinci basamak antimikrobiyaller olarak kullanılmasını desteklemektedir. Ayrıca izolatların %27,8'i (5/18) eritromisine ve %61,1'i (11/18) tetrasikline dirençli bulundu. Tetrasikline karşı saptanan yüksek direnç oranı, bu antimikrobiyalın *S. uberis* enfeksiyonları tedavisinde etkili bir seçenek olmadığını göstermektedir. Dört farklı AMR profili saptandı. "Gentamisin, streptomisin, eritromisin, tetrasiklin" (CN-S-E-TE) direnç profiline sahip izolatlar çoklu ilaç dirençli (MDR) izolatlar (%16,7, 3/18) olarak tanımlandı. MDR izolatlarının varlığı, sığır mastitisinin tedavisine başlanmadan önce AMR testlerinin yapılmasının gerekliliğini vurgulamaktadır. Sonuç olarak, bu bulgular mevcut literatür ile uyumludur ve sığır mastitisinin yönetiminde kanıta dayalı ve sürdürülebilir yaklaşımları yönlendirmek amacıyla düzenli izleme çalışmalarının önemini ortaya koymaktadır.

Anahtar kelimeler: Antimikrobiyal direnç izleme, Penisilin, Sığır mastiti, *Streptococcus uberis*, Tetrasiklin

Introduction

Bovine mastitis is a frequently observed infection in the global dairy industry, generally caused by

inadequate hygiene practices and environmental contamination (Kaczorek et al. 2017). Its incidence can be reduced through various measures, such as

improved hygiene, balanced diet, and reduction of stress factors. When these measures are insufficient, therapeutic treatment becomes necessary, in which case it is crucial to identify the causative bacterial isolate and determine its antimicrobial resistance (AMR) profile (Denamiel et al. 2005). However, this is not always possible due to limitations in cost and time. The resulting misuse or overuse of antimicrobials can increase antimicrobial resistant bacteria (Kaczorek et al. 2017). This situation is also observed in dairy farms (Saini et al. 2012). Beyond animal health, it is critical for public health to monitor the AMR profiles of pathogens originating from food-producing animals, given the potential of pathogenic bacteria to transmit to the food chain (Durso and Cook 2014). Therefore, several AMR monitoring programs have been applied across Europe to monitor pathogens originating from food animals, while the European Commission Action Plan also prioritizes AMR monitoring systems in veterinary medicine (EC 2011; Boireau et al. 2018; de Jong et al. 2018).

Streptococcus uberis (*S. uberis*) is one of the major causes of bovine mastitis, with high prevalences in affected populations (Cameron et al. 2016; Abd El-Aziz et al. 2021). It is commonly observed as causative agent of both subclinical and clinical mastitis in dairy cows (Sorge et al. 2021). As an environmental pathogen, *S. uberis* can be transmitted through various sources, including bedding materials, feces, flies, pasture, water, and soil (Zhang et al. 2021; Tsuyuki et al. 2024). It leads to substantial economic losses due to reduced milk production, cost of therapeutic treatments, and additional workload for managing affected animals (Rato et al. 2013). *S. uberis* is also a frequent cause of antimicrobial use in dairy cattle (Zouharova et al. 2023). Previous studies have emphasized the significance of surveilling these bacteria for AMR and using antimicrobials prudently to treat the infections they cause, given the emergence of resistant *S. uberis* isolates (Cameron et al. 2016; Thomas et al. 2024). The majority of previous studies conducted in our country have primarily focused on the investigation of *Staphylococcus* spp. in bovine mastitis cases. However, only a limited number of studies have addressed the isolation of *S. uberis*, as one of the major causative agents of bovine mastitis (Bal et al. 2010; Ocak et al. 2024).

This study conducted to investigate AMR patterns of *S. uberis* isolates recovered from bovine milk samples for guiding therapeutic treatment strategies.

Materials and Methods

Study samples

The present study analyzed 120 milk samples collected in 2025 from cows with mastitis on a dairy farm. The mastitis cases were confirmed through physical examination and the California Mastitis Test (CMT). Following aseptic procedures, 15 ml of milk sample was taken horizontally from each teat into a sterile tube (Krukowski et al. 2020). The samples were transported under cold chain conditions and stored at -20°C until bacteriological analyses.

Isolation and identification of *S. uberis*

The milk samples were analyzed using standard bacteriological analyses. Initially, the samples were inoculated onto 5% sheep blood agar. Following their incubation at 37°C for 24-48 h, bacterial colonies were evaluated based on their morphological characteristics and each colony was individually transferred to new plate to obtain pure culture. The presence of *S. uberis* isolates was investigated using Gram staining, catalase test, oxidase test, oxidation-fermentation test, and Christie-Atkins-Munch-Petersen (CAMP) test. Edwards agar (Oxoid, UK) media was used to determine the cultural properties of the isolates (Markey et al. 2013).

Molecular confirmation of *S. uberis*

Confirmation of *S. uberis* isolates was performed by sequence analysis of the 16S *rRNA* gene (Zhang et al. 2021). For this purpose, DNA samples were extracted using the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, USA). The DNA samples were then used as templates in polymerase chain reaction (PCR) analyses targeting the 16S *rRNA* gene, with universal 27F and 1492R primers used for amplification (Zhang et al. 2020). Amplification was conducted as described by Zhang et al. (2020). *S. uberis* ATCC 700407 isolate was used as positive control. After 1.5% agarose gel electrophoresis (Thermo Scientific, USA), the products were examined using a UV transilluminator. Positive amplicons were purified by Exosap-IT (Thermo Fisher Scientific, USA). Sequence analysis was performed using the BigDye Direct Cycle Sequencing Kit (Applied Biosystems, USA). For purification of sequencing amplicons, the Sephadex G-50 (Oxoid, UK) gel filtration method was used. ABI 3500 Genetic Analyzer System (Applied Biosystems, USA) was used for Sanger sequencing analysis. All sequences were analyzed using CLC Main Workbench v. 8 software (Qiagen, USA). The

obtained sequences were compared with reference sequences in the GenBank database and species-level identification was performed using the BLASTn tool with threshold of $\geq 99.0\%$ sequence similarity.

Determination of antimicrobial resistance patterns

All isolates were tested for AMR by the Kirby-Bauer disc diffusion method (Bauer et al. 1959). The bacterial suspensions were prepared based on the 0.5 McFarland turbidity standard. All suspensions were spread onto Mueller–Hinton agar supplemented with 5% sheep blood, with sterile cotton swabs to ensure uniform inoculation. The antimicrobial agents were selected according to two primary criteria, approval for the treatment of bovine mastitis or use in human medicine (Rato et al. 2013). Six antimicrobials from five antimicrobial classes were used: aminoglycosides (gentamicin CN: 10 µg and streptomycin S: 10 µg), beta-lactams (penicillin P: 10 µg), glycopeptides (vancomycin VA: 30 µg), macrolides (erythromycin E: 15 µg), and tetracyclines (tetracycline TE: 30 µg). The findings were evaluated according to the Clinical and Laboratory Standards Institute (CLSI 2008) guidelines. All different AMR patterns were recorded. Multidrug-resistant (MDR) isolates were defined as described by Magiorakos et al. (2012).

Results

A total of 18 (15.0%, 18/120) *S. uberis* isolates were identified by both standard bacteriological analyses and sequence analysis of the 16S *rRNA* gene. No isolate was found susceptible to all tested antimicrobials. Moreover, none was resistant to all antimicrobials. All *S. uberis* isolates (100.0%, 18/18) exhibited resistance to gentamicin and streptomycin from aminoglycosides. In contrast, all isolates (100.0%, 18/18) were susceptible to both penicillin and vancomycin. Additionally, 27.8% (5/18) of the isolates were found to be resistant to erythromycin, while 61.1% (11/18) were resistant to tetracycline. Four different AMR patterns were observed, as CN-S-E-TE (16.7%, 3/18), CN-S-E (11.1%, 2/18), CN-S-TE (44.4%, 8/18), and CN-S (27.8%, 5/18) (Figure 1). Isolates with the CN-S-E-TE pattern were defined as MDR (16.7%, 3/18).

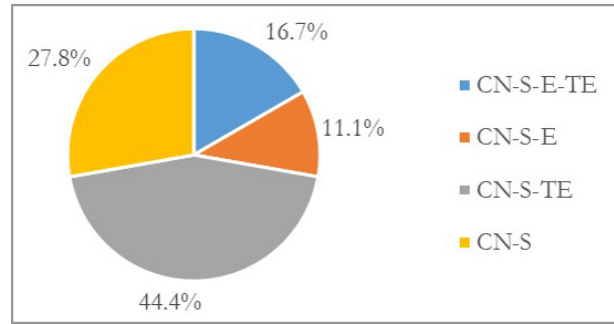


Figure 1. Distribution of *S. uberis* isolates based on AMR patterns.

Discussion and Conclusion

The annual cost of bovine mastitis is estimated to be approximately €20 billion worldwide (Hassan et al. 2023). Streptococci are among the major causes of bovine mastitis (Shome et al. 2012). The present study aimed to determine AMR patterns of *S. uberis* isolates obtained from bovine milk samples to help guide therapeutic treatment strategies. The bacteriological analyses indicated that 15.0% of the milk samples were positive for *S. uberis*. Previous studies have indicated that *S. uberis* is a common species among mastitis-causing agents. For example, Krukowski et al. (2020) found that 14.8% of bovine milk samples with mastitis in Poland were positive for *S. uberis*. Hsieh et al. (2019) and Hassan et al. (2023) reported that *S. uberis* was the dominant species among Streptococci isolated from bovine mastitis in Taiwan and Bangladesh, respectively. Similar findings have also been reported from Turkey. For example, Turutoglu et al. (2002) isolated *S. uberis* in 6.43% of bacteria isolated from bovine mastitic milk samples. The authors found that *S. uberis* was the dominant species among *Streptococcus* isolates. Similarly, Bal et al. (2010) identified *S. uberis* as the dominant species among Streptococci recovered from bovine mastitis cases. Recently, Ocak et al. (2024) also reported that *S. uberis* (18.3%) was the dominant species among Streptococci isolates obtained from bovine milks with mastitis. Additionally, all isolates were confirmed by sequence analysis of the 16S *rRNA* gene in this study. This finding is line with the finding of Shome et al. (2012) that sequence analysis of the 16S *rRNA* gene is a useful tool to identify *S. uberis*. Similarly, Hassan et al. (2023) identified *Streptococcus* species in bovine mastitis cases by sequence analysis of the 16S *rRNA* gene.

In this study, all *S. uberis* isolates (100.0%) were resistant to gentamicin and streptomycin. Similarly,

Rato et al. (2013) reported that 80.0% and 100.0% of *S. uberis* isolates obtained from bovine mastitis were resistant to gentamicin and streptomycin, respectively. Abd El-Aziz et al. (2021) reported a high level of resistance to streptomycin (86.96%), while a low level of resistance to gentamicin (20.28%). Recently from Turkey, Guner et al. (2024) found that 20.0% of *S. uberis* isolates from bovine mastitis were resistant to gentamicin. The authors clearly found that *S. uberis* had a higher rate of resistance gentamicin compared to all other bacterial species recovered from bovine mastitis.

Beta-lactam antimicrobials are frequently used for treating bovine mastitis caused by *S. uberis* (Zouharova et al. 2023). They are considered the first-line antimicrobials for *Streptococcus* infections (Cameron et al. 2016). In this study, all isolates (100.0%) were susceptible to penicillin, which confirms similar findings from various countries investigating the AMR of *S. uberis* isolated from bovine mastitis. For example, Rato et al. (2013) found that all *S. uberis* isolates sampled in Portugal were susceptible to penicillin, while De Jong et al. (2018) found no penicillin-resistant isolates in their cross-European monitoring study. Zhang et al. (2021) and Thomas et al. (2024) reported that all *S. uberis* isolates sampled in Thailand and Germany, respectively, were susceptible to penicillin. The finding of the present study supports Sorge et al. (2021), who argue that penicillin should be used as a first-line antimicrobial for treating *S. uberis* infections in the bovine industry.

As a result of AMR test, all *S. uberis* isolates (100.0%) were found to be susceptible to vancomycin, consistent with the findings of Rato et al. (2013), Hassan et al. (2023), and Thomas et al. (2024), who all reported 100% susceptibility to vancomycin in *S. uberis* isolates from Portugal, Bangladesh, and Germany, respectively. In contrast, the isolates in this study showed a higher resistance rate to erythromycin (27.8%) compared to several previous reports. For instance, Zhang et al. (2021) and Zouharova et al. (2023) reported erythromycin resistance rates of 8.33% and 4.0% in *S. uberis* isolates from bovine mastitis in the Czech Republic and Thailand, respectively. However, several other studies have reported higher resistance rates to erythromycin, such as 20.0%, 20.2%, 22.0%, and 26.75%, in France, Europe, France, and Portugal respectively (Schmitt-Van de Leemput et al. 2007; Rato et al. 2013; Boireau et al. 2018; de Jong et al. 2018). Additionally, Ocak et al. (2024) reported from Turkey that 38.1% of *S. uberis* isolates recovered from bovine mastitis were

resistant to erythromycin. Consistent with these studies, the high resistance to erythromycin detected in this study can be associated with its frequent use for treatment of streptococcal mastitis.

The present study revealed that the highest resistance rate (61.1%) was to tetracycline. Tetracyclines are widely used for treating cattle infections (Abd El-Aziz et al. 2021). However, they degrade slowly in both the organism and the environment, so their use leads to increased resistance in pathogens, as a result of extended exposure (Zouharova et al. 2023). Previous studies have also found high resistance to tetracycline in *S. uberis* isolates originating from bovine mastitis. For example, Rato et al. (2013) reported that 60.0% of *S. uberis* isolates were resistant to tetracycline, while Cameron et al. (2016) and De Jong et al. (2018) reported resistance rates of 44.0% and 36.7%, respectively. Abd El-Aziz et al. (2021) reported that 65.22% of *S. uberis* isolates were resistant to tetracycline, while Zouharova et al. (2023) and Thomas et al. (2024) detected resistance rates of 59.0% and 42.6%, respectively. Additionally, Boireau et al. (2018) conducted AMR surveillance in major pathogens isolated from bovine mastitis in France. They specifically identified an increase in tetracycline resistance of *S. uberis* isolates between 2006 and 2016. Similarly, Zhang et al. (2021) reported a marked increase in tetracycline resistance between 2010 and 2017 to a notably high resistance rate of 82.02% in Thailand. From Turkey, Ocak et al. (2024) also detected a high rate of resistance to tetracycline (64.3%) for *S. uberis* isolates recovered from bovine mastitis.

The AMR test identified four different AMR patterns: CN-S-E-TE, CN-S-E, CN-S-TE, and CN-S. Only the CN-S-E-TE pattern represented MDR *S. uberis* isolates, accounting for 16.7% of the total. The prevalence of MDR *S. uberis* from bovine mastitis reported in previous studies varies widely. For example, Diana et al. (2024) did not identify any MDR *S. uberis* isolates in Uruguay, while Magagula et al. (2023) reported a rate of 6.4% in South Africa. Tsuyuki et al. (2024) reported a 13.7% MDR prevalence of *S. uberis* from bovine milk samples in Japan, while Boireau et al. (2018) reported 14.5% in France. Zouharova et al. (2023) identified a 19.5% MDR *S. uberis* prevalence in Czech Republic, whereas Monistero et al. (2021) detected 25.4% in Italy. Ocak et al. (2024) reported from Turkey that 28.6% of *S. uberis* isolates recovered from bovine mastitis were MDR, while Kang et al. (2022) strikingly reported from Korea that 73.2% of *S. uberis* isolates were MDR.

In conclusion, this study provides crucial new insights into AMR patterns of *S. uberis* isolates from bovine mastitis in Turkey, thereby offering guidance for developing effective therapeutic strategies. The fact that all isolates were susceptible to penicillin supports the use of beta-lactam antimicrobials as first-line antimicrobials for treating *S. uberis* infections. In contrast, given the high resistance rate detected in this study, tetracycline is unlikely to be effective for treating *S. uberis* infections. Finally, the high resistance rate to erythromycin is considered to result from its frequent use in animals across the world. The identification of MDR isolates demonstrates that it is essential to conduct AMR testing prior to initiating antimicrobial treatment for bovine mastitis. Overall, these findings align with the existing literature and confirm the importance of regular surveillance to inform evidence-based and sustainable approaches in bovine mastitis management.

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Author contribution: SSI carried out study design, investigation, methodology, writing, and reviewing. Author read and approved the finalized form of the manuscript.

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