Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
32 (3): 429-437, 2026
DOI: 10.9775/kvfd.2026.36569

Research Article

Molecular Characterization, Virulence Determinants, Antimicrobial Resistance, and Genotyping of *Streptococcus uberis* Isolated from Mastitic Dairy Cows in the Aegean Region of Türkiye

Çağatay NUHAY [1(*)], Deha Ali DENİZ [1], Serap SAVAŞAN [2]

[1] İzmir Bornova Veterinary Control Institute, Department of Bacteriology, TR-35040 İzmir - TÜRKİYE
[2] Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Veterinary Microbiology Department, TR-09100 Aydın - TÜRKİYE

(*) **Corresponding author:**
Çağatay Nuhay
Phone: +90 232 388 0010
Cellular phone: +90 544 933 6467
Fax: +90 232 388 5052
E-mail: cnuhay@gmail.com

How to cite this article?

Nuhay Ç, Deniz DA, Savaşan S: Molecular Characterization, Virulence Determinants, Antimicrobial Resistance, and Genotyping of *Streptococcus uberis* Isolated from Mastitic Dairy Cows in the Aegean Region of Türkiye. *Kafkas Univ Vet Fak Derg*, 32 (3): 429-437, 2026
DOI: 10.9775/kvfd.2026.36569

Article ID: KVFD-2026-36569
Received: 11.03.2026
Accepted: 15.06.2026
Published Online: 19.06.2026

Abstract

Bovine mastitis remains one of the most prevalent and economically damaging diseases of dairy herds, and *Streptococcus uberis* is a leading environmental pathogen implicated in both clinical and subclinical infections. In this study, we characterized *S. uberis* isolates from mastitis-suspected dairy cows in the Aegean Region of Türkiye using phenotypic and molecular identification, virulence and antimicrobial resistance (AMR) gene profiling, antimicrobial susceptibility testing, and ERIC-PCR genotyping. Overall, 167 milk samples were cultured and *S. uberis* was isolated from 11 samples (6.6%). Identification was performed with VITEK® 2 and confirmed by 16S rRNA PCR (1.400bp). Virulence genes were widely distributed, including capsule genes (*hasB* 91%, *hasC* 82%, *hasA* 73%) and adhesion/invasion-related loci (*gapC* and *oppF* 82% each, *skc* 91%, *sua* 64%, *cfu* 73%), whereas *lbp* showed lower prevalence (27%). AMR gene screening revealed high carriage of penicillin-binding protein genes (*pbp1A*, *pbp2A*, *pbp2B* 100%; *pbp1B* 90.9%), *blaTEM* (72.7%), *blaZ* (9.1%), *ermA/ermB* (100%), *tetM* (54.5%), and *gyrA/parC* (27.3% each). Phenotypically, resistance was frequent, notably to tetracycline (100%) and penicillin G (63.6%), with heterogeneous patterns to other agents, indicating multidrug resistance. ERIC-PCR demonstrated marked genetic heterogeneity, identifying two small clusters (2 and 3 isolates) and six unique profiles at a 70% similarity cut-off, consistent with multiple environmental sources rather than a single dominant clone. These findings underscore the need for improved environmental hygiene and susceptibility-guided therapy, and support further molecular surveillance of circulating strains and resistance mechanisms.

Keywords: Antimicrobial resistance, Bovine mastitis, ERIC-PCR, *Streptococcus uberis*, Virulence genes

Introduction

Bovine mastitis remains one of the most common and economically detrimental diseases affecting dairy herds worldwide. It reduces milk yield, alters milk composition, increases culling rates, and results in substantial therapeutic and veterinary expenses. Annual global losses attributable to mastitis are estimated to reach billions of dollars, driven largely by reduced productivity, discarded milk, treatment costs, and premature culling [1-4]. Subclinical mastitis is particularly challenging because it often remains unnoticed, leading to chronic inflammation and sustained milk losses over time.

Mastitis-associated pathogens are broadly classified as contagious or environmental. Contagious agents primarily spread during milking; however, improved milking hygiene practices and dry-cow therapy have decreased their relative importance in many herds [5,6]. In parallel, environmental pathogens have become increasingly predominant, emphasizing the role of housing conditions, bedding, manure contamination, and overall farm management in mastitis epidemiology.

Among environmental pathogens, *Streptococcus uberis* is recognized as a major etiological agent of bovine mastitis [1]. Its prevalence varies substantially across regions and production systems, ranging from approximately 15-40% in Europe and North America and reaching higher levels in pasture-based systems [5,7,8]. In Türkiye, *S. uberis* has been increasingly reported in dairy herds, and humid, pasture-influenced areas such as the Aegean Region may provide favorable conditions for persistence and transmission [9]. The pathogenic success of *S. uberis* is linked to a diverse set of virulence factors, including capsule-associated determinants that support immune evasion, adhesion-related factors that facilitate colonization and persistence

This article is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0)

within the mammary gland, and additional components involved in tissue invasion and cytolytic activity [9,10].

Antimicrobial therapy remains a cornerstone of mastitis control; nevertheless, treatment failure is increasingly reported due to escalating antimicrobial resistance. In *S. uberis*, β-lactam resistance has been associated with alterations in penicillin-binding proteins (e.g., *pbp1A, pbp2A, pbp2B*) and, less frequently, β-lactamase-related mechanisms (e.g., *blaTEM, blaZ*) [11]. Macrolide resistance is largely mediated by *ermA* and *ermB*, whereas tetracycline resistance is frequently linked to tetM. Emerging fluoroquinolone resistance has also been associated with mutations in key target genes such as gyrA and parC [12-15]. Genotyping studies frequently demonstrate high genetic diversity among *S. uberis* isolates, supporting the concept that many infections arise from environmental reservoirs rather than direct cow-to-cow [6]. Therefore, the present study aimed to characterize *S. uberis* isolates obtained from mastitis-suspected (CMT-positive) dairy cows in the Aegean Region of Türkiye through phenotypic and molecular identification, virulence gene profiling, antimicrobial susceptibility testing, detection of resistance determinants, and assessment of genetic diversity using ERIC-PCR.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Local Ethics Committee of the Bornova Veterinary Control Institute (Date: 01.04.2025, Decision No: 2025/2).

Sample Size Justification and Bacterial Identification

Assuming an expected prevalence of approximately 10% for *S. uberis* in mastitic milk, the sample size was calculated under a binomial framework to estimate prevalence with acceptable precision [16]. A total of 167 CMT-positive quarter milk samples were collected from mastitis-suspected dairy cows located in eight provinces of the Aegean Region of Türkiye, namely İzmir, Aydın, Manisa, Muğla, Denizli, Uşak, Kütahya, and Afyonkarahisar.

A descriptive, cross-sectional sampling strategy was used. Seven dairy farms were included from each province, resulting in a total of 56 farms. Three CMT-positive quarter milk samples were collected from each farm, except for one farm in Afyonkarahisar, from which two samples were obtained. Thus, the final sample size consisted of 167 milk samples. Quarter selection was based on the California Mastitis Test (CMT), and samples were collected from quarters with a CMT score ≥1+ [17,18].

Samples were submitted to the Bornova Veterinary Control Institute. Each sample was cultured on 5% ovine blood agar (Oxoid®, UK) and incubated aerobically at 37°C for 24-48 h [19,20]. After incubation, colonies with streptococcal morphology were selected and examined by Gram staining; isolates showing Gram-positive cocci in chains and a negative catalase reaction with 3% H_2O_2 were considered consistent with streptococci [5,20]. Isolates were identified using the VITEK® 2 Compact system (bioMérieux, France) with GP identification cards according to the manufacturer's instructions [9,19]. Genomic DNA was extracted from purified *S. uberis* isolates using the High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. The extracted DNA samples were stored at −20°C until further PCR analysis. Molecular confirmation was performed by PCR targeting the 16S rRNA gene using published primers (Forward, 5′-GAGAGTTTGATCCTGGCTCAGGA-3′; Reverse, 5′-CGGGTGTTACAAACTCTCGTGGT-3′); the expected 1,400bp amplicon was interpreted as positive [20].

Detection of Virulence Genes

Virulence-associated genes were screened in all *S. uberis* isolates by PCR using genomic DNA extracted as described above. The following loci were targeted: *hasA, hasB, hasC, gapC, oppF, skc, sua, cfu* and *lbp,* which are associated with adhesion, immune evasion, and persistence in the bovine mammary gland [9,20]. Published primer sets and expected amplicon sizes were used [20] *(Table 1)*.

Table 1. *Primers used for amplification of virulence-associated genes in S. uberis isolates*

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Amplicon (bp)	Annealing Temperature (°C)	Ref.
cfu	TATCCCGATTTGCAGCCTAC	CCTGGTCAACTTGTGCAACTG	205	59	[20]
hasA	GAAAGGTCTGATGCTGAT	TCATCCCCTATGCTTACAG	600	58	[20]
hasB	TCTAGACGCCGATCAAGC	TGAATTCCYATGCGTCGATC	300	58	[20]
hasC	TGCTTGGTGACGATTTGATG	GTCCAATGATAGCAAGGTACAC	300	58	[20]
gapC	GCTCCTGGTGGAGATGATGT	GTCACCAGTGTAAGCGTGGA	200	55	[20]
lbp	CGACCCTTCAGATTGGATTC	TAGCAGCATCACGTTCTTCG	698	53	[20]
oppF	GGCCTAACCAAAACGAAACA	GGCTCTGGAATTGCTGAAAG	419	54	[20]
skc	CTCCTCTCCAACAAAGAGG	GAAGGCCTTCCCCTTTGAAA	800	52	[20]
sua	ACGCAAGGTGCTCAAGAGTT	TGAACAAGCGATTCGTCAAG	776	58	[20]

PCR was performed in 25 µL reaction volumes containing Xpert Fast Hotstart Mastermix (2×, GRiSP®, Portugal), gene-specific primers, nuclease-free water, and template DNA. All PCR assays were performed as separate single-target reactions for each gene; no multiplex PCR protocol was used in this study. Cycling conditions consisted of an initial denaturation at 95°C for 3min, followed by 35-40 cycles of 95°C for 30 s, gene-specific annealing for 30 s, and extension at 72°C for 30-60 s, with a final extension at 72°C for 5min. Amplicons were resolved by electrophoresis on 1.5% agarose gels and visualized under UV illumination. The presence of each gene was recorded based on the expected product size *(Table 1)*.

Detection of Antimicrobial Resistance Genes

Antimicrobial resistance (AMR) genes were screened in all *S. uberis* isolates by PCR using genomic DNA prepared as described above. Eleven resistance-associated loci representing major antimicrobial classes were targeted: penicillin-binding protein genes *(pbp1A, pbp1B, pbp2A, pbp2B)*, β-lactamase genes *(blaTEM, blaZ)*, macrolide resistance genes *(ermA, ermB)*, the tetracycline resistance gene *(tetM)*, and quinolone resistance-associated genes *(gyrA, parC)* [13,21-23].

However, for *pbp*, *gyrA*, and *parC*, PCR positivity was interpreted only as the presence of the corresponding target locus/amplicon. Since sequence analysis was not performed, resistance-conferring mutations or allelic variations in these loci could not be demonstrated.

PCR was performed in 25 µL reaction volumes containing Xpert Fast Hotstart Mastermix (2×, GRiSP®, Portugal), gene-specific primer pairs, nuclease-free water, and template DNA. All PCR assays were performed as separate single-target reactions for each gene; no multiplex PCR protocol was used in this study. Cycling conditions followed published protocols: initial denaturation at 95°C for 3 min; 35-40 cycles of 95°C for 30 s, gene-specific annealing for 30s, and 72°C extension for 30-60 s; and a final extension at 72°C for 5 min [18,21,24-26]. Amplicons were resolved on 1.5% agarose gels and visualized under UV illumination. The presence of each gene was recorded according to the expected product size. Primer sequences and amplicon sizes are presented in *Table 2*.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of all *S. uberis* isolates was determined by the disk diffusion method on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [29]. Bacterial suspensions were prepared in sterile saline and adjusted to 0.5 McFarland, and plates were inoculated by swabbing the entire agar surface. Commercial antimicrobial disks (Oxoid®, UK) were applied, and plates were incubated aerobically at 37°C for 18-24 h.

The antimicrobial disks tested were penicillin G (10 units), ampicillin (10 µg), erythromycin (15 µg), tetracycline (30 µg), oxytetracycline (30 µg), cefquinome (30 µg), trimethoprim–sulfamethoxazole (1.25/23.75 µg), and chloramphenicol (30 µg). After incubation, inhibition zone diameters were measured in millimetres. Isolates were categorized as susceptible, intermediate, or resistant according to CLSI M100, 34th edition, 2024, where applicable. For antimicrobial agents without directly applicable CLSI interpretive criteria for *S. uberis*, interpretation was performed using published criteria from previous studies on mastitis-associated streptococci, as clearly indicated in *Table 4* [26]. Streptococcus pneumoniae ATCC 49619 was used for quality control according to CLSI recommendations [29]. Phenotypic resistance results were compared descriptively with AMR-associated locus profiles.

***Table 2.** Primers used for amplification of antimicrobial resistance genes in S. uberis isolates*

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Amplicon (bp)	Annealing Temperature (°C)	Ref.
pbp1A	GGCGACATCTGGATGAAAAT	GCCATTGTTCCAACATAATCA	718	52	[21]
pbp1B	CTTTGGCGGTTTGCTAGATG	GGATGGCGTTGGCTAGATTA	890	52	[21]
pbp2A	AGGGCTTGTTGGTCGTGTTA	CGGTCTTGTTAAAACCGATCC	734	52	[21]
pbp2B	CTATGTCGGGCTTGTCTCGT	TGGCAACAGCTACTTCAGGA	879	52	[21]
blaZ	TAAGAGATTTGCCTATGCTT	TTAAAGTCTTACCGAAAGCAG	377	52	[23]
blaTEM	ATGAGTATTCAACATTTTCGTG	TTACCAATGCTTAATCAGTGAG	860	58	[27]
ermA	TCAGGAAAAGGACATTTTACC	ATACTTTTTGTAGTCCTTCTT	432	58	[28]
ermB	TTTTTGAAAGCCATGCGTCT	CTGTGGTATGGCGGGTAAGT	201	60	[13]
tetM	GTGGACAAAGGTACAACGAG	CGGTAAAGTTCGTCACACAC	406	55	[25]
gyrA	CGATGTCGGTCATTGTTG	ACTTCCGTCAGGTTGTGC	496	50	[22]
parC	TGGGTTGAAGCCGGTTCA	CAAGACCGTTGGTTCTTTC	361	58	[24]

Table 3. *Provincial and farm-level distribution of milk samples and S. uberis isolates*

Province	No. of Farms Sampled	No. of Milk Samples	No. of *S. uberis*-Positive Farms	No. of *S. uberis*-Positive İsolates	Sample-Level Positivity (%)	Farm-Level Positivity (%)
İzmir	7	21	3	3	14.3	42.9
Aydın	7	21	2	2	9.5	28.6
Manisa	7	21	2	2	9.5	28.6
Muğla	7	21	1	1	4.8	14.3
Denizli	7	21	1	1	4.8	14.3
Uşak	7	21	1	1	4.8	14.3
Kütahya	7	21	0	0	0.0	0.0
Afyonkarahisar	7	20	1	1	5.0	14.3
Total	**56**	**167**	**11**	**11**	**6.6**	**19.6**

Genotyping by ERIC-PCR

Genetic relatedness among the isolates was assessed by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using the ERIC-2 primer (5′-AAGTAAGTGACTGGGGTGAGCG-3′), and amplification was performed according to a previously described method [30]. Briefly, a 25 µL ERIC-PCR reaction mixture was prepared containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5U Taq DNA polymerase, 25 pmol primer, and 5 µL template DNA. Thermal cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 3 min, with a final extension at 72°C for 7 min [30]. Amplification products were separated on 1.5% agarose gels containing ethidium bromide (2 µg/mL) and visualized under UV illumination. ERIC-PCR banding patterns were analyzed to construct dendrograms using the unweighted pair-group method with arithmetic averages (UPGMA), and genetic relatedness was interpreted using a 70% similarity cut-off.

Results

Isolation and identification of Streptococcus uberis

A total of 167 CMT-positive quarter milk samples collected from mastitis-suspected dairy cows in eight provinces of the Aegean Region of Türkiye were examined. *S. uberis* was isolated from 11 samples, corresponding to an overall sample-level positivity rate of 6.6% and a farm-level positivity rate of 19.6% (11/56).

Among the 11 *S. uberis*-positive samples, three isolates were obtained from three different farms in İzmir, two isolates from two farms in Aydın, and two isolates from two farms in Manisa. In addition, one isolate each was recovered from a single farm in Muğla, Denizli, Uşak, and Afyonkarahisar. No *S. uberis* isolate was recovered from Kütahya. *S. uberis* was isolated from 11 samples (6.6%) *(Table 3)*.

On 5% ovine blood agar, isolates produced 1-3 mm, opaque S-type colonies. Gram staining revealed Gram-positive cocci in chains, and all isolates were catalase-negative. Species identification was performed using the

Table 4. *Antimicrobial susceptibility profiles of S. uberis isolates (n = 11)*

Antimicrobial Agent	Disk Content	Interpretive Reference	Resistant (n)	Susceptible (n)	Resistance (%)	Detected Related AMR Genes/loci
Penicillin G	10 units	[29]	7	4	63.6	*pbp1A, pbp1B, pbp2A, pbp2B, blaTEM, blaZ*
Ampicillin	10 µg	[29]	1	10	9.1	*pbp1A, pbp1B, pbp2A, pbp2B, blaTEM, blaZ*
Erythromycin	15 µg	[26,29]	5	6	45.5	*ermA, ermB*
Tetracycline	30 µg	[26,29]	11	0	100.0	*tetM*
Oxytetracycline	30 µg	[26]	10	1	90.9	*tetM*
Cefquinome	30 µg	[26]	11	0	100.0	*pbp1A, pbp1B, pbp2A, pbp2B, blaTEM, blaZ*
TMP-SMX	1.25/23.75 µg	[26,29]	11	0	100.0	Not investigated
Chloramphenicol	30 µg	[26,29]	11	0	100.0	Not investigated

VITEK® 2 GP system and molecular confirmation was conducted by PCR targeting the 16S rRNA gene, yielding the expected 1.400bp amplicon in all 11 isolates *(Fig. 1)*.

The overall sample-level positivity rate was 6.6% (11/167; 95% Wilson CI: 3.7-11.4%). Provincial positivity rates ranged from 0.0% to 14.3%, with the highest rate observed in İzmir (3/21; 14.3%; 95% CI: 5.0-34.6%), followed by Aydın and Manisa (2/21 each; 9.5%; 95% CI: 2.7-28.9%). Lower rates were detected in Muğla, Denizli, Uşak, and Afyonkarahisar, whereas no *S. uberis* isolate was recovered from Kütahya. Although numerical differences were observed among provinces, the difference in sample-level positivity was not statistically significant by chi-square test (χ^2 = 4.52, df = 7, P=0.719). Because of the small number of positive isolates and low expected cell counts, the result was also checked using the Fisher-Freeman-Halton exact test with Monte Carlo simulation, which similarly showed no significant provincial difference (P=0.778).

At the farm level, *S. uberis* was detected in 11 of 56 sampled dairy farms, corresponding to a farm-level positivity rate of 19.6% (95% Wilson CI: 11.3-31.8%). Farm-level positivity varied from 0.0% in Kütahya to 42.9% in İzmir. However, this variation was not statistically significant among provinces either by chi-square test (χ^2 = 5.32, df = 7, P=0.621) or by Fisher-Freeman-Halton exact test with Monte Carlo simulation (P=0.783). Therefore, although İzmir showed the highest numerical positivity, the present data do not support a statistically significant provincial clustering of *S. uberis* positivity.

Distribution of Virulence Genes

All *S. uberis* isolates were screened for virulence-associated genes. Capsular synthesis–related genes were frequently detected *(hasB* 91%, *hasC* 82%, *hasA* 73%). Adhesion-associated genes were also common *(gapC* 82%, *oppF* 82%, *sua* 64%). The *skc* gene was present in 91% of isolates, whereas *cfu* was detected in 73%. The *lbp* gene showed the lowest prevalence (27%). Overall, most isolates carried multiple virulence determinants *(Fig. 2)*.

Detection of Antimicrobial Resistance Genes

All *S. uberis* isolates were screened for 11 AMR-associated genes. Penicillin-binding protein genes were frequently detected: *pbp1A, pbp2A,* and *pbp2B* were present in all isolates (100%, 11/11), whereas *pbp1B* was detected in 90.9% (10/11). Among β-lactamase genes, *blaTEM* was identified in 72.7% (8/11), while *blaZ* was detected in 9.1% (1/11). Macrolide resistance genes *(ermA* and *ermB)* were detected in all isolates (100%, 11/11), and the tetracycline resistance gene *tetM* was present in 54.5% (6/11). Quinolone resistance-associated genes *gyrA* and *parC* were each detected in 27.3% (3/11). Overall, most isolates carried multiple AMR determinants *(Fig. 3)*.

Antimicrobial Susceptibility Results

Antimicrobial susceptibility testing demonstrated heterogeneous resistance patterns among the *S. uberis* isolates *(Table 4)*. Resistance to penicillin G was observed

Fig 1. Agarose gel electrophoresis image of PCR amplification of the 16S rRNA gene, showing the expected 1,400bp amplicon. Lanes 1-5: test samples/isolates, P: positive control, N: negative control, M: DNA marker

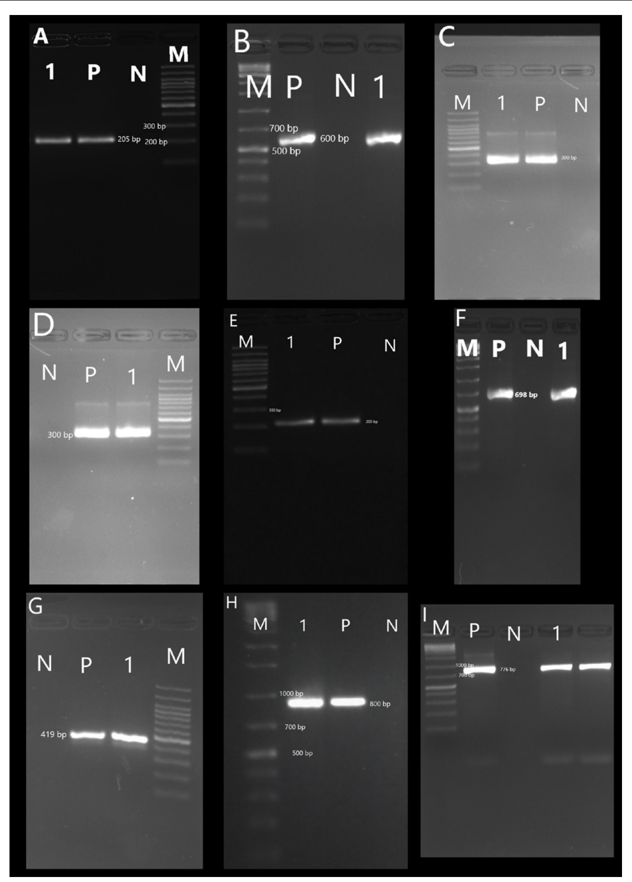

Fig 2. Agarose gel electrophoresis images of PCR amplicons for the virulence genes investigated in this study. **A**: *cfu* (205bp), **B**: *hasA* (600bp), **C**: *hasB* (300bp), **D**: *hasC* (300bp), **E**: *gapC* (200bp), **F**: *lbp* (698bp), **G**: *oppF* (419bp), **H**: *skc* (800bp), **I**: *sua* (776bp). P: positive control, N: negative control, 1: test sample/isolate, M: DNA marker

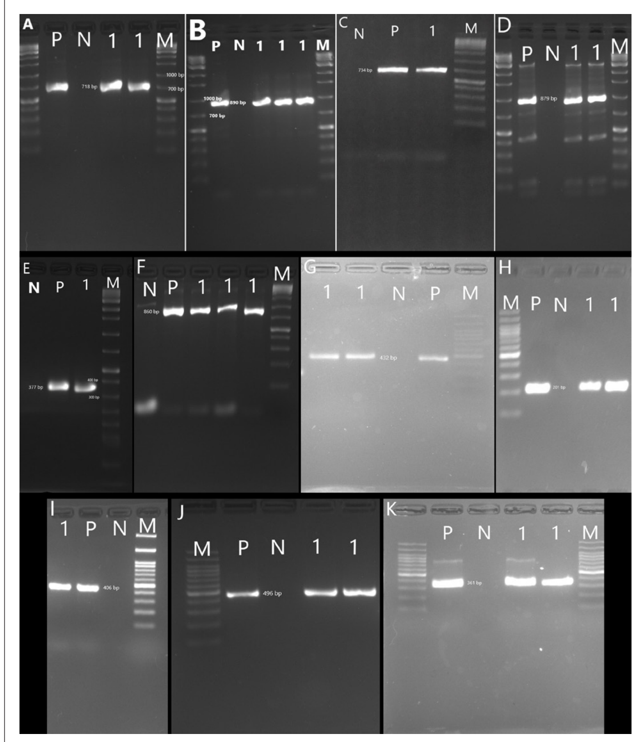

Fig 3. Agarose gel electrophoresis images of PCR amplicons targeting β-lactam resistance, macrolide resistance, tetracycline resistance, and quinolone resistance-associated genes. Panels: **A**: *pbp1A*, *718bp*; **B**: *pbp1B*, *890bp*; **C**: *pbp2A*, *734bp*; **D**: *pbp2B*, *879bp*, **E**: *blaZ*, *377bp*; **F**: *blaTEM*, *860bp*; **G**: *ermA*, *432bp*; **H**: *ermB*, 201bp; **I**: *tetM*, *406bp*; **J**: *gyrA*, *496bp*; **K**: *parC*, *361bp*. P: positive control; N: negative control; 1: test isolate/sample; M: DNA size marker

in 63.6% (7/11) of isolates, whereas most isolates remained susceptible to ampicillin (90.9%, 10/11). All isolates were resistant to tetracycline, cefquinome, trimethoprim-sulfamethoxazole, and chloramphenicol (each 100%, 11/11), and resistance to oxytetracycline was also high (90.9%, 10/11). Erythromycin resistance was detected in 45.5% (5/11). Collectively, the susceptibility profiles indicate a high prevalence of multidrug resistance in the study population *(Table 4)*. Molecular results indicate PCR detection of related AMR genes/loci in the isolate collection and do not necessarily indicate phenotypic resistance in each isolate.

ERIC-PCR Genotyping Results

ERIC-PCR fingerprinting generated clear and reproducible banding patterns *(Fig. 4)*. At a 70% similarity cut-off, two clusters were identified: one comprising two isolates (RG1 and RG2) and another comprising three isolates (RH1-RH3). The remaining six isolates (RA-RF) exhibited unique profiles and did not cluster with other isolates at this threshold. Overall, ERIC-PCR indicated marked genetic heterogeneity among the isolates, consistent with the presence of multiple genotypes rather than a single dominant clone *(Fig. 4)*.

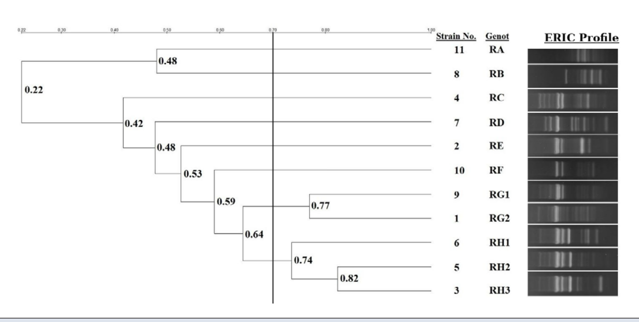

Fig 4. ERIC-PCR banding patterns and corresponding dendrogram of *S. uberis* isolates

Discussion

This study determined a *S. uberis* isolation rate of 6.6% from 167 milk samples collected from mastitis-suspected (CMT-positive) dairy cows in the Aegean Region of Türkiye. This rate is close to the lower bound of the 10-25% range reported in some previous studies from Türkiye and may reflect differences in herd type, sampling strategy, seasonal/environmental conditions, and management practices [9]. A previous study from Aydın Province, located within the Aegean Region, reported the detection of virulence genes in *S. uberis* isolates from bovine mastitis cases [9]. The presence of this earlier regional report supports the relevance of *S. uberis* as a mastitis-associated pathogen in the Aegean Region. However, the present study differs by including isolates obtained from multiple provinces of the region and by evaluating virulence-associated genes together with antimicrobial susceptibility, resistance-associated loci, and ERIC-PCR genotyping. Therefore, our findings expand the available regional data beyond a single-province observation. Globally, *S. uberis* prevalence has been reported to vary widely, ranging approximately from 15% to 40% in Europe and North America, and reaching higher levels in pasture-based systems [5,6,8]. Therefore, the relatively low isolation rate observed in the present study does not diminish the regional relevance of this pathogen; rather, its virulence potential and treatment challenges highlight that it remains economically and clinically important in mastitis management.

The high detection frequencies of virulence-associated genes in our isolates suggest that *S. uberis* circulating in the region may possess substantial genetic capacity for mammary gland colonization and persistence. In particular, the capsule synthesis-related genes *hasA* (73%), *hasB* (91%), and *hasC* (82%) were widely distributed. This distribution is in line with previous studies from Türkiye and other regions, which have shown a high prevalence of capsule-associated genes and support the importance of the capsule as a conserved virulence determinant involved in immune evasion and persistence [9,20,30-32].

The detection of adhesion-associated genes *oppF* (82%), *gapC* (82%), and *sua* (64%) indicates a strong potential for early adherence to the mammary epithelium and persistence during intramammary infection. These findings are consistent with previous reports demonstrating the presence of adhesion-related genes in *S. uberis* isolates and support their possible role in colonization and persistence during infection [9]. In addition, the detection of *sua* and *gapC* may indicate a potential association with a prolonged clinical course and a tendency toward chronic mastitis [33]. Taken together, the adhesion gene patterns observed here suggest that, despite its predominantly environmental origin, *S. uberis* may establish persistent infections in mammary tissue.

Virulence determinants associated with tissue invasion and cellular damage were also frequently detected, including *skc* (91%) and *cfu* (73%). This profile suggests a notable virulence potential among the isolates. The high prevalence of *cfu* in the present study is in agreement with previous findings reporting this gene at high frequency among *S. uberis* isolates, further supporting its potential role in the pathogenesis of bovine mastitis [9,20]. In contrast, the relatively low prevalence of *lbp* (27%) may reflect strain-to-strain variability in iron acquisition mechanisms and possible regional differences in adaptation. Overall, the co-occurrence of multiple virulence genes in most isolates indicates a multifactorial pathogenic profile and further supports the continuing importance of *S. uberis* as a mastitis pathogen [9,10,19].

Phenotypic antimicrobial susceptibility results indicated a high resistance burden, particularly against tetracyclines and several other agents. These findings highlight the limitations of empirical therapy in mastitis cases and underscore the need for treatment strategies guided by isolation, identification, and susceptibility testing. For β-lactams, resistance to penicillin (63.6%) together with largely preserved susceptibility to ampicillin suggests that resistance may not be explained solely by classical β-lactamase presence, but could involve target alterations and/or differences in breakpoint interpretation. In this context, the presence of PBP-related genes alone may be insufficient to predict phenotype; rather, allelic variation/mutations and expression or regulatory differences are likely to be more decisive [21,32-35]. Local data from Türkiye reporting frequent detection of *pbp* genes, yet incomplete concordance with phenotypic resistance, are compatible with this interpretation [36,37].

For macrolides, erythromycin resistance was 45.5%, whereas all isolates carried *ermA/ermB,* indicating that gene presence and phenotypic resistance may not fully coincide. This discrepancy may be related to inducible expression of *erm* genes, regulatory variation, or contributions of non-erm mechanisms such as efflux systems [13,14,22,32,38,39]. Similarly, the observation of 100% phenotypic tetracycline resistance despite *tetM* positivity of 54.5% suggests that additional genes (e.g., *tetO/tetL)* or efflux mechanisms may contribute. Detection of fluoroquinolone target gene alterations *(gyrA* and *parC)* at 27.3% may reflect a limited but present selection pressure in the region; given their potential contribution to treatment failure, these markers warrant continued monitoring [14,22]. Therefore, the molecular findings for *pbp, gyrA*, and *parC* should be interpreted with caution, as sequencing was not performed to confirm resistance-associated mutations.

Finally, ERIC-PCR genotyping revealed marked genetic heterogeneity among the isolates. As shown in *Fig. 4*, the presence of two small clusters at the 70% similarity threshold (RG1-RG2 and RH1-RH3) together with multiple unique profiles indicates that cases were associated with circulation of multiple genotypes rather than a single dominant clone. This finding is consistent with the prevailing concept that *S. uberis* mastitis frequently arises from environmental sources via multiple introductions and that substantial molecular diversity may occur within and between herds [22,40].

This study has some limitations. First, although the study provides regional data on *S. uberis* isolates from mastitic dairy cows in the Aegean Region of Türkiye, it was designed as a descriptive, cross-sectional study and should not be interpreted as a randomized prevalence survey representing all dairy herds in the region. Second, the methods used, including bacterial isolation, PCR-based gene screening, antimicrobial susceptibility testing, and ERIC-PCR, are conventional and do not provide the resolution of sequencing-based approaches. In particular, sequence analysis of *pbp, gyrA*, and *parC* loci was not performed; therefore, resistance-associated mutations or allelic variations could not be confirmed. Further studies using larger isolate collections and whole-genome sequencing are needed to clarify the epidemiology, virulence potential, and resistance mechanisms of *S. uberis* in Türkiye.

In conclusion, this study demonstrated that *S. uberis* isolates recovered from bovine mastitic dairy cows in the Aegean Region of Türkiye harbor a substantial repertoire of virulence-associated genes, exhibit pronounced antimicrobial resistance patterns, and display marked genetic heterogeneity. Collectively, these findings support the role of *S. uberis* as an important pathogen in environmentally acquired intramammary infections and suggest a considerable potential for persistence within the mammary gland. The high resistance observed to several commonly used antimicrobials underscores the need to curb indiscriminate antibiotic use and highlights the importance of susceptibility-guided therapy. Although

the study does not provide methodological novelty, it contributes regional baseline data by evaluating phenotypic characteristics, virulence-associated genes, antimicrobial susceptibility profiles, resistance-associated loci, and ERIC-PCR genotypes of *S. uberis* isolates from mastitic dairy cows in the Aegean Region of Türkiye. Accordingly, improving environmental hygiene, implementing appropriate herd management practices, and selecting antimicrobials based on susceptibility testing should be prioritized for effective mastitis control. Further molecular and genomic investigations are warranted to better elucidate the epidemiology of circulating strains and the underlying resistance mechanisms.

Declarations

Availability of Data and Materials: The data and materials that support the findings of this study are available from the corresponding author, CN, upon reasonable request.

Acknowledgements: None.

Funding Support: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing Interests: The authors declare that they have no competing interests.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: CN designed the research study. Laboratory work and the remaining parts of the study were performed by CN, DAD and SS. The manuscript was written by CN. All authors reviewed and approved the final version of the manuscript.

References

1. **Kebede N, Tilahun A:** A review on dairy cow mastitis and its economic impact. *Int J Adv Res Biol Sci,* 10 (2): 109-125, 2023.
2. **Morales-Ubaldo AL, Rivero-Perez N, Valladares-Carranza B, Velázquez-Ordoñez V, Delgadillo-Ruiz L, Zaragoza-Bastida A:** Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches. *Vet Anim Sci,* 21:100306, 2023. DOI: 10.1016/j.vas.2023.100306
3. **Diana L, Mastroianni L, Diana V, Puentes R:** *Streptococcus* spp. isolated from bovine mastitis: Antimicrobial sensitivity studies and disagreement evaluation between routine phenotypic diagnosis and molecular identification. *Rev Argent Microbiol,* 56 (4): 351-358, 2024. DOI: 10.1016/j.ram.2024.07.006
4. **Celebi D, Celebi O, Baser S, Ari B, Ektas Kalayci S, Kara A:** Efficacy of pyocyanin isolated from *Pseudomonas aeruginosa* and *Lactobacillus plantarum* against methicillin resistant *Staphylococcus aureus* caused bovine mastitis. *Kafkas Univ Vet Fak Derg,* 31 (2): 237-245, 2025. DOI: 10.9775/kvfd.2024.33429
5. **Bradley AJ, Leach KA, Breen JE, Green LE, Green MJ:** Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet Rec,* 160 (8): 253-257, 2007. DOI: 10.1136/vr.160.8.253
6. **Zadoks RN, Gillespie BE, Barkema HW, Sampimon OC, Oliver SP, Schukken YH:** Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. *Epidemiol Infect,* 130 (2): 335-349, 2003. DOI: 10.1017/S0950268802008221
7. **Avanus K, Yılmaz A, Güneş H, Altınel A, Ekiz B, Yalçıntan H, Keçici D, Doğan N:** CXCR1 gene SNP variability that affects mastitis resistance in Holstein cows in Türkiye. *Kafkas Univ Vet Fak Derg,* 31 (1):119-124, 2025. DOI: 10.9775/kvfd.2024.32948
8. **Rüegsegger F, Ruf J, Tschuor A, Sigrist Y, Rosskopf M, Hässig M:** Antimicrobial susceptibility of mastitis pathogens of dairy cows in Switzerland. *Schweiz Arch Tierheilkd,* 156 (10): 483-488, 2014. DOI: 10.1024/0036-7281/a000635
9. **Parın U, Kırkan Ş, Çiçek E, Yüksel HT:** Detection of virulence genes in *Streptococcus uberis* isolated from bovine mastitis in Aydın Province by multiplex polymerase chain reaction. *Fırat Üniv Sağ Bil Vet Derg,* 31 (3): 213-219, 2017.
10. **Shivaee A, Kazemi F, Navidifar T, Golab N, Parvizi E, Mofid M, Sholeh M, Beig M:** Global trends in macrolide and lincosamide resistance in *Streptococcus* species: A comprehensive systematic review and meta-analysis. *BMC Infect Dis,* 25 (1):1577, 2025. DOI: 10.1186/s12879-025-11884-5
11. **McDougall S:** Efficacy of two antibiotic treatments in curing clinical and subclinical mastitis in lactating dairy cows. *N Z Vet J,* 46 (6): 226-232, 1998. DOI: 10.1080/00480169.1998.36094
12. **Grundmann H, Hori S, Tanner G:** Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. *J Clin Microbiol,* 39 (11): 4190-4192, 2001. DOI: 10.1128/JCM.39.11.4190-4192.2001
13. **Pyatov V, Vrtková I, Knoll A:** Detection of selected antibiotic resistance genes using multiplex PCR assay in mastitis pathogens in the Czech Republic. *Acta Vet Brno,* 86 (2): 167-174, 2017. DOI: 10.2754/avb201786020167
14. **Zhang T, Niu G, Boonyayatra S, Pichpol D:** Antimicrobial resistance profiles and genes in *Streptococcus uberis* associated with bovine mastitis in Thailand. *Front Vet Sci,* 8:705338, 2021. DOI: 10.3389/fvets.2021.705338
15. **Miotti C, Cicotello J, Suarez Archilla G, Neder V, Alvarado Lucero W, Calvinho L, Signorini M, Camussone C, Zbrun MV, Molineri AI:** Antimicrobial resistance of *Streptococcus uberis* isolated from bovine mastitis: Systematic review and meta-analysis. *Res Vet Sci,* 164:105032, 2023. DOI: 10.1016/j.rvsc.2023.105032
16. **Lwanga SK, Lemeshow S:** Sample size determination in health studies. Geneva: World Health Organization, 1991.
17. **Schalm OW, Noorlander DO:** Experiments and observations leading to development of the California mastitis test. *J Am Vet Med Assoc,* 130 (5): 199-204, 1957.
18. **Kivaria FM, Noordhuizen JPTM, Nielen M:** Interpretation of California mastitis test scores using *Staphylococcus aureus* culture results for screening of subclinical mastitis in low yielding smallholder dairy cows in the Dar es Salaam region of Tanzania. *Prev Vet Med,* 78 (3-4): 274-285, 2007. DOI: 10.1016/j.prevetmed.2006.10.011
19. **Abd El-Aziz NK, Ammar AM, El Damaty HM, Abd Elkader RA, Saad HA, El-Kazzaz W, Khalifa E:** Environmental *Streptococcus uberis* associated with clinical mastitis in dairy cows: Virulence traits, antimicrobial and biocide resistance, and epidemiological typing. *Animals,* 11 (7):1849, 2021. DOI: 10.3390/ani11071849
20. **Reinoso EB, Lasagno MC, Dieser SA, Odierno LM:** Distribution of virulence-associated genes in *Streptococcus uberis* isolated from bovine mastitis. *FEMS Microbiol Lett,* 318 (2): 183-188, 2011. DOI: 10.1111/j.1574-6968.2011.02258.x
21. **Haenni M, Galofaro L, Ythier M, Giddey M, Majcherczyk P, Moreillon P, Madec JY:** Penicillin-binding protein gene alterations in *Streptococcus uberis* isolates presenting decreased susceptibility to penicillin. *Antimicrob Agents Chemother,* 54 (3): 1140-1145, 2010. DOI: 10.1128/AAC.00915-09
22. **Reinoso EB:** Bovine mastitis caused by *Streptococcus uberis*: Virulence factors and biofilm. *J Microb Biochem Technol,* 9 (5): 237-243, 2017. DOI: 10.4172/1948-5948.1000371
23. **Turner AM, Li L, Taiaroa G, Monk IR, Mansell P, Howden BP, Gorrie CL, Carter GP:** Global analysis of the genomic diversity, antimicrobial resistance and potential vaccine candidates carried by

the major global bovine pathogen *Streptococcus uberis*. *Microb Genom,* 11 (7):001441, 2025. DOI: 10.1099/mgen.0.001441

24. **de Toro M, Rojo-Bezares B, Vinué L, Undabeitia E, Torres C, Sáenz Y:** *In vivo* selection of *aac(6')-Ib-cr* and mutations in the *gyrA* gene in a clinical *qnrS1*-positive *Salmonella enterica* serovar Typhimurium DT104B strain recovered after fluoroquinolone treatment. *J Antimicrob Chemother,* 65 (9): 1945-1949, 2010. DOI: 10.1093/jac/dkq262

25. **Malhotra-Kumar S, Lammens C, Piessens J, Goossens H:** Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. *Antimicrob Agents Chemother,* 49 (11): 4798-4800, 2005. DOI: 10.1128/AAC.49.11.4798-4800.2005

26. **Schmitt-Van de Leemput E, Zadoks RN:** Genotypic and phenotypic detection of macrolide and lincosamide resistance in *Streptococcus uberis*. *J Dairy Sci,* 90 (11): 5089-5096, 2007. DOI: 10.3168/jds.2007-0101

27. **Shen J, Wu X, Yang Y, Lv Y, Li X, Ding X, Wang S, Yan Z, Yan Y, Yang F, Li H:** Antimicrobial resistance and virulence factor of *Streptococcus dysgalactiae* isolated from clinical bovine mastitis cases in Northwest China. *Infect Drug Resist,* 14, 3519-3530, 2021. DOI: 10.2147/IDR.S327924

28. **Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L:** Detection of erythromycin resistant determinants by PCR. *Antimicrob Agents Chemother,* 40 (11): 2562-2566, 1996. DOI: 10.1128/AAC.40.11.2562

29. **CLSI:** Performance Standards for Antimicrobial Susceptibility Testing. 34th ed., CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2024.

30. **Versalovic J, Koeuth T, Lupski R:** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res,* 19 (24): 6823-6831, 1991. DOI: 10.1093/nar/19.24.6823

31. **Hunter PR, Gaston MA:** Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *J Clin Microbiol,* 26 (11): 2465-2466, 1988. DOI: 10.1128/jcm.26.11.2465-2466.1988

32. **Zouharova M, Nedbalcova K, Matiaskova K, Slama P, Matiasovic J:** Antimicrobial susceptibility and resistance genes in *Streptococcus uberis* isolated from bovine mastitis in the Czech Republic. *Antibiotics,* 12 (10):1527, 2023. DOI: 10.3390/antibiotics12101527

33. **Boonyayatra S, Tharavichitkul P, Oliver S:** Virulence-associated genes and molecular typing of *Streptococcus uberis* associated with bovine mastitis in northern Thailand. *Turk J Vet Anim Sci,* 42 (1): 73-81, 2018. DOI: 10.3906/vet-1704-75

34. **Dowson CG, Coffey TJ, Kell C, Whiley RA:** Evolution of penicillin resistance in *Streptococcus pneumoniae*: The role of *Streptococcus mitis* in the formation of a low affinity PBP2B in *S. pneumoniae*. *Mol Microbiol,* 9 (3): 635-643, 1993. DOI: 10.1111/j.1365-2958.1993.tb01723.x

35. **Sá-Leão R, Vilhelmsson SE, de Lencastre H, Kristinsson KG, Tomasz A:** Diversity of penicillin-nonsusceptible *Streptococcus pneumoniae* circulating in Iceland after the introduction of penicillin-resistant clone Spain6B-2. *J Infect Dis,* 186 (7): 966-975, 2002. DOI: 10.1086/343769

36. **Sarıçam İnce S:** Antimicrobial resistance patterns of *Streptococcus uberis* isolates recovered from bovine mastitis. *Etlik Vet Mikrobiyol Derg,* 36 (2): 101-106, 2026. DOI: 10.35864/evmd.1776357

37. **Kaymaz Kanat HZ:** Mastit olgularından izole edilen *Streptococcus uberis* izolatlarının antibiyotik direnç profillerinin moleküler yöntemlerle incelenmesi. *Doktora Tezi,* Lisansüstü Eğitim Enstitüsü, İstanbul Üniversitesi-Cerrahpaşa, 2025.

38. **Pitkälä A, Koort J, Björkroth J:** Identification and antimicrobial resistance of *Streptococcus uberis* and *Streptococcus parauberis* isolated from bovine milk samples. *J Dairy Sci,* 91 (10): 4075-4081, 2008. DOI: 10.3168/jds.2008-1040

39. **Tian XY, Zheng N, Han RW, Ho H, Wang J, Wang YT, Yu ZN:** Antimicrobial resistance and virulence genes of *Streptococcus* isolated from dairy cows with mastitis in China. *Microb Pathog,* 131, 33-39, 2019. DOI: 10.1016/j.micpath.2019.03.035

40. **Tomazi T, Freu G, Alves BG, de Souza Filho AF, Heinemann MB, Veiga Dos Santos M:** Genotyping and antimicrobial resistance of *Streptococcus uberis* isolated from bovine clinical mastitis. *PLoS One,* 14 (10):e0223719, 2019. DOI: 10.1371/journal.pone.0223719