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ORIGINAL ARTICLE

Isolation, Antimicrobial Susceptibility Determination and Genotyping of *Sphingomonas paucimobilis* from Milk Samples of Cows with Mastitis

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ABSTRACT

Mastitis is one of the most economically significant diseases affecting dairy cattle worldwide, with its subclinical form causing greater production losses than the clinical form. *Sphingomonas paucimobilis*, a Gram-negative, yellow-pigmented, aerobic bacillus, has recently been recognized as an emerging environmental pathogen in cases of mastitis. This study aimed to isolate *S. paucimobilis* from milk samples of cows with mastitis in the İzmir and Aydın regions, determine its antimicrobial susceptibility, and perform genotyping. A total of 100 milk samples were collected from cows with mastitis (50 clinical and 50 subclinical cases). Conventional bacteriological methods, VITEK® Compact 2, and PCR were used for identification. Eight (8%) *S. paucimobilis* isolates were obtained, including six from subclinical mastitis samples and two from clinical mastitis samples. Antimicrobial susceptibility testing by the Kirby-Bauer disk diffusion method revealed high resistance rates to amoxicillin-clavulanic acid (87.5%), ampicillin-sulbactam (100%), and cefquinome (100%), while all isolates were susceptible to cefoperazone and gentamicin. Genotyping using the $(GTG)_5$-PCR method showed that all isolates had distinct patterns, with genetic similarity ranging from 32% to 98%. The findings indicate that *S. paucimobilis* should be considered among potential environmental mastitis pathogens and highlight the need for advanced diagnostic methods and effective antimicrobial stewardship to prevent resistance development.

Keywords: *Antibiotic Resistance, Genotype, Mastitis, Sphingomonas paucimobilis.*

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Introduction

Mastitis is considered the most important disease affecting dairy farms economically worldwide. Among cattle diseases, mastitis is also one of the most significant in terms of animal welfare economic impact. It negatively affects farmers' profits and causes substantial production losses in the global dairy industry (Aghamohammadi et al., 2018; Babacan, 2024; Bardhan, 2013; Das et al., 2018; Izquierdo et al., 2017; Sinha et al., 2014).

One reason the importance of mastitis increases is that its subclinical form is more prevalent than the clinical form. Research indicates that production losses associated with subclinical mastitis are about three times those from clinical cases. Accordingly, mastitis is considered responsible for nearly 60-70% of the total economic impact of infectious diseases in dairy cattle (De Vliegher et al., 2012; Nuhay, 2025; Sinha et al., 2014). Since farmers cannot immediately detect subclinical mastitis, the disease can persist in the animal for a long time, leading to irreversible udder damage. Bovine mastitis has been known for many years. Numerous studies have been conducted and continue to be carried out to eliminate the causative agents. However, due to the complex etiology of mastitis, a definitive solution has not yet been achieved. Therefore, in order to minimize the losses caused by mastitis, it is crucial to determine the distribution and risk factors of mastitis in the region where the animals are raised (Dego, 2020; Nuhay et al. 2025).

Many mastitis-causing agents have been identified, including bacterial, fungal, and viral pathogens. Physiologically and economically, bacterial mastitis is regarded as the most critical form (Blowey & Edmondson, 2010). More than 150 bacterial species have been implicated in the pathogenesis of mastitis (Kuang et al., 2009). Commonly isolated bacteria include *Escherichia coli*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus dysgalactiae* (Bray & Shearer, 1994). In addition to well-known pathogens, many new mastitis agents have been identified in recent years. One such agent is *S. paucimobilis* (Kenar et al., 2019).

The bacterium *S. paucimobilis* is an aerobic, Gram-negative rod, notable for its yellow pigmentation, inability to ferment carbohydrates, and the presence of a polar flagellum (Tada & Inoue, 2000). Its ability to adapt to optimal growth temperatures, wide distribution in the environment, and ability to attach to dust particles and remain suspended in the air for long periods enable it to colonize both animal and human tissues. Such features predispose the organism to a variety of infections, and respiratory involvement is particularly frequent (Hsueh et al., 1998; Koskinen et al., 2010; Tokajian et al., 2008). Furthermore, recent studies have confirmed the high pathogenic potential of this microorganism and revealed that it can cause serious diseases in different organs and systems, accompanied by various clinical manifestations (Maragakis et al., 2009).

In this study, we aimed to isolate *S. paucimobilis*, a newly identified mastitis pathogen, from mastitic milk, determine its antimicrobial susceptibility, and perform genotyping.

Material and Method

Bacteriological identification of *S. paucimobilis*

Since our study was conducted on materials submitted for clinical diagnostic purposes, it falls outside the scope of ethical approval under Article 8(k) of the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees.

To investigate the presence of *S. paucimobilis*, 100 milk samples were collected from cows with mastitis in the regions of İzmir and Aydın, including 50 clinical mastitis samples and 50 subclinical mastitis samples. Clinical mastitis cases were defined based on visible udder and/or milk abnormalities, whereas subclinical mastitis cases were identified using the California Mastitis Test (CMT). The milk samples were aseptically collected, placed in sterile tubes, and transported under cold-chain conditions to the Routine Diagnostic Laboratory. They were kept at +4 °C until subjected to bacteriological isolation. Upon reaching the laboratory, milk samples were streaked onto blood agar plates containing 5% sheep blood and incubated under aerobic conditions at 37 °C for 24-48 h. After incubation,

the resulting colonies were Gram-stained, and those exhibiting Gram-negative bacillary morphology were selected for further analysis.

S. paucimobilis is characterized by the production of yellow-green pigment on blood agar. Therefore, isolates producing yellow-green pigment on blood agar and testing positive for catalase and oxidase reactions were subcultured onto Tryptone Soya Agar (TSA) to obtain pure cultures (Cengiz et al., 2015).

For definitive identification, the VITEK® Compact 2 automated bacterial identification system and polymerase chain reaction (PCR) were used (Tokajian et al., 2008).

Molecular detection (PCR) of *S. paucimobilis*

For molecular identification, the boiling method was used for DNA extraction. Briefly, isolates were inoculated onto TSA and incubated at 37 °C for 24-48 hours. Colonies were suspended in deionized water in Eppendorf tubes, boiled at 100 °C for 25 minutes, and centrifuged at 12,000 rpm for 4 minutes to obtain DNA (Savaşan & Göksoy, 2018). The obtained target DNA samples were aliquoted (0.5 µl) under sterile conditions and stored at -20 °C.

Nucleic acid samples were aliquoted into 0.5 µl portions under aseptic conditions and stored at -20 °C until analysis. PCR was performed in a 25 µl reaction mixture containing primers (F: AAGTCGTAACAAGGTAACC, R: GGGTTBCCCCATTCRG), nucleotides, $MgCl_2$, buffer, and 2 µl of template DNA. The amplification profile included an initial denaturation, 40 cycles of denaturation, annealing at 48 °C, and extension, followed by a final elongation step, as previously described by Cengiz et al. (2014). PCR amplicons were resolved on 1.5% agarose gels containing ethidium bromide, and bands were visualized using UV illumination (Tokajian et al., 2008).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *S. paucimobilis* was determined using the standardized disc-diffusion method (Kirby-Bauer) on Mueller-Hinton agar in accordance with the CLSI (2006) guidelines. After overnight culture in Brain Heart Infusion broth, suspensions were adjusted to the 0.5 McFarland standard and used to prepare confluent lawns. The test panel comprised amoxicillin-clavulanate (20/10 µg), ampicillin-sulbactam (10/10 µg), cefoperazone (30 µg), cefquinome (30 µg), gentamicin (10 µg), enrofloxacin (5 µg), and enrofloxacin. Plates were incubated at 37 °C for 18-24 h, and zone diameters were interpreted using CLSI breakpoints.

Genotyping of isolates by (GTG)5-PCR

Genotyping was carried out by $(GTG)_5$-PCR using the primer sequence 5'-GTGGTGGTGGTGGTG-3', following the method of Huang et al. (2013). Amplification was performed in 25 µl reaction mixtures and run under a standard thermal profile including denaturation, annealing at 52 °C, and extension at 65 °C. PCR products were separated on agarose gels and recorded with an imaging system. Dendrograms of banding profiles were generated with the UPGMA clustering method using CHEF-DR® III and Quantity One® software (Bio-Rad, Hercules, CA), and genetic relatedness among isolates was evaluated with a 90% similarity threshold (Köstereli & Onuk, 2019; Yaman et al., 2025).

Results

A total of eight *S. paucimobilis* isolates (8%) were obtained from the examined milk samples, including six isolates from subclinical mastitis samples and two isolates from clinical mastitis samples. Species identification was confirmed by PCR, and all isolates generated specific bands within the 350-500 bp range. Representative agarose gel electrophoresis results are presented in Figure 1.

Antimicrobial susceptibility testing was carried out by the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar, and inhibition zones were interpreted in line with CLSI (2006) standards. The findings demonstrated considerable resistance: seven isolates (87.5%) showed resistance to amoxicillin-clavulanic acid, while complete resistance (100%) was observed against ampicillin-sulbactam and cefquinome. Only one isolate (12.5%) was susceptible to amoxicillin-clavulanic acid. In contrast, all isolates (100%) were susceptible to cefoperazone, cefquinome, and gentamicin (Table 1).

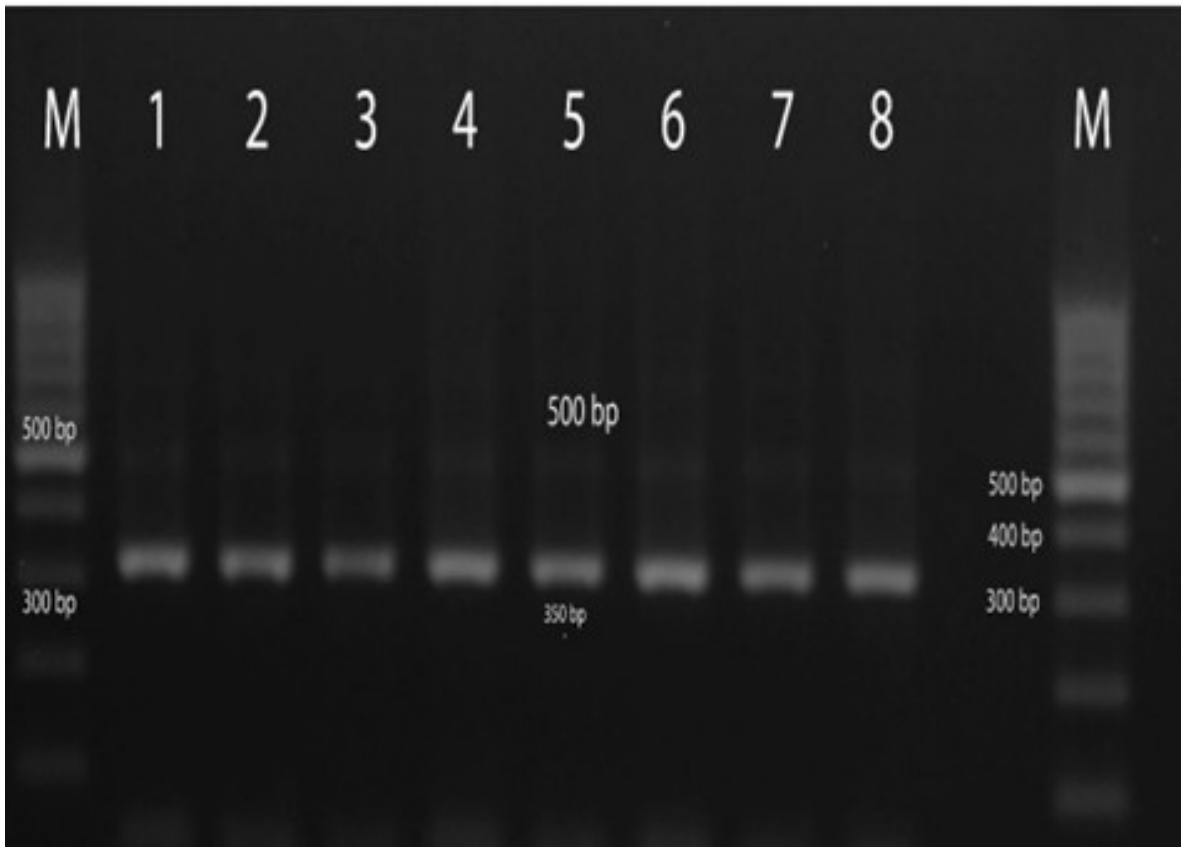


Figure 1. PCR profiles of the *S. paucimobilis* isolates examined. Lane M: molecular size marker (DNA ladder); lane 1: positive control; lanes 2-8: examined isolates (samples).

Table 1. Antibiogram results of the *S. paucimobilis* isolates.

***S. paucimobilis* isolates**	**Amoxicillin-Clavulanic acid (30 µg)**	**Ampicillin-Sulbactam (20 µg)**	**Cefoperazone (30 µg)**	**Cefquinome (30 µg)**	**Enrofloxacin (5 µg)**	**Gentamicin (10 µg)**
1	R	R	S	R	S	S
2	R	R	R	R	S	S
3	R	R	R	R	S	S
4	R	R	R	R	S	S
5	R	R	R	R	S	S
6	R	R	R	R	S	S
7	R	R	R	R	S	S
8	S	R	S	R	S	S

To assess the genetic diversity of the S. paucimobilis isolates, three different molecular typing methods were applied. RAPD-PCR (primers M13, P5, and P6) and ERIC-PCR (primer ERIC-2) failed to produce bands; therefore, genotyping could not be performed with these methods. In contrast, the $(GTG)_5$-PCR method generated distinct and reproducible banding patterns for all eight isolates, with each isolate representing a unique genotype (Figure 2). Phylogenetic analysis revealed genetic similarity coefficients ranging from 32% to 98% among the genotypes (Figure 3).

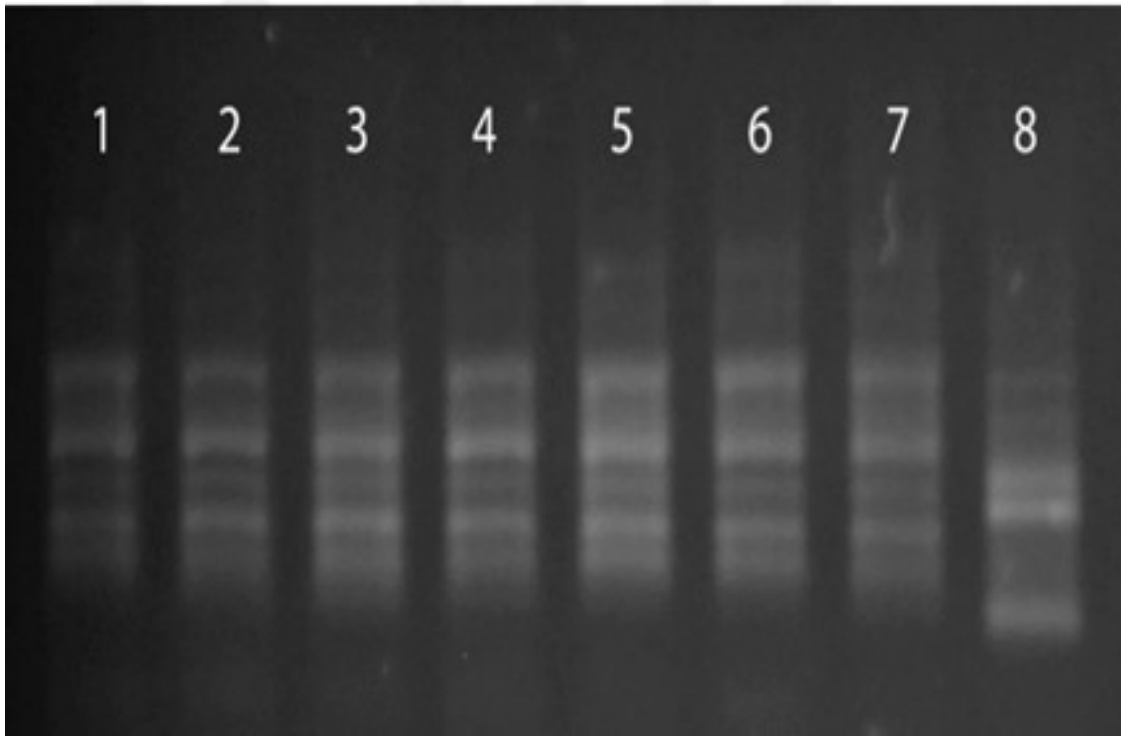


Figure 2. GTG5 PCR profiles of the *S. paucimobilis* isolates examined.

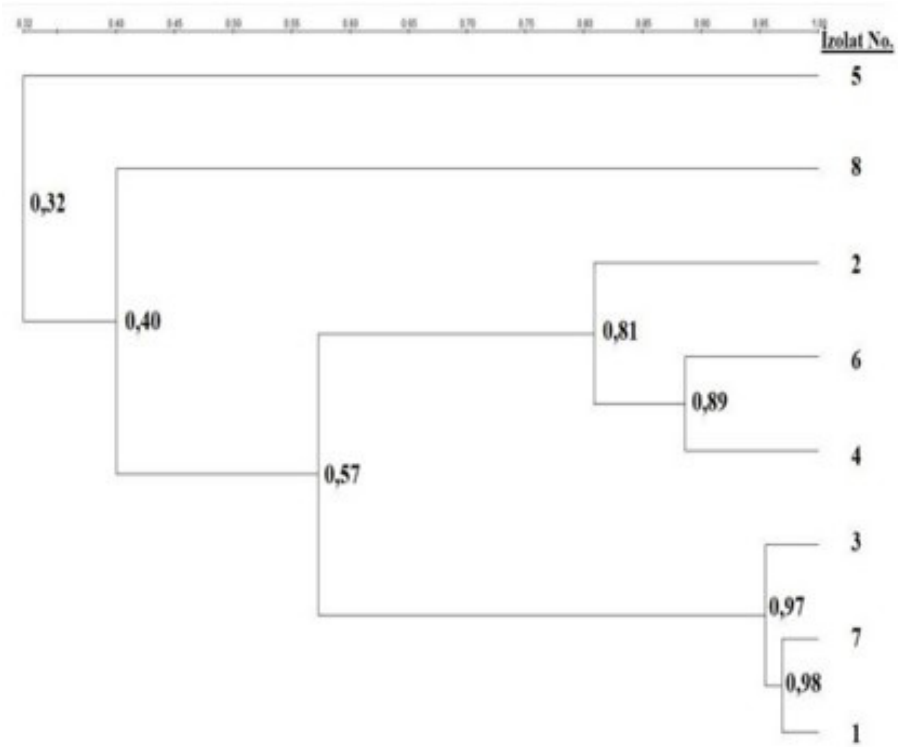


Figure 3. Phylogenetic relatedness analysis of the *S.paucimobilis* isolates examined.

Discussion

Mastitis is an infectious disease in dairy cattle characterized by inflammation of the mammary gland, leading to reduced milk yield and quality, and causing significant economic losses in the dairy industry. While its clinical form presents with obvious symptoms, the most economically devastating form in herds is subclinical mastitis. Due to the absence of overt clinical signs, subclinical mastitis often persists undetected for extended periods, resulting in decreased milk production, altered milk composition, increased somatic cell count, and reduced milk prices (Ruegg et al., 2017). Furthermore, this form can lead to permanent productivity losses throughout lactation, increased treatment costs, greater labor requirements, and higher culling rates (Seegers et al., 2003). Therefore, early detection, routine surveillance, and the implementation of effective control strategies for subclinical mastitis are critical to maintaining sustainable production and profitability in dairy farming (Bradley, 2002).

S. paucimobilis is an environmental, Gram-negative, aerobic, non-fermentative, and oxidase-positive bacillus (Hsueh et al., 1998; Ryan & Adley, 2010). It is commonly found in diverse environments, including soil, water, plant surfaces, and hospital settings. Its ability to adhere to dust particles and remain airborne facilitates its potential to cause infections in both humans and animals (Koskinen et al., 2010; Tokajian et al., 2008). In humans, *S. paucimobilis* is generally regarded as an opportunistic pathogen, but in immunocompromised individuals or in nosocomial settings, it can cause severe clinical conditions such as bacteremia, meningitis, wound infections, and respiratory tract infections (Maragakis et al., 2009; Ryan & Adley, 2010).

Although less frequently reported in veterinary medicine, *S. paucimobilis* has been isolated as a causative agent of infections in various animal species. Being environmentally derived, its likelihood of transmission increases in facilities with poor water, feed, and hygiene conditions. Although rarely reported as a mastitis pathogen in dairy cattle, its identification in such cases highlights the need to consider it among potential pathogens in environmental mastitis (Cengiz et al., 2014). In the subclinical form of mastitis, the pathogen can persist undetected for extended periods, causing substantial economic losses due to its negative effects on both milk yield and quality. Therefore, determining the potential role of *S. paucimobilis* as a mastitis pathogen in dairy cattle is important for both veterinary medicine and food safety

In the present study, the isolation, antimicrobial susceptibility, and genotyping of *S. paucimobilis* identified as a novel mastitis pathogen were investigated using milk samples from mastitis cases. Screening for *S. paucimobilis* identified eight (8%) isolates from 100 milk samples.

Cengiz et al. (2015) reported the first animal origin *S. paucimobilis* infection cases in Turkey, isolating the bacterium from cows, a calf, and a lamb, and identifying it using VITEK Compaq® 2 and PCR methods. The consistency among clinical history, necropsy, and bacteriological findings emphasized that *S. paucimobilis* should be considered as a potential pathogen in respiratory tract infections, septicemia, and antibiotic-resistant foot infections.

Cengiz et al. (2014) identified *S. paucimobilis* from various animal species using multiple identification methods. The isolates, obtained from two cows, one calf, and one lamb from different herds, were associated with septicemia in the calf, antibiotic-resistant pneumonia in one cow, antibiotic-resistant foot infection in the other cow, and severe diarrhea and fever in the lamb. The susceptibility assay showed a uniform resistance pattern, as every isolate was non-susceptible to lincomycin, gentamicin, cefquinome, ampicillin-sulbactam, and cefoperazone. In contrast, all strains remained sensitive to enrofloxacin and amoxicillin-clavulanic acid.

Kenar et al. (2019) examined the antimicrobial susceptibility of *S. paucimobilis* isolates obtained from mastitic cows between 2014 and 2018. According to their findings, 2018 isolates were resistant to penicillin but remained sensitive to the rest of the antimicrobials. Of the two isolates from 2017, one was susceptible to all antibiotics, while the other was resistant to

gentamicin and showed intermediate susceptibility to oxytetracycline.

Kurt and Eşki (2021) conducted a study in the Adana region to isolate mastitis pathogens from milk samples of clinically affected cows, using microculture techniques to identify various bacterial species. Among the Gram-negative isolates, *S. paucimobilis* was identified and reported as pathogens. It was detected in four cases (2.7%) out of a total of 146 isolates.

The findings showed that seven out of eight isolates (87.5%) did not respond to amoxicillin-clavulanic acid, and resistance was universal against ampicillin-sulbactam and cefquinome. In contrast, one isolate (12.5%) was susceptible to amoxicillin-clavulanic acid, and all isolates were inhibited by cefoperazone, cefquinome, and gentamicin.

Roque et al. (2016) conducted a comprehensive study in Korea between 2012 and 2015 to identify potential airborne pathogenic microorganisms in pig, poultry, and cattle farms with different ventilation conditions. Air samples collected from the farms underwent preliminary screening using Gram staining, oxidase, and catalase tests, followed by definitive identification using the VITEK 2 automated microbial identification system according to the manufacturer's instructions. The study found that S. paucimobilis was isolated from three of seven pig farms and four of thirteen poultry farms, indicating its presence in farm air and its potential to pose a respiratory exposure risk to both animals and farm workers.

Kühn et al. (2013) conducted a microbiome analysis of milk samples from the udders of clinically mastitic cows in the United States. Their findings revealed that Sphingomonas and Stenotrophomonas species were more prevalent in clinically mastitic udders, suggesting that the possible association of these species with mastitis can also be demonstrated through non-culture-based molecular methods.

Conclusion

Mastitis is among the leading diseases causing the greatest economic losses in cattle farming worldwide. This infection, which can occur in clinical or subclinical form and may present with acute or chronic progression, results in substantial economic damage due to reduced milk yield, product losses, treatment expenses, and labor costs, and also poses a potential risk to public health. In this investigation, eight *S. paucimobilis* isolates were obtained from a total of 100 milk samples collected from both healthy and mastitic cows in the İzmir and Aydın provinces. Antimicrobial susceptibility testing was carried out by the Kirby-Bauer disk diffusion assay, while genotyping and phylogenetic evaluation were conducted using the $(GTG)_5$-PCR method.

The results demonstrated that *S. paucimobilis* is a newly recognized bacterium among mastitis pathogens, and highlighted the importance of employing advanced diagnostic techniques such as VITEK Compact® 2 and PCR in addition to conventional bacteriological methods for accurate identification. Furthermore, ensuring proper housing conditions, improving hygiene practices, and carefully planning antibiotic usage strategies are considered critical to preventing the development of resistance in this environmentally derived bacterium. This study provides a foundation for future, more comprehensive investigations aimed at understanding the role of *S. paucimobilis* in the epidemiology of mastitis and developing effective prevention strategies.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

Since our study was conducted on materials submitted for clinical diagnostic purposes, it falls outside the scope of ethical approval under Article 8(k) of the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees.

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