Phenotypic and Genotypic Characterization of *Corynebacterium pseudotuberculosis* and *Staphylococcus aureus* subsp. *anaerobius* from Abscesses in Small Ruminants in the Aegean Region of Türkiye

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ABSTRACT

Abscesses in small ruminants cause significant economic losses and animal welfare concerns, making the identification of their etiological agents crucial for effective control. This study was aimed to investigate the phenotypic and genotypic characteristics of *Corynebacterium pseudotuberculosis* and *Staphylococcus aureus* subsp. *anaerobius* isolated from abscesses in sheep and goats raised in the Aegean Region of Türkiye. A total of 100 subcutaneous abscess samples collected from sheep and goats between 2021 and 2023 were examined using conventional bacteriological, phenotypic and molecular methods. Isolates were identified by culture characteristics, biochemical analysis, VITEK 2 Compact system, and PCR, while genotypic relationships were evaluated by ERIC-PCR. Of the 100 samples examined, 17 *C. pseudotuberculosis* and 31 *S. aureus* subsp. anaerobius isolates were identified. Among the isolates obtained, *C. pseudotuberculosis* was detected in 20% of sheep samples and 14% of goat samples, whereas *S. aureus* subsp. *anaerobius* was identified in 32% of sheep samples and 30% of goat samples. ERIC-PCR analysis revealed similarity ranges of 41%-100% among *C. pseudotuberculosis* isolates and 27%-100% among *S. aureus* subsp. *anaerobius* isolates. Statistical analysis using Fisher's exact test showed no significant differences in isolation rates between sheep and goats for either pathogen (*C. pseudotuberculosis*: p=0.59; *S. aureus* subsp. *anaerobius*: p=0.84), with the proportion of isolates presented alongside 95% confidence intervals. These findings emphasize that, in the Aegean Region of Türkiye, abscesses in small ruminants involve both *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius*, underscoring the need to integrate both pathogens into regional diagnostic protocols and herd health programs. As epidemiological patterns show considerable variation worldwide, these results specifically reflect the situation observed in Türkiye. Routine molecular typing and surveillance will enhance early detection, support targeted interventions, and mitigate economic losses in the region.

Keywords: Abscess; *Corynebacterium pseudotuberculosis*; Goat; PCR; Sheep; *Staphylococcus aureus subsp. anaerobius*; ERIC-PCR.

INTRODUCTION

Small ruminant farming is exceedingly important for utilizing poor pastures, fallow lands, and non-arable areas. Products obtained from small ruminant farming, such as meat, milk, wool, and leather, have broad industrial applications in our country (20, 25). Small ruminant husbandry in Türkiye is traditionally practiced based on pasture grazing. Due to Türkiye's topography, climate, vegetation, and geographical structure, small ruminant farming is carried out in a migratory manner in some regions (9).

Corynebacterium pseudotuberculosis is the causative agent of a disease called Caseous Lymphadenitis, which occurs when abscesses in the superficial lymph nodes rupture and discharge. Caseous lymphadenitis is also referred to as "Thin Ewe Syndrome" due to its association with weight loss (14). Caseous lymphadenitis leads to significant economic losses as it causes reduced fertility, weight loss, and deterioration in skin quality in sheep and goats (17).

Morel's disease is an infection caused by *Staphylococcus aureus* subsp. *anaerobius*, characterized by the formation of abscesses in the lymph nodes, on the skin surface, and within the muscles of sheep and goats. Another name for Morel's disease is abscess disease. Morel's disease causes significant economic losses in herds by leading to a decrease in meat yield, deterioration in hide quality, and a reduction in the market value of the animals (11, 17).

C. pseudotuberculosis is a Gram-positive bacterium that appears as coccobacilli or rod-shaped forms, measuring 0.5-0.6 µm in width and 1-3 µm in length. It is non-spore-forming, non-capsulated, non-motile, and is facultatively intracellular (2). When incubated on culture media at 37°C, *C. pseudotuberculosis* forms sparse colonies within the first 24 hours, while after 48-72 hours, both the number and size of colonies increase, reaching a diameter of 1-2 mm. The colonies typically appear as dry, opaque, cream-colored R-type colonies (8).

S. aureus subsp. *anaerobius* has been reported to produce small haemolytic colonies when incubated in a microaerophilic environment at 37°C on Columbia agar supplemented with 5% sheep blood for 24-48 hours (11). Since the phenotypic identification of *S. aureus* subsp. *anaerobius* is challenging, molecular identification is crucial (18).

This study aimed to isolate and to phenotypically and genotypically characterize *Corynebacterium pseudotuberculosis* and *Staphylococcus aureus* subsp. *anaerobius* from abscesses obtained from sheep and goats raised in the Aegean Region in Türkiye. Furthermore, the genetic relationships of the obtained isolates were determined using the ERIC-PCR method.

MATERIAL AND METHODS

Materials

The material of this study consisted of a total of 100 tissue samples obtained from subcutaneous abscesses observed in sheep and goats raised in the Aegean Region of Türkiye and submitted to the Routine Diagnostic Laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, for etiological diagnosis. The samples were submitted from small ruminant farms located in the provinces of Aydın, Izmir, Kütahya, Manisa, Denizli, Uşak, Muğla, and Afyonkarahisar. Samples were taken from animals that had clinically apparent abscess lesions. Abscess samples were sent to the laboratory for diagnosis.

Of the samples, 50 were of sheep origin and 50 were of goat origin. Equal numbers of samples from both species were included from each province. In this context, 8 sheep and 8 goat samples were obtained from İzmir, whereas 6 sheep and 6 goat samples from each of Aydın, Manisa, Muğla, Denizli, Uşak, Kütahya, and Afyonkarahisar were included in the study. The study included a total of 16 samples from Izmir (8 sheep and 8 goats) and 12 samples from other provinces (6 sheep and 6 goats from each province).

Ethical Approval

The study was approved by the Bornova Veterinary Control Institute Directorate Local Ethics Committee for Animal Experiments (HADYEK) with official letter number 71705440-770 dated February 17, 2022.

Bacterial Isolation and Phenotypic Identification

Abscess materials delivered in sterile syringes were transferred into sterile Petri dishes. A loopful of each abscess sample was inoculated onto 5% sheep blood agar and incubated under both aerobic and microaerophilic conditions at 37°C for 48 h.

Following incubation, colonies suspected to be *C. pseudotuberculosis*, characterized by a small, white, dry, and easily fragmented appearance, were subjected to Gram staining. Gram-positive small coccobacilli were selected and sub-cultured. Colonies showing catalase-positive and oxidase-negative reactions were considered presumptive *C. pseudotuberculosis* isolates and stored for further analysis (3, 13).

For the detection of *S. aureus* subsp. *anaerobius*, colonies producing small haemolytic zones after incubation were evaluated by Gram staining. Gram-positive cocci were selected, and colonies showing weak or variable catalase activity and oxidase-negative reactions were considered presumptive

S. aureus subsp. *anaerobius* isolates and stored for further analysis (11).

Presumptive isolates of *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* were further identified phenotypically using the VITEK 2 system (bioMérieux, Marcy-l'Étoile, France). *C. pseudotuberculosis* isolates were identified using the appropriate VITEK 2 identification card for Gram-positive or coryneform bacteria, while *S. aureus* subsp. anaerobius isolates were identified via biochemical profiles using the VITEK 2 GP kit.

Molecular confirmation

Molecular confirmation was performed on *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* isolates identified by phenotypic and biochemical methods. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Germany), according to the manufacturer's instructions.

For molecular confirmation of *C. pseudotuberculosis*, amplification of a 551 bp fragment was carried out using the *PIP* F (5'-AACTGCGGCTTTCTTTATTC-3') and *PIP* R (5'-GACAAGTGGGAACGGTATCT-3') primers (10).

For molecular confirmation of *S. aureus* subsp. *anaerobius*, the *nuc* gene was first amplified using the primers 5'-GCGATTGATGGTGATACGGTT-3' and 5'-AGCCAAGCCTTGACGAACTAAAGC-3', yielding a 279 bp amplicon. Isolates positive for the *nuc* gene were subsequently subjected to PCR targeting the *S. aureus* subsp. *anaerobius*-specific *cat* gene region using the *cat808*F (5'-CTCCATTTTAGAACGCAACAA-3') and *cat1583*R (5'-TGGGTCAGCTTTGTAACA-3') primers (5, 23).

Genotypic analysis

The isolates confirmed by PCR were genotyped by ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-PCR). Genotypic profiles were generated using ERIC-PCR with the ERIC-2 primer (5'-AAGTAAGTGACTGGGGTGAGCG-3'), and amplification was performed as previously described (24).

Each ERIC-PCR reaction was prepared in a final volume of 25 µL, containing 1×PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 U Taq DNA polymerase, 25 pmol primer, and 5 µL template DNA. The amplification protocol consisted of an initial denaturation step 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.

PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) (2 µg/mL) and visualized under a UV transilluminator (Vilber Lourmat, Marne-la-Vallée, France). Banding patterns were analyzed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method in Quantity One software.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics software (version 26.0; IBM Corp., Armonk, NY, USA). Isolation rates of *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* in sheep and goats were compared using Fisher's exact test (26, 27). A p-value of <0.05 was considered statistically significant. In addition, proportions isolates were presented with 95% confidence intervals (95% CI), calculated using the Wilson score method (28).

RESULTS

Phenotypic findings

Bacterial growth was observed in 48 (48%) of the 100 abscess samples cultured on blood agar. Seventeen of the cultures exhibited colony morphology, Gram staining properties, and biochemical reactions consistent with *C. pseudotuberculosis*. In addition, 31 isolates exhibited phenotypic characteristics compatible with *S. aureus* subsp. *anaerobius*.

Biochemical identification performed using the VITEK 2 Compact system confirmed all 17 isolates as *C. pseudotuberculosis*. Similarly, the biochemical profiles of the 31 isolates analysed with the VITEK 2 GP kit were found to be consistent with *S. aureus* subsp. *anaerobius*.

PCR analysis findings

The 17 isolates identified as *C. pseudotuberculosis* by conventional methods and the VITEK 2 Compact system yielded the expected 551 bp amplicon targeting the *PIP* gene region, confirming all isolates as *C. pseudotuberculosis*. A representative agarose gel electrophoresis image is presented in Figure 1.

Similarly, all 31 isolates identified phenotypically as *S. aureus* subsp. *anaerobius* were positive for the *nuc* gene and produced the expected 279 bp amplicon, confirming their

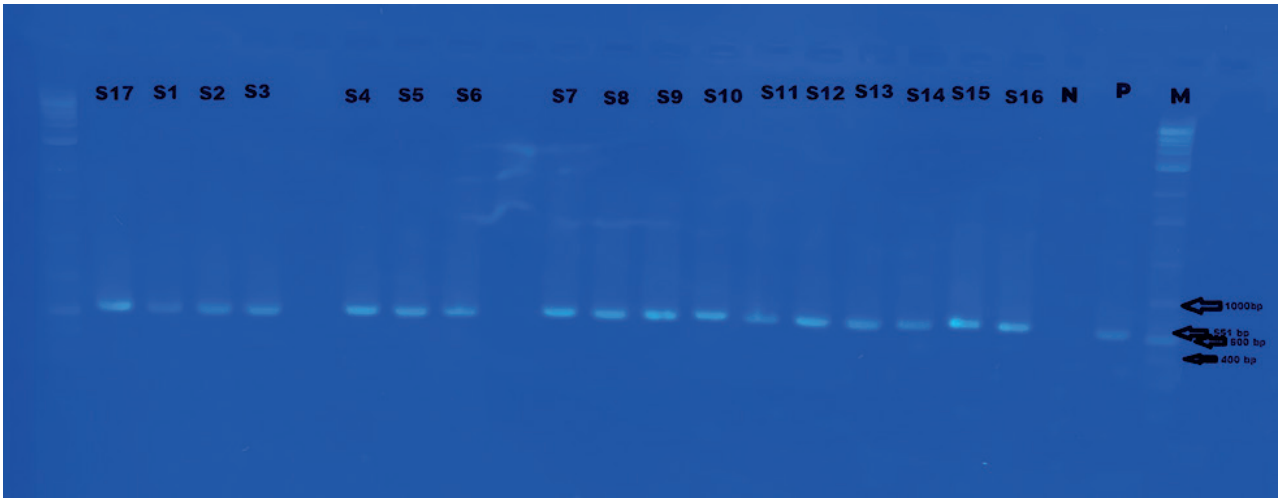

Figure 1. Agarose gel electrophoresis image of the *PIP* gene PCR product (551 bp) for *C. pseudotuberculosis*. PC: *C. pseudotuberculosis* NCTC 3450 positive control; NC: negative control (sterile ddH_2O); S1–S17: *C. pseudotuberculosis*-positive samples; M: GRS Universal Ladder.

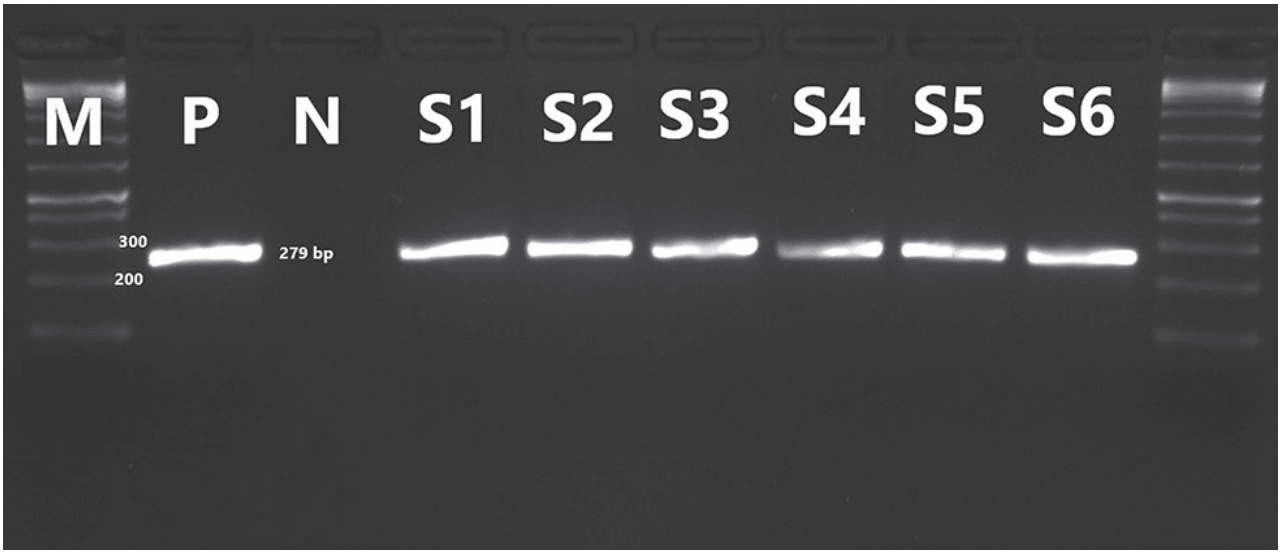

Figure 2. Agarose gel electrophoresis of the PCR product targeting the *nuc* gene specific for *S. aureus* (279 bp). PC: *S. aureus* ATCC 25923 positive control; NC: negative control (sterile ddH_2O); S1-S6: *S. aureus* subsp. *anaerobius* isolates; M: GRS Universal Ladder.

identity as *S. aureus*. A representative agarose gel electrophoresis image is shown in Figure 2.

PCR with the subspecies-specific primers *cat808F* and *cat1583R* produced the expected 771 bp amplicon in all 31 isolates, confirming their identity as *S. aureus* subsp. *anaerobius*. A representative agarose gel electrophoresis image is presented in Figure 3.

Genotypic analysis findings (ERIC-PCR analysis)

For phylogenetic analysis, ERIC-PCR genotyping was performed on *C. pseudotuberculosis* isolates. The isolates showed 41%-100% similarity (Figure 4). Based on phylogenetic analysis, four different genotypes (RA, RB, RC, RD) were identified. The RD genotype was found to be dominant, with 13 isolates showing 100% similarity. The RA and RC genotypes each contained one isolate, while the RB genotype included two isolates.

ERIC-PCR genotyping was performed to assess the genetic relatedness of *S. aureus* subsp. *anaerobius* isolates. The isolates exhibited 27%-100% similarity (Figure 5). Cluster analysis based on ERIC-PCR profiles identified 16 distinct genotypic patterns (RA-RP). The dominant RK genotype contained five isolates, all showing 100% similarity. The RB, RD, RF, RH, RO, and RP genotypes

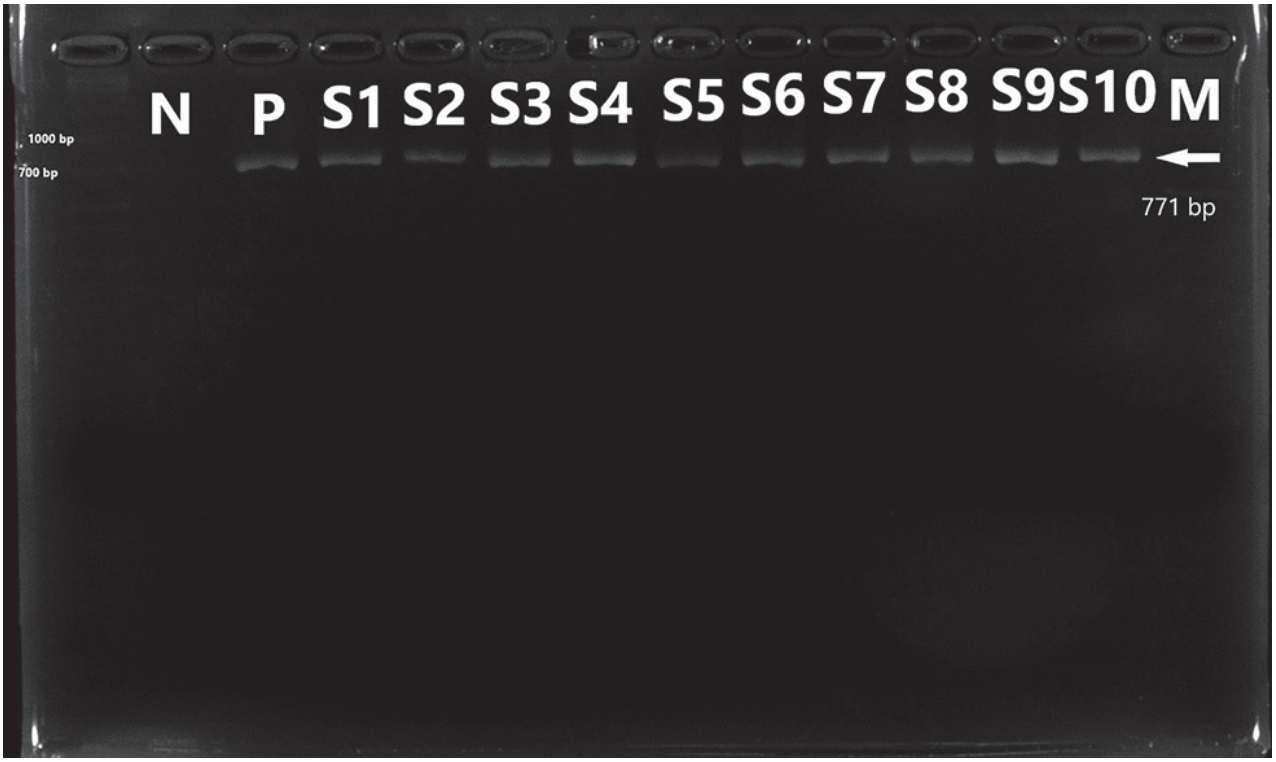

Figure 3. Agarose gel electrophoresis image of the 771 bp subspecific PCR amplicon of *S. aureus* subsp. *anaerobius*. M: GRS Universal Ladder; PC: synthetic DNA positive control; NC: negative control (sterile ddH_2O); S1-S10: *S. aureus* subsp. *anaerobius* isolates.

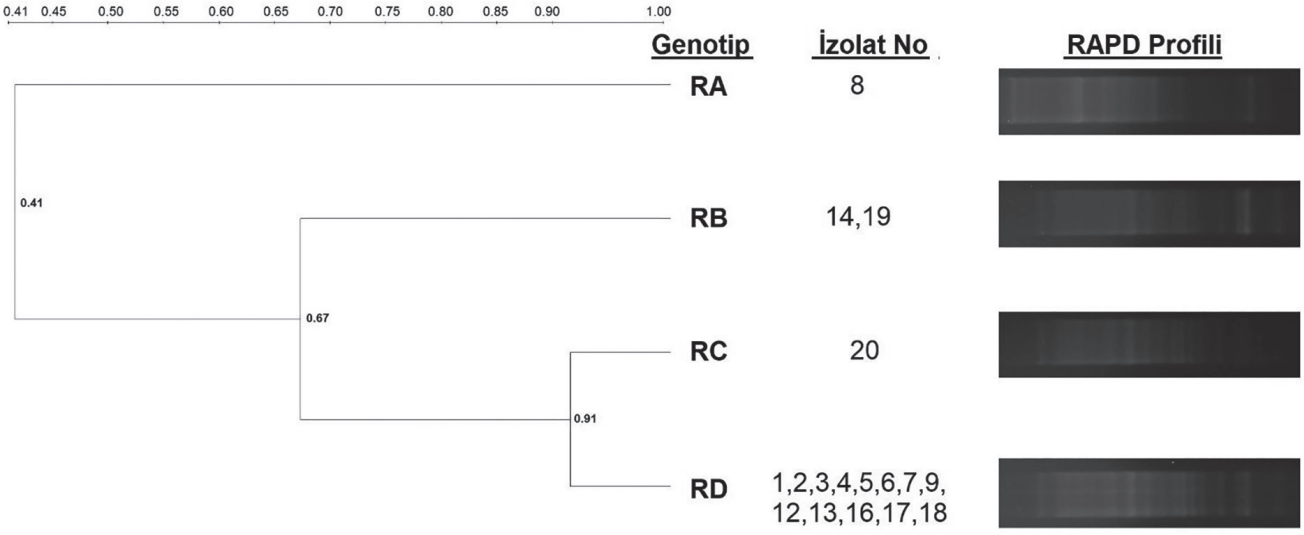

Figure 4. ERIC-PCR gel image and UPGMA dendrogram of *C. pseudotuberculosis* isolates showing genotypic relationships among isolates.

each contained one isolate. Additionally, the RA, RC, RE, RG, RJ, RL, and RN genotypes contained two isolates each, while the RI and RM genotypes contained three isolates each.

Statistical findings

The isolation rate of *C. pseudotuberculosis* was 20% (95% CI: 11%-31%) in sheep and 14% (95% CI: 7%-24%) in goats, with no statistically significant difference between

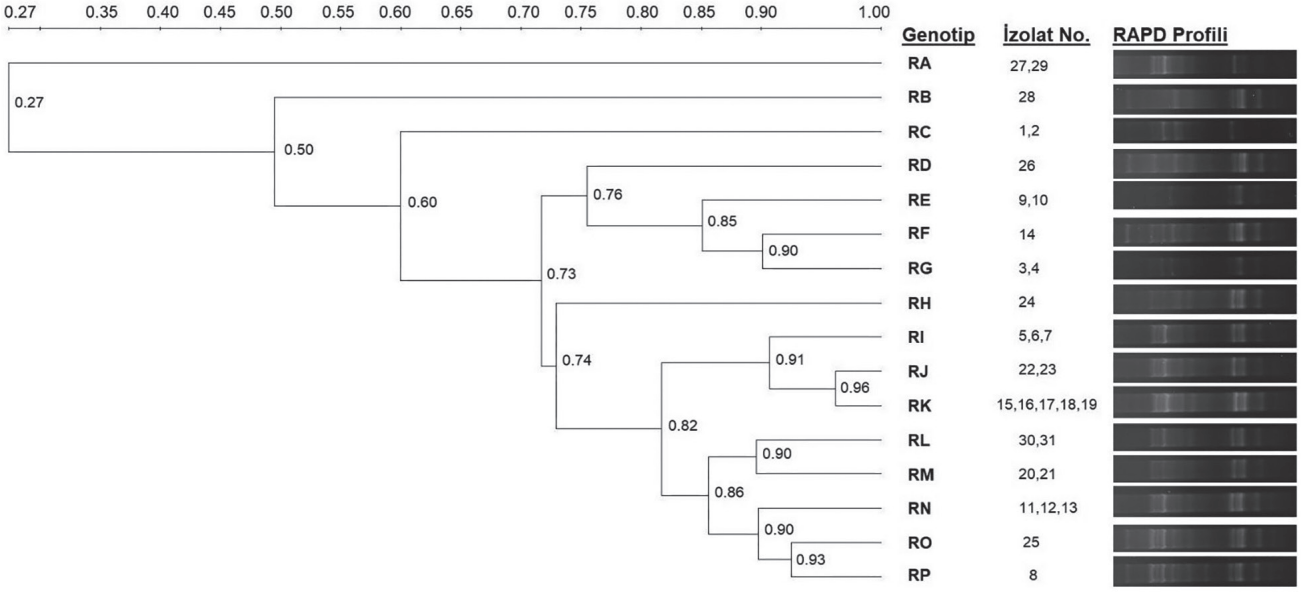

Figure 5. ERIC-PCR gel image and UPGMA dendrogram of *S. aureus* subsp. *anaerobius* isolates illustrating the genetic relationships among isolates.

the two species (p=0.59). Similarly, the detection rate of *S. aureus* subsp. *anaerobius* was 32% (95% CI: 22%-44%) in sheep and 30% (95% CI: 20%-42%) in goats, and no statistically significant difference was observed between species (p=0.84).

Distribution of study results by province and host species

The distribution of *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* isolates according to province and host species is presented in Table 1.

DISCUSSION

In the present study, *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* were isolated from subcutaneous abscess samples obtained from sheep and goats raised in the Aegean Region of Türkiye. The overall isolation rate of *C. pseudotuberculosis* was 17.0%, with species-specific rates of 20.0% in sheep and 14.0% in goats. For *S. aureus* subsp. *anaerobius*, the overall isolation rate was 31.0%, with rates of 32.0% in sheep and 30.0% in goats. No significant difference was observed between sheep and goats, suggesting a similar distribution of both pathogens in the study population.

The isolation rate of *C. pseudotuberculosis* observed in this study is in agreement with previous reports from different regions of Türkiye (6,21,22). In particular, the isolation rate reported by Parın *et al*. in Aydın appears comparable to the overall isolation rate found in the present study. Worldwide, the reported isolation rate of *C. pseudotuberculosis* infection varies considerably, ranging from 3% to 54% (1.7, 19). Such variation may be related to differences in sample type, animal population, husbandry practices, diagnostic approach, and regional epidemiological conditions. In the present study, the use of abscess material may also have influenced the isolation rate compared with studies based on lymph node or post-mortem samples (12, 15).

The isolation rate of *S. aureus* subsp. *anaerobius* in the present study was also consistent with previous reports for Türkiye (11,16), indicating that Morel's disease remains an important health problem in small ruminants. Although some studies have suggested that this infection may occur more frequently in young animals (1), age-related data were not evaluated in the present study; therefore, no direct conclusion can be drawn regarding age predisposition in the sampled population. Nevertheless, the relatively high isolation rate observed here supports the view that *S. aureus* subsp. *anaerobius* is widely distributed in small ruminant flocks in the region. The relatively low culture positivity rate may be associated with prior antimicrobial treatment, the presence of fastidious or anaerobic microorganisms, or potential limitations related to sample handling and transport conditions.

Mixed infections were not specifically evaluated in this

Table 1. Distribution of *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* by province and host species.

Province	Sheep			Goat		
	No. of Samples	*C. pseudotuberculosis* Positive	*S. aureus subsp. anaerobius* Positive	*No. of Samples*	*C. pseudotuberculosis* Positive	*S. aureus subsp. anaerobius Positive*
İzmir	8	2	4	8	2	3
Aydın	6	2	3	6	1	2
Manisa	6	1	1	6	1	2
Muğla	6	1	1	6	-	2
Denizli	6	1	2	6	1	1
Uşak	6	1	-	6	1	3
Kütahya	6	1	2	6	-	1
Afyonkarahisar	6	1	3	6	1	1
Total	50	10	16	50	7	15

–, no isolate detected.

study, which may represent a limitation in interpreting the etiology of abscess formation.

The persistence and spread of both *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* in sheep and goat flocks may be associated with management-related factors such as hygiene status, animal density, environmental contamination, and the movement of infected animals between herds. Although these factors were not directly investigated, they may help explain the persistence of caseous lymphadenitis and Morel's disease under field conditions.

Both diseases are of considerable economic importance in small ruminant production because they may lead to reduced productivity, carcass and skin damage, and additional treatment and control costs (4). In this context, rapid and accurate diagnosis is essential for effective disease management. The molecular confirmation of the isolates in the present study demonstrates the usefulness of PCR-based methods as reliable tools for confirming etiological agents associated with abscess formation in small ruminants. Isolates showing 100% similarity in ERIC-PCR profiles suggest the presence of clonally related strains, indicating a possible common source of infection or transmission within or between flocks in the region.

Our investigation demonstrates that abscesses in small ruminants in the Aegean Region of Türkiye are associated with both *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius*. This regional evidence underlines the importance of incorporating both pathogens into diagnostic protocols and herd health strategies to improve disease management and reduce economic losses. While global reports indicate considerable variation in the prevalence and distribution of these agents across different countries, the present findings provide a clear picture of the epidemiological situation in Türkiye and contribute valuable data for regional surveillance and control programs.

In conclusion, the present study provides regional data on the occurrence of *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* in sheep and goats raised in the Aegean Region of Türkiye. The findings indicate that both caseous lymphadenitis and Morel's disease continue to be relevant for small ruminant health and production. Further large-scale epidemiological studies incorporating herd-level risk factors, animal age, management practices, and molecular typing data are needed to clarify transmission dynamics and to guide effective control strategies.

ORIGINALITY STATEMENT

This article has not been published previously and is not under consideration for publication elsewhere, in whole or in part.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

Ç.N.: Conceptualization, methodology, investigation, data collection, laboratory analyses, data curation, interpretation of data, and drafting the manuscript.
S.S.: Supervision, methodology, validation, interpretation of data, and critical revision of the manuscript.

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