Journal of Anatolian Environmental and Animal Sciences
(*Anadolu Çevre ve Hayvancılık Bilimleri Dergisi*)
DOI: https://doi.org/10.35229/jaes.1629149

JAES
Year: 10, No: 2, 2025 (202-207)
AÇEH
Yıl: 10, Sayı: 2, 2025 (202-207)

ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Investigation of the Presence and Antimicrobial Susceptibilities of *Corynebacterium pseudotuberculosis* in Subclinical Mastitis in Cattle Raised in the Aegean Region

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Received: *29.01.2025* ***Accepted:*** *14.03.2025* ***Published:*** *25.03.2025*
How to cite: **Nuhay, Ç. (2025).** Investigation of the Presence and Antimicrobial Susceptibilities of *Corynebacterium pseudotuberculosis* in Subclinical Mastitis in Cattle Raised in the Aegean Region. *J. Anatol. Env. Anim. Sci.*, ***10***(2), 202-207. https://doi.org/10.35229/jaes.1629149
Atıf yapmak için: **Nuhay, Ç. (2025).** Ege Bölgesinde Yetiştirilen Subklinik Mastitisli Sığırlarda *Corynebacterium pseudotuberculosis* Varlığının Araştırılması ve Antimikrobiyal Duyarlılıkların Belirlenmesi. *Anadolu Çev. Hay. Bil. Derg.*, ***10***(2), 202-207. https://doi.org/10.35229/jaes.1629149

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Abstract: This study aims to investigate the presence of *Corynebacterium pseudotuberculosis* and its antimicrobial susceptibility in cattle with subclinical mastitis in the Aegean Region. In 2024, 200 milk samples were collected from dairy farms in the region where subclinical mastitis was identified using the California Mastitis Test. The samples were analyzed through culture, Gram staining, and biochemical testing. Suspected isolates were confirmed using the VITEK 2 system and molecularly identified by PCR targeting the PIP gene. Antimicrobial susceptibility was assessed via the Kirby-Bauer Disk Diffusion Method. Seven *Corynebacterium pseudotuberculosis* isolates were identified in the analysis. Antimicrobial susceptibility tests showed 71% sensitivity to enrofloxacin and gentamicin, 57% to amoxicillin-clavulanic acid, and 43% to oxytetracycline. In conclusion, although the detection rate of *Corynebacterium pseudotuberculosis* was low, it remains a significant pathogen in cattle with subclinical mastitis. Further studies on antibiotic resistance are essential for developing effective treatment strategies.

Keywords: Antimicrobial resistance, cattle, *Corynebacterium pseudotuberculosis*, PCR, subclinical mastitis.

Ege Bölgesinde Yetiştirilen Subklinik Mastitisli Sığırlarda *Corynebacterium pseudotuberculosis* Varlığının Araştırılması ve Antimikrobiyal Duyarlılıkların Belirlenmesi

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Öz: Bu çalışmanın amacı, Ege Bölgesi'nde subklinik mastitisli sığırlarda *Corynebacterium pseudotuberculosis* varlığını ve bu bakterinin antimikrobiyal duyarlılığını araştırmaktır. 2024 yılında Ege Bölgesi'ndeki süt çiftliklerinden, California Mastitis Testi ile subklinik mastitis saptanan 200 süt örneği toplandı. Örnekler kültür, Gram boyama ve biyokimyasal testlerle analiz edildi. Şüpheli izolatlar, VITEK 2 sistemi kullanılarak doğrulandı ve PIP geni için PCR ile moleküler olarak tanımlandı. İzolatların antimikrobiyal duyarlılığı Kirby-Bauer Disk Difüzyon Yöntemi ile değerlendirildi. Yapılan analizlerde 7 adet *Corynebacterium pseudotuberculosis* izolatı tespit edildi. Antibiyogram sonuçlarına göre izolatların enrofloksasin ve gentamisine %71, amoksisilin-klavulanik aside %57, oksitetrasikline %43 duyarlı olduğu belirlendi. Sonuç olarak, *Corynebacterium pseudotuberculosis* düşük tespit oranlarına sahip olmasına rağmen subklinik mastitisli sığırlarda önemli bir patojen olarak değerlendirilmiştir. Antibiyotik direnci ile ilgili daha fazla çalışma yapılması, etkili tedavi stratejileri geliştirilmesi açısından büyük önem taşımaktadır.

Anahtar kelimeler: Antimikrobiyal direnç, *PCR, sığır, subklinik sastitis, Corynebacterium pseudotuberculosis*

INTRODUCTION

Mastitis is an infectious disease of the mammary glands in mammals caused by various microorganisms, characterized by inflammation and pus formation. As a significant health issue in lactating animals, mastitis leads to a decrease in milk production, alterations in milk composition, and complications that can result in death if left untreated. This disease, commonly observed in dairy cattle farming, is reported to cause one of the largest economic losses in the industry (Dufour et al., 2019; İlhan, 2018; Sharun et al., 2021).

Mastitis is classified into clinical and subclinical forms based on its progression (Schukken et al., 2014). Visible changes in milk, such as clots, flakes, serum, or even blood, are the primary indicators of clinical mastitis. Whether the disease manifests as clinical or subclinical is generally determined by the type and strain of the pathogen. One of the main reasons mastitis causes significant economic losses is its tendency to progress predominantly in a subclinical form (Gökdağ & Çiftci, 2021; Reyher et al., 2011, Savasan et al., 2023). One of the subclinical mastitis agents in cattle is *C. pseudotuberculosis* (Yeruham et al., 1996). Additionally, *Corynebacterium bovis*, *Corynebacterium mastitis*, *Corynebacterium amycolatum*, *Corynebacterium minutissimum*, and *Corynebacterium ulcerans* have also been reported to cause mastitis in livestock (Hadimli et al., 2006).

This study aims to investigate the presence of *C. pseudotuberculosis* and determine its antimicrobial susceptibility in milk samples collected from subclinical mastitis cattle raised in the Aegean Region.

MATERIAL AND METHOD

The study material consisted of 200 milk samples collected in 2024 from dairy cattle farms operating in the Aegean Region, where subclinical mastitis was detected using the California Mastitis Test. The samples, transported to the laboratory under a cold chain, were inoculated onto Columbia Agar (5% sheep blood, Liofilchem) and incubated at 37°C for 24-48 hours. Following the incubation period, small, white, dry, and easily disintegrating colonies were Gram-stained. Gram-positive small coccobacilli were selected and subcultured. Colonies testing positive for catalase and negative for oxidase in biochemical tests were identified as *C. pseudotuberculosis* suspects and confirmed using the CBC diagnostic kit designed for the VITEK 2 (bioMérieux) system.

The molecular diagnostic method was used for the identification of C. pseudotuberculosis. The DNA extractions of isolates identified as *C. pseudotuberculosis* were performed using a commercial DNA extraction kit (High Pure PCR Template Preparation Kit, Roche). Molecular confirmation was carried out using primers specific to the PIP gene of *C. pseudotuberculosis* (PIP F: 5'-AACTGCGGCTTTCTTTATTC-3'; PIP R: 5'-GACAAGTGGGAACGGTATCT-3') (D'Afonseca et al., 2010).

PCR mixtures (12.5 µL Xpert Fast Hotstart Mastermix (2×, GRiSP), 2 µL forward primer, 2 µL reverse primer, and 3.5 µL water) underwent amplification under the following conditions: an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 94°C for 15 seconds (denaturation), 54°C for 30 seconds (annealing), and 72°C for 15 seconds (extension), with a final extension at 72°C for 3 minutes. PCR reactions were performed using the Techne TC-412 thermal cycler (Keison Products) with Xpert Fast Hotstart Mastermix (2×, GRiSP) as the master mix. (D'Afonseca et al., 2010).

Antimicrobial susceptibilities of the obtained *C. pseudotuberculosis* isolates were determined by Kirby-Bauer Disk Diffusion Method. A 0.5 McFarland suspension was prepared from the isolates in fresh cultures in physiological saline, transferred to Mueller Hinton Agar (MHA) and left for incubation. For antimicrobial susceptibility testing, tetracycline (30 µg, Oxoid), oxytetracycline (30 µg, Bioanalyse), enrofloxacin (5 µg, Bioanalyse), ampicillin/sulbactam (10/10 µg) (Bioanalyse), amoxicillin/clavulanic acid (2/1) (30 µg, Oxoid), trimethoprim/sulfamethoxazole (1. 25 µg/23.75 µg) and gentamicin (10 µg, Oxoid) disks were used. Antibiotic disks were placed in the media and incubated at 37°C for 24 h. Zone diameters formed after the incubation period were measured and evaluated according to CLSI 2013 (Bauer et al., 1966, CLSI 2013).

RESULTS

Out of 200 subclinical mastitis milk samples analyzed, 7 *C. pseudotuberculosis* isolates were identified, which corresponds to 3.5% of the total samples. Isolates yielded a specific 551 bp band for *C. pseudotuberculosis* (Figure 1).

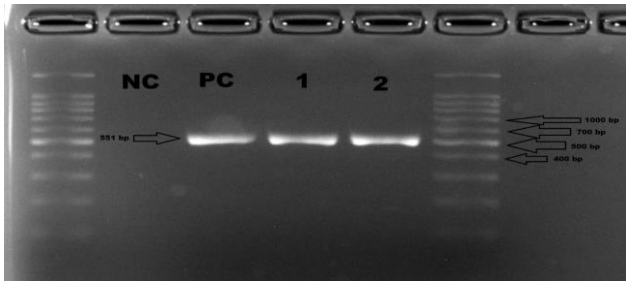

Figure 1. PIP gene (551 bp) PCR analysis electrophoresis image of *Corynebacterium pseudotuberculosis*. PC: *Corynebacterium pseudotuberculosis* positive control NCTC 3450; NC: Negative control (sterilised ddH2O); 1-2: *Corynebacterium pseudotuberculosis* positive samples Marker: GRS Universal Ladder.

Antimicrobial susceptibility testing of the isolates was conducted using the Kirby-Bauer disk diffusion method. The results are summarized in. Table 1. As a result of antibiotic susceptibility tests, Enrofloxacin (71%) and Gentamicin (71%) showed the highest susceptibility, while Oxytetracycline (43%), Tetracycline (43%), Amoxicillin/Clavulanic Acid (57%), and Ampicillin/Sulbactam (29%) exhibited varying degrees of susceptibility, with the highest resistance observed in Trimethoprim/Sulfamethoxazole (86%) and Ampicillin/Sulbactam (71%), and the evaluations were conducted according to the CLSI 2013 reference.

Table 1. Antimicrobial resistance profiles of isolates.

Antibiotic Name	Susceptible	Intermediate	Resistant	Referans
Enrofloxacin	5(%71)	0	2(%29)	CLSI 2013
Gentamicin	5(%71)	1(%14)	1(%14)	CLSI 2013
Oxytetracycline	3(%43)	1(%14)	3(%43)	CLSI 2013
Tetracycline	3(%43)	0	4(%57)	CLSI 2013
Trimethoprim/Sulfamethoxazole	1(%14)	0	6(%86)	CLSI 2013
Amoxicillin/Clavulanic Acid	4(%57)	0	3(%43)	CLSI 2013
Ampicillin/Sulbactam	2(%29)	0	5(%71)	CLSI 2013

DISCUSSION AND CONCLUSION

Mastitis cases in cattle are generally associated with *Staphylococcus aureus*, *Streptococcus* spp., *Escherichia coli*, *Trueperella pyogenes*, and *Corynebacterium* spp. (Cantekin et al., 2015). Among the Corynebacterium species associated with mastitis in livestock, *C. pseudotuberculosis*, *C. bovis*, *C. mastitis*, *C. amycolatum*, *C. minutissimum*, and *C. ulcerans* have been frequently reported (Hadimli et al., 2006).

In this study, 200 milk samples collected from subclinical mastitis cattle raised in the Aegean Region were examined, and *C. pseudotuberculosis* was isolated from 3.5% (7/200) of the samples. Antimicrobial susceptibility testing of the isolates revealed 71% susceptibility to enrofloxacin and gentamicin, 43% to oxytetracycline, and 57% to amoxicillin-clavulanic acid. Susceptibility to tetracycline was 43%, while susceptibility to trimethoprim-sulfamethoxazole and ampicillin-sulbactam was found to be 14% and 29%, respectively.

In Türkiye, a study conducted in Afyon province on 500 milk samples from cattle with clinical and subclinical mastitis reported the isolation rates of *C. ulcerans* at 6.45% and *C. bovis* at 5.65% (Özenç et al., 2019). Another study in Konya province involving 500 milk samples from goats with clinical and subclinical mastitis found a 23% isolation rate of *Corynebacterium* spp. (Çiftçi, 1996). In 2021, a study conducted in Söke district of Aydın province on milk samples from subclinical mastitis cattle reported a 17.97% isolation rate of *Corynebacterium* spp. (Çelik, 2021). A study in Yozgat province involving 238 milk samples from cattle with clinical mastitis reported the isolation of *Corynebacterium* spp. at a rate of 13.79% (24 samples) (Özavcı et al., 2017). Additionally, a study conducted on mastitis milk samples from cattle in Konya, Kayseri, and Afyon provinces reported a 3,12% isolation rate of *C. pseudotuberculosis*, which remains the only study in Türkiye identifying this pathogen in mastitis cases (Hadimli et al., 2006).

In general, studies conducted in Türkiye have focused on *Corynebacterium* spp. without extending identification to the subspecies level. Isolation rates of *Corynebacterium* spp. in mastitis cases have been observed to vary between 11% and 23%. These differences are thought to arise from variations in livestock management practices across regions and differences in sample selection methods used in the studies. When compared to the only study in Türkiye identifying subspecies, which reported a 3.12% detection rate for *C. pseudotuberculosis*, the 3.5% detection rate found in the present study shows a similar result. (Hadimli et al., 2006).

Globally, cases of mastitis caused by *C. pseudotuberculosis* have been reported in sheep, goats, and cattle. A study conducted in Italy involving 120 milk samples from mastitic sheep reported a 7% detection rate of *C. pseudotuberculosis* (Rossi & Bianchi, 2016). Similarly, a 2013 study in Brazil found that 14% of mastitis cases in goats were caused by C. pseudotuberculosis (Silva & Santos, 2013). In Egypt, *C. pseudotuberculosis* was isolated from 12% of milk samples collected from 100 dairy goats (Abdelsalam & Ahmed, 2017). Additional studies on mastitic goat milk reported isolation rates of *C. pseudotuberculosis* at 8% in Spain in 2019 and 10% in South Africa in 2021 (Van der Merwe & Botha, 2021; Gonzalez & Garcia, 2019).

Studies focusing on cattle have also highlighted cases of *C. pseudotuberculosis* mastitis. For instance, in 2021, a mastitis case in a cow in England was attributed to *C. pseudotuberculosis* (Thompson & Wright, 2020). Experimental research in Canada observed clinical mastitis symptoms caused by *C. pseudotuberculosis* in 85% of experimentally infected cows (Dorella et al., 2008). A 2015 study in New Zealand detected *C. pseudotuberculosis* in 5% of mastitis cases in cattle (McDonald & Jeffers, 2015). In Australia, *C. pseudotuberculosis* was identified in 15% of samples from 200 dairy cows (Connor & Quirie, 2010). A 2011 study in the United States examined 29 cattle herds and reported a 10% detection rate of *C. pseudotuberculosis*, emphasizing the potential role of vectors in the transmission of the infection (Baird & Fontaine, 2011).

One of the most significant reports came from Israel, where a mastitis outbreak caused by *C. pseudotuberculosis* occurred in the summer of 2004. The outbreak affected 32 cows, with the pathogen isolated from 25% of milk samples (Yeruham et al., 2004).

The isolation of *C. pseudotuberculosis* in mastitis cases is a significant issue both in Türkiye and globally. Although the limited number of studies conducted in Türkiye have reported low detection rates of this pathogen in mastitis cases, it is considered an important pathogen, especially when evaluated alongside other *Corynebacterium* species. Globally, reports of *C. pseudotuberculosis* isolation in sheep, goats, and cattle have shown varying prevalence across different regions. This demonstrates that the pathogen exhibits diverse distribution and impact among species and geographical areas. The mastitis outbreak reported in Israel highlights the potential of this pathogen to cause epidemics, emphasizing the necessity for effective control strategies.

A study conducted in Balıkesir province on *C. pseudotuberculosis* isolates reported that the isolates were susceptible to neomycin/bacitracin/tetracycline (81.2%), amoxicillin/clavulanic acid (68.7%), cloxacillin (62.5%), oxytetracycline (68.7%), tetracycline (56.2%), ampicillin/sulbactam (31.2%), penicillin/novobiocin (75.0%), trimethoprim/sulfamethoxazole (18.7%), and enrofloxacin (87.5%), while all isolates were resistant to streptomycin (İlhan, 2020).

In Konya province, a study on *C. pseudotuberculosis* isolates obtained from sheep abscesses found that the isolates were susceptible to ampicillin (37.5%), florfenicol (98.6%), cloxacillin (55.5%), telithromycin (91.6%), penicillin G (83.3%), oxytetracycline (81.9%), rifampicin (81.9%), amoxicillin (77.7%), ampicillin/sulbactam (76.3%), erythromycin (69.4%), spiramycin (58.3%), gentamicin (81.9%), and enrofloxacin (83.3%) (Sakmanoğlu et al., 2015).

A 2024 study reported that four isolated strains of *C. pseudotuberculosis* were susceptible to tilmicosin, tetracycline, clindamycin, and ciprofloxacin (Babacan, 2024).

In Egypt, a study on *C. pseudotuberculosis* isolates reported that the isolates were susceptible to ciprofloxacin (96.2%), amikacin (90.4%), neomycin (88.5%), and streptomycin (80.8%), while they showed resistance to penicillin (96.2%) and erythromycin (92.3%) (Algammal, 2016).

In a study by Robaj et al. (2017), *C. pseudotuberculosis* isolates were found to be susceptible to penicillin and erythromycin (100%), tetracycline (95%), gentamicin (90%), cephalothin (85%), streptomycin (80%), amoxicillin (75%), ceftriaxone (70%), ciprofloxacin (65%), and trimethoprim/sulfamethoxazole (60%).

In another study conducted by El Damaty et al. (2023) in Egypt, *C. pseudotuberculosis* strains were found to be resistant to florfenicol and bacitracin (100%), penicillin (92.6%), and erythromycin (92.6%), but fully susceptible to norfloxacin.

In this study, the antibiotic susceptibility profiles of *C. pseudotuberculosis* isolates were evaluated (CLSI, 2013). The isolates demonstrated susceptibility rates of 71% for enrofloxacin and gentamicin, 43% for oxytetracycline, and 57% for amoxicillin/clavulanic acid. Susceptibility to tetracycline was found to be 43%, while it was 14% for trimethoprim/sulfamethoxazole and 29% for ampicillin/sulbactam.

The susceptibility and resistance profiles of *C. pseudotuberculosis* strains to various antibiotics appear to be highly variable. This variability can be attributed to factors such as the country, region, and environmental conditions where the study was conducted, as well as the diversity and frequency of antibiotic treatments applied to the animal population from which the isolates were obtained. In Türkiye, the increase in antibiotic resistance against agents like *C. pseudotuberculosis* may be linked to the indiscriminate and unnecessary use of antibiotics. The lack of guidelines for therapeutic and prophylactic antibiotic use, as well as non-compliance with treatment durations, are among the factors contributing to the spread of resistant strains.

Therefore, raising awareness about the rational use of antibiotics, adopting treatment approaches based on susceptibility testing, and implementing resistance monitoring programs are of great importance. Additionally, studying resistance mechanisms could contribute to the development of effective treatment strategies.

In conclusion, it was determined that subclinical mastitis cases in cattle raised in the Aegean Region could be caused by *C. pseudotuberculosis*, and the antimicrobial susceptibility data obtained in this study should be considered when implementing treatment strategies for affected animals.

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