Antibiotic Resistance Profiles and *pld* Gene Distribution in *Corynebacterium pseudotuberculosis* Isolates from Small Ruminants in Razavi Khorasan Province, Iran

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Abstract

Introduction: Caseous Lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis*, is a serious infectious disease in small ruminants with limited treatment options and significant economic implications for livestock production.

Objective: This study investigated antibiotic resistance patterns and toxin production in resistant isolates of *C. pseudotuberculosis* based on the *Pld* gene in Razavi Khorasan Province.

Materials and Methods: *C. pseudotuberculosis* samples were systematically collected from 350 central slaughterhouses in Razavi Khorasan Province from small ruminant carcasses displaying characteristic lesions. Antimicrobial sensitivity testing, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) for commonly used antibiotics were determined using standardized protocols. ERIC-PCR was employed to identify the type of *Pld* gene among the isolated strains and compare the results with antibiotic sensitivity profiles to establish genotype-phenotype correlations.

Results: Out of the samples tested, 40 were confirmed to be infected with the target bacteria through cultivation on specific selective media and routine biochemical tests including catalase and urease assays. The isolated *corynebacteria* displayed varying degrees of resistance and sensitivity to the tested antibiotics with distinct patterns. Notably, the highest resistance rates were observed against vancomycin (75%), tetracycline (72.5%), and cefotaxime (60%). Among the isolates, 45.8% were classified as multi-drug resistant (MDR), representing a concerning public health issue. ERIC-PCR dendrogram analysis revealed significant genetic similarities between isolates from sheep and goats, suggesting potential cross-species transmission pathways.

Conclusion: This comprehensive study enhances the epidemiological understanding of *C. pseudotuberculosis* and highlights the importance of ERIC-PCR as a reliable

molecular method for genotyping and characterizing this pathogen, which can inform evidence-based selection of appropriate antibiotic treatments.

Keywords: Antimicrobial Agents; Caseous Lymphadenitis; *Corynebacterium pseudotuberculosis*; ERIC PCR.

1. Introduction

Skin abscesses in some ruminants are caused by *Corynebacterium pseudotuberculosis*, a pathogen sometimes mistaken for *Mycobacterium tuberculosis*, which primarily affects humans. Although human cases are rare, several reports suggest possible zoonotic risk [1]. In veterinary medicine, however, *C. pseudotuberculosis* is a major concern as the causative agent of caseous lymphadenitis (CLA), a chronic condition marked by abscess formation in lymph nodes and other tissues. Transmission generally occurs through abrasions or wounds, either from environmental sources or direct contact with infected animals, and insects such as flies can facilitate bacterial entry. Once the bacteria penetrate the skin, they can multiply and induce abscess formation as a result of the host's immune response. Characteristic abscesses are most often seen in the lymph nodes of small ruminants [2], especially in the parotid and retropharyngeal nodes and internal organs, though some infected sheep display only internal abscesses with minimal external symptoms. *C. pseudotuberculosis* has a broad host range, causing disease in cattle, horses, pigs, deer, camels, and laboratory animals [3]. In cattle, abscesses may form in the neck, shoulders, or other traumatized areas. Diagnosis relies mainly on clinical signs, with laboratory confirmation via culture and sensitivity testing of pus samples [4].

A central factor in the pathogenesis of *C. pseudotuberculosis* is the pld gene, which encodes phospholipase D (PLD), a potent exotoxin and the principal virulence determinant in this bacterium. PLD enzymatically hydrolyzes phospholipids (phosphatidylcholine and sphingomyelin) in host cell membranes, thereby disrupting membrane integrity. This activity not only leads to local tissue necrosis and the accumulation of pus but also promotes bacterial dissemination from primary infection sites to regional lymph nodes and internal organs. PLD also facilitates immune evasion by impairing host defense cells and contributes to the chronic nature of CLA [5–9]. Recent genomic studies have further identified additional virulence-associated factors, including the iron acquisition operon (fag A–D) [10]. Given its role in disease progression, the pld gene is a key target for molecular epidemiology, diagnosis, vaccine development, and novel therapeutic strategies in veterinary medicine [11–14].

Increased antibiotic resistance among *C. pseudotuberculosis* isolates particularly to commonly used drugs such as beta-lactams, macrolides, tetracyclines, and sulfonamides poses a growing challenge in livestock management [15–19]. Factors such as overuse of

antibiotics, genetic transfer of resistance genes, and insufficient management practices contribute to this trend [3, 16]. To counteract the spread of resistant strains and safeguard the health of affected animals, regular monitoring of antimicrobial susceptibility, prudent antibiotic use, and research into alternative treatments or vaccines are essential [5, 20]. Understanding the molecular and epidemiological characteristics of *C. pseudotuberculosis*, especially those related to the pld gene, is thus vital for effective disease control and prevention in veterinary practice.

2. Materials and Methods

2.1. Sample Collection and Bacterial Isolation

From April 2022 to February 2023, a total of 350 samples were collected from small ruminant carcasses (sheep and goats) at the central slaughterhouse of Razavi Khorasan province, Iran. Samples were obtained from carcasses presenting visible caseous abscesses in lymph nodes specifically from the submandibular, pharyngeal, mediastinal, pelvic mesenteric lymph nodes, and lymphatic vessels of motor organs in line with clinical suspicion of caseous lymphadenitis (CLA) [1,2]. Approximately 1 cm^3 of tissue was excised from the lesions using sterile technique and immediately transferred into sterile containers with transport medium at 4°C. All samples were transported to the microbiology laboratory of the Razi Vaccine and Serum Research Institute for processing within 24 hours [1,4].

Under aseptic conditions, each sample was inoculated onto Nutrient Agar and Brain Heart Infusion (BHI) Agar, then incubated aerobically and microaerophilically at 37°C for 48–72 hours. Suspected colonies were identified based on colony morphology and further confirmed by Gram staining and standard biochemical tests: oxidase, catalase, urease, nitrate reduction, motility, triple sugar iron (TSI) agar, and CAMP test.

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the Kirby–Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI VET01/S, 2023) guidelines. Bacterial suspensions were adjusted to 0.5 McFarland standard and spread onto Mueller Hinton agar plates. The following antibiotics and concentrations were used:

tetracycline (30 µg), penicillin (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amikacin (30 µg), chloramphenicol (30 µg), rifampicin (10 µg), and vancomycin (30 µg). After 18 hours incubation at 37°C, inhibition zones were measured and susceptibility was classified as sensitive, intermediate, or resistant according to CLSI breakpoints [10,18].

2.3. Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for all 40 confirmed *C. pseudotuberculosis* isolates using the broth microdilution method [10]. Antibiotics displaying the lowest and highest resistance rates in disk diffusion testing were selected for MIC/MBC analysis: tetracycline, vancomycin, ciprofloxacin, penicillin, rifampicin, and gentamicin. Serial two-fold dilutions of each antibiotic were prepared in Mueller Hinton broth across 96-well microtiter plates. Standardized bacterial suspensions (~10^5 CFU/mL) were added, and plates were incubated at 37°C for 24 hours. MIC was defined as the lowest concentration with no visible bacterial growth. For MBC determination, aliquots from wells with no growth were subcultured on BHI agar; MBC was defined as the lowest concentration with 99.9% reduction in CFU [10,18].

2.4. Statistical Analysis

To compare resistance rates between antibiotics, Chi-square or Fisher's exact tests were performed as appropriate, with statistical significance set at $p < 0.05$. Statistical analyses were carried out using SPSS software (version 26).

2.5. Molecular Detection of the *pld* Gene and ERIC-PCR Strain Typing

For molecular confirmation, DNA was extracted from bacterial cultures using the SinaClon extraction kit (Iran). The presence of the *pld* gene was detected by PCR using previously published primers (Table 1), with amplicons visualized by electrophoresis on 1% agarose gels. PCR reaction mixtures (25 µL) included 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, 1 U Taq polymerase, and 2 µL template DNA. Cycling conditions were: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; final extension at 72°C for 5 min [7].

ERIC-PCR was performed for strain typing using ERIC-1R and ERIC-2 primers. PCR products were resolved on 1.5% agarose gels and photographed. DNA banding patterns were analyzed using Dice similarity coefficients and dendrograms constructed with the unweighted pair group method with arithmetic mean (UPGMA) using GelJ software.

Table 1: Primers used in this research

		To check for the presence of toxins
Genes	**Primer Name**	**sequence (5-3)**
pld gene detection	*Pld* F	ATAAGCGTAAGCAGGGAGCA
	Pld R	ATCAGCGGTGATTGTCTTCCAGG
ERIC-PCR fingerprint	*ERIC* 1	ATGTAAGCTCCTGGGGATTCAC
	ERIC 2	AAGTAAGTGACTGGGGTGAGC

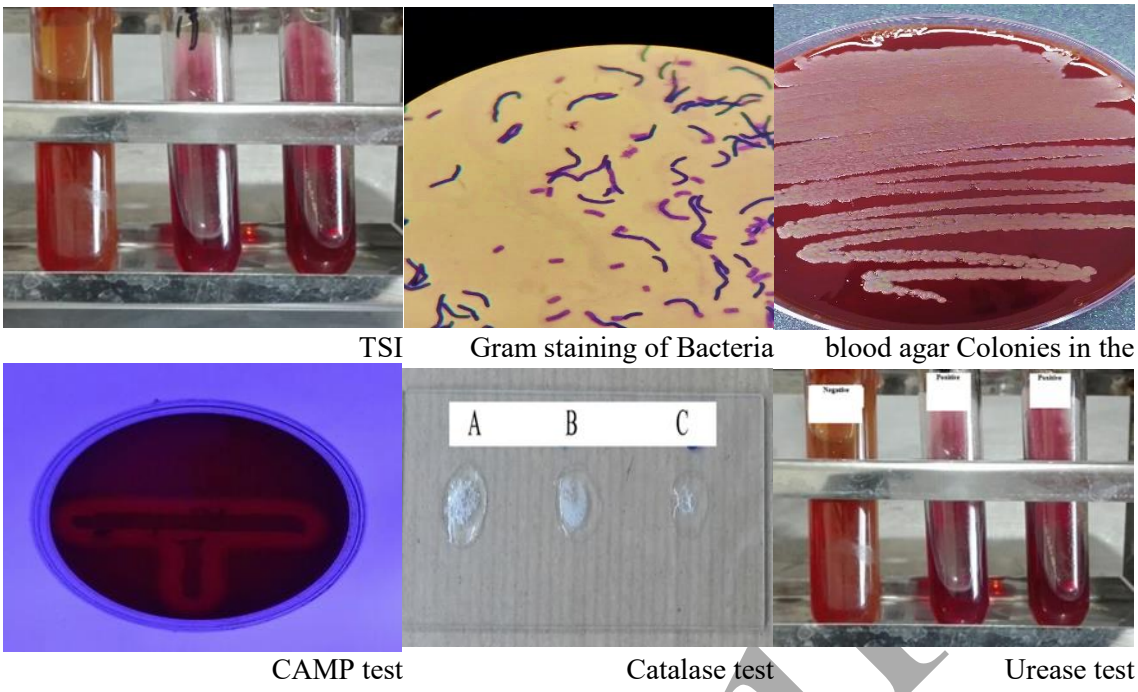
3. Results

3.1. Isolation and Identification of *Corynebacterium pseudotuberculosis*

Out of 350 clinical samples collected from the central slaughterhouse of Razavi Khorasan province, 40 (11.4%) were confirmed as *C. pseudotuberculosis* based on colony morphology and standard biochemical tests. Following 48–72 hours of incubation on blood agar, the colonies appeared small, dry, granular, and white to cream-colored, with a narrow zone of beta-hemolysis consistent with classic descriptions for this species.
A breakdown of isolates by anatomical source is detailed in Table 1, highlighting the tropism of *C. pseudotuberculosis* for specific lymph node groups in small ruminants.

Table 2. Distribution of positive *C. pseudotuberculosis* isolates by sample source

SAMPLE SOURCE	NUMBER OF SAMPLES	NUMBER POSITIVE	PERCENTAGE POSITIVE (%)
Submandibular lymph node	120	14	4.0
Pharyngeal lymph node	80	8	2.3
Mediastinal lymph node	80	7	2
Pelvic mesenteric lymph node	35	6	1.7
Lymphatic vessels of motor organs	35	5	1.4
Total	350	40	11.4



TSI Gram staining of Bacteria blood agar Colonies in the

CAMP test Catalase test Urease test

Figure 1. Set of biochemical and bacteriological tests performed to identify *C. pseudotuberculosis* strains

All 40 isolates were positive for catalase, urease, and nitrate reduction, and negative for oxidase and motility. These biochemical profiles summarized in Table 3 are consistent with the identification of *C. pseudotuberculosis*. Weak CAMP reaction was also observed in all isolates.

Table 3. Biochemical characteristics of *C. pseudotuberculosis* isolates

TEST	RESULT
Catalase	Positive
Urease	Positive
Nitrate reduction	Positive
Oxidase	Negative
Motility	Negative
CAMP	Weakly positive

3.2. Determining Microbial Sensitivity by Disc Diffusion Method

The antibiotic resistance of the strains was investigated using the disk diffusion method to determine the percentage of sensitivity and resistance to selected antibiotics. All

isolated *Corynebacterium* strains showed varying degrees of resistance or sensitivity to the tested antibiotics, as illustrated in Table 2, and Figure 2.

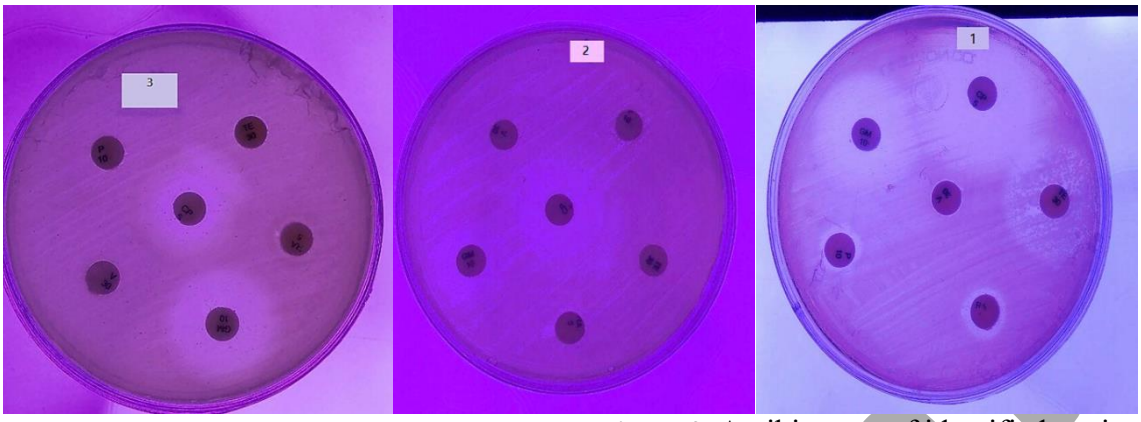


Figure 2. Antibiogram of identified strains

3.2. Antimicrobial Susceptibility Profiles

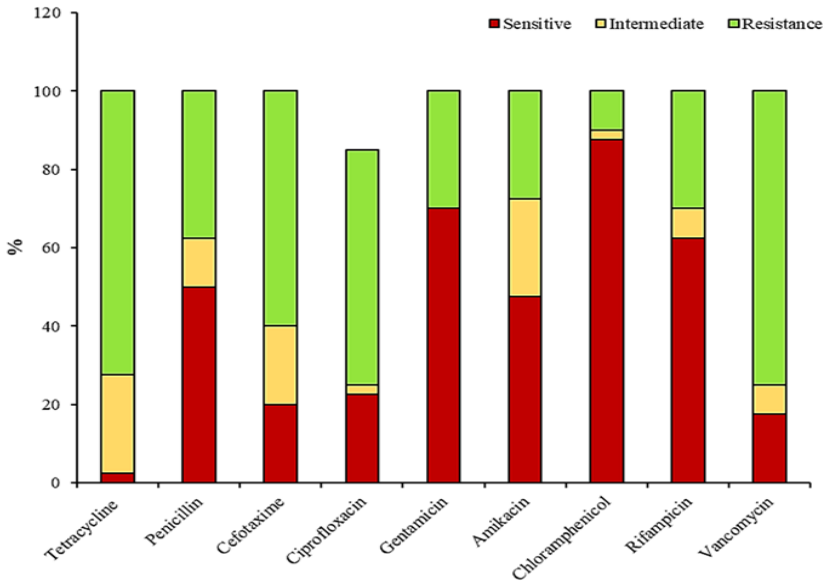
Disk diffusion testing revealed varying degrees of resistance to all antibiotics examined (Table 2). The highest rates of resistance were observed for vancomycin (75%), tetracycline (72.5%), and cefotaxime (60%), and the lowest for chloramphenicol.

Table 2. Antimicrobial susceptibility of isolated *C. pseudotuberculosis* (40 strains)

Antibiotics	**Antibiogram Results (%)**		
	Sensitive%	**Semi-sensitive%**	**Resistant%**
Tetracycline (TE)	2.5	25.0	72.5
Penicillin (PEN)	50.0	12.5	37.5
Cefotaxime (C)	20.0	20.0	60.0
Ciprofloxacin (CIP)	22.5	2.5	60.0
Gentamicin	70.0	-	30.0
Amikacin (Ak)	47.5	25.0	27.5
Chloramphenicol (CH)	87.5	2.5	10.0
Rifampicin (RIF)	62.5	7.5	30.0
Vancomycin (VAN)	17.5	7.5	75.0

Statistical analysis (Chi-square test) revealed that resistance to vancomycin, tetracycline, and cefotaxime was significantly higher compared to other antibiotics ($p = 0.0004$). Multidrug resistance (MDR, defined as non-susceptibility to ≥3 classes of antibiotics) was detected in 18/40 isolates (45%).

Figure 2. Determination of antibiotic sensitivity of clinical *C. pseudotuberculosis* strain



The antibiogram results from 40 *C. pseudotuberculosis* isolates revealed diverse resistance patterns among the isolates. Using this phenotypic method, resistance was observed in varying proportions. Notably, resistance to vancomycin, tetracycline and cefotaxime was observed in 75%, 72.5%, and 60% of the samples, respectively, showing significantly higher resistance levels (p-value = 0.0004). Among the *C. pseudotuberculosis* isolates, 45.8% (83/38) were identified as multidrug-resistant (MDR).

3.3. Determination of MIC and MBC of Antibiotics in Resistant Bacteria (MDR)

The MIC_{50}, MIC_{90}, MBC_{50}, and MBC_{90} values of each tested antibiotic are summarized in Table 3. These results reveal the relatively high resistance profiles among the MDR *C. pseudotuberculosis* isolates, especially to vancomycin, ciprofloxacin, penicillin, and gentamicin.

Tabele 3. MIC and MBC of Antibiotics

ANTIBIOTIC	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MBC_{50} (µg/ml)	MBC_{90} (µg/ml)
Tetracycline	1	4	16	32
Vancomycin	2	16	16	64
Ciprofloxacin	1	16	8	64
Penicillin	8	16	32	64
Rifampicin	1	16	4	16
Gentamicin	4	16	32	64

3.5. Molecular Study of C. pseudotuberculosis Strains Presence of Toxin Production Gene

PCR assays targeting the *pld* gene detected this gene in all 40 isolates (100%), confirming the genetic potential for phospholipase D (PLD) toxin production, although actual toxin expression or activity was not determined.

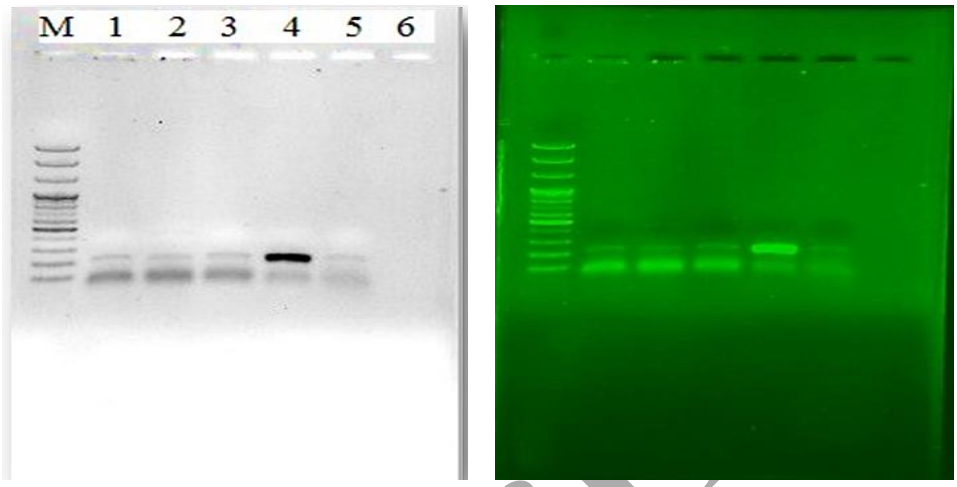


Figure 9: PCR amplification of the *pld* gene (203 bp) in representative clinical isolates. The 50 bp DNA marker is shown. Lanes 1–6 correspond to clinical samples that also exhibited resistance in antibiotic susceptibility testing. The presence of the 203 bp band confirms a positive result for the *pld* gene of *pld*

3.6. Genetic Diversity (ERIC-PCR Typing)

The assessment of genetic diversity by ERIC-PCR (Figure 10) grouped the 40 isolates into seven major clusters in the dendrogram. This clustering pattern reflects a moderate to high level of genetic diversity among the isolates, suggesting that these strains do not originate from a single source and may represent multiple epidemiological lineages.

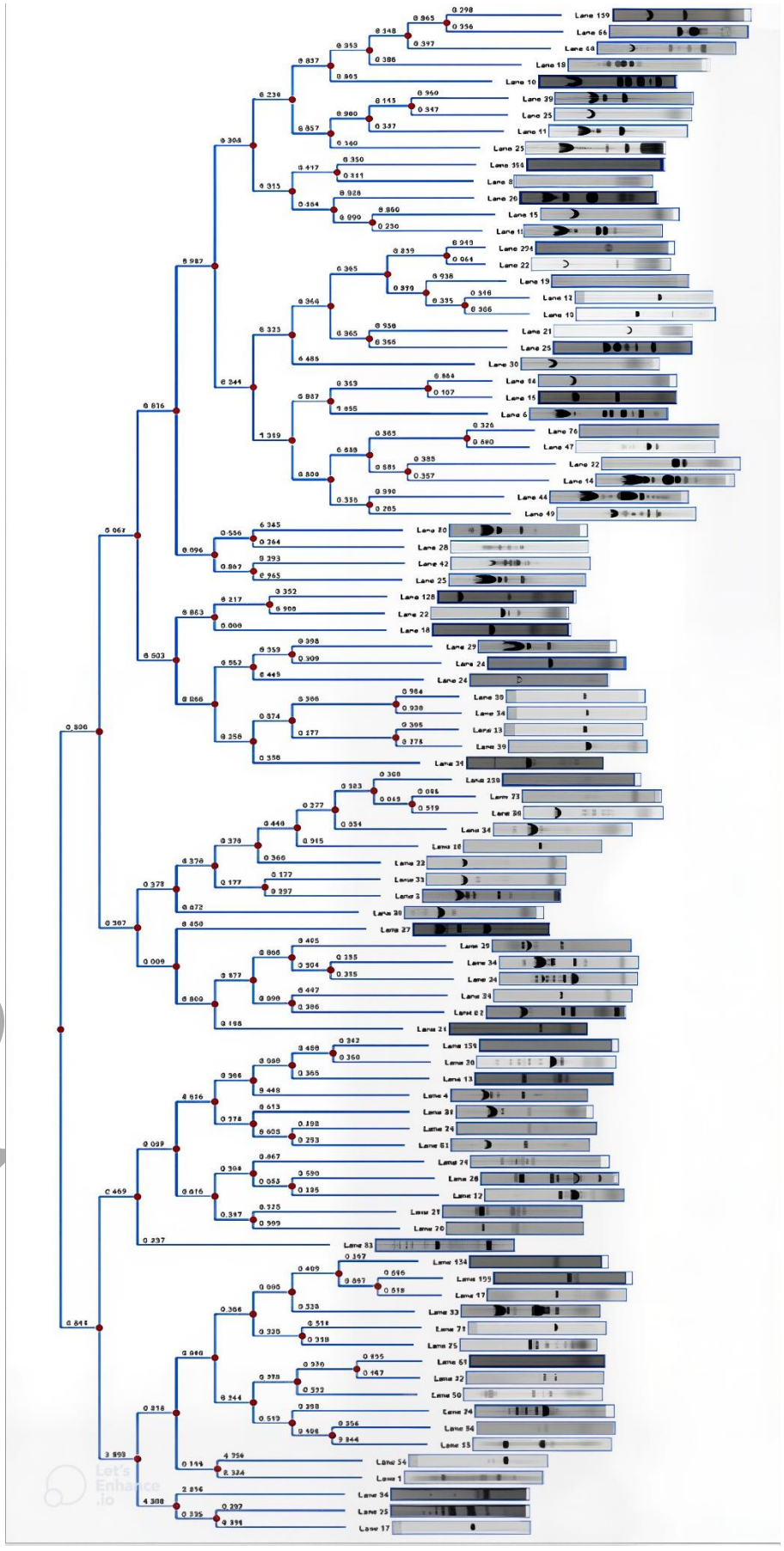


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Figure 10. ERIC-PCR isolate *C. pseudotuberculosis*. (left) ERIC-PCR fingerprint in agarose gel containing 1.5% agarose. (right) A dendrogram showing the association of the isolates.

4. Discussion

This study showed that *Corynebacterium pseudotuberculosis* is an important cause of caseous lymphadenitis (CLA) in small ruminants in Razavi Khorasan province, with a prevalence of 11.4% among clinically suspicious samples (Table 2). This finding is consistent with earlier studies from Iran and other countries, which reported prevalences ranging from 8% to 22% in similar populations [1,2]. The relatively high percentage observed in this study underlines the urgent need for increased clinical and laboratory vigilance in the region.

A major finding was the widespread antibiotic resistance among the isolates, especially to commonly used antibiotics such as vancomycin (75%), tetracycline (72.5%), and cefotaxime (60%) (Table 4, Figure 2). Notably, 45.8% of isolates were classified as multidrug resistant (MDR), surpassing the rates reported in some previous studies [10,17]. Such high resistance likely reflects the excessive or inappropriate use of antibiotics in livestock management in this province, a pattern that has also been observed elsewhere [3,16]. These resistance patterns complicate the treatment of CLA and signal the need for strict antibiotic stewardship and the development of alternative therapeutic strategies [18–20].

All 40 isolates in this study carried the pld gene, confirming the presence of the principal virulence factor phospholipase D in CLA strains [5–9,11,12]. The universal presence of pld underscores its critical role in the pathogenesis and transmission of C. pseudotuberculosis, supporting findings from previous molecular epidemiological research [10,14].

Molecular typing by ERIC-PCR showed that isolates from both sheep and goats were widely distributed across the dendrogram's clusters, with no clear separation by animal species or geographic origin (Figure 10). This suggests potential cross-species transmission of closely related strains, echoing similar observations by previous authors [15,17]. The high level of genetic similarity further points toward ongoing circulation of endemic strains among local herds, emphasizing the importance of comprehensive, multi-species control programs.

The persistence of CLA in endemic regions is partly attributed to the environmental resilience of *C. pseudotuberculosis*. The bacterium can survive in the environment for up to six months, while infected animals act as long-term carriers, spreading the agent through purulent secretions [1,3,21]. Ineffective vaccination, frequent physical trauma, and poor farm management further facilitate the maintenance and dissemination of infection [21,23]. The identification of similar ERIC-PCR patterns in this and other studies highlights the vital role of molecular methods for epidemiological tracking and outbreak control [7,15].

This study demonstrates that *Corynebacterium pseudotuberculosis* is a principal etiological agent of caseous lymphadenitis (CLA) among small ruminants in Razavi Khorasan province, with a prevalence of 11.4% in clinically suspicious samples. This rate is consistent with previous reports from Iran and other regions, where prevalence ranged from 8% to 22% (1,2). Differences in prevalence across studies may reflect variations in

sampling criteria, herd management, or control measures in different regions. A key finding of this study is the high level of antimicrobial resistance in local *C. pseudotuberculosis* isolates. The most notable resistance rates were observed to vancomycin (75%), tetracycline (72.5%), and cefotaxime (60%). The proportion of multidrug-resistant (MDR) isolates was 45%, which is higher than some domestic and international studies [10,17] but comparable to rates reported in settings with widespread antimicrobial usage [3,16]. These elevated resistance patterns likely result from excessive and, at times, indiscriminate antibiotic use in livestock, a phenomenon previously reported in Iran and other countries. Such levels of resistance complicate the clinical management of CLA and highlight the urgent need for systematic antibiotic stewardship and exploration of alternative treatments [18,20].

Genotypic analysis revealed that all isolates harbored the pld gene, confirming the presence of the major virulence determinant phospholipase D (PLD), in agreement with similar molecular epidemiology studies [5,9,11,12,14]. Although PCR analysis reliably determines the genetic potential for toxin production, it does not address actual pld gene expression or phospholipase D activity; further studies using gene expression assays or toxin detection methods would be required to fully characterize the virulence profile of isolates. This limitation must be considered when interpreting the clinical significance of the findings.

ERIC-PCR typing showed no clear separation of isolates by animal species or sampling location, and most clusters comprised strains from both sheep and goats. This pattern suggests potential interspecies transmission and circulation of endemic clones a finding echoed in previous regional and international studies [15,17]. Ongoing presence of closely related strains may be due to insufficient control measures, as well as the environmental robustness of *C. pseudotuberculosis*. Previous work has shown the bacterium can persist in the environment for months, with long-term carrier animals acting as important reservoirs [1,3,21]. Inadequate vaccination, physical trauma, and poor biosecurity greatly facilitate the perpetuation and spread of disease at the herd and regional levels [21,23].

In agreement with prior studies, the use of ERIC-PCR provided valuable insights into the genetic relatedness among isolates, facilitating epidemiological tracing and improved outbreak management [7,15]. Molecular typing should be considered an integral part of surveillance programs for CLA.

The main limitation of the present study is that genetic potential for toxin production was assessed based solely on the PCR detection of the pld gene; no assessment of actual gene expression or phospholipase D enzyme activity was performed. In addition, this study was limited to samples from one province; future research is needed to characterize the epidemiology and antibiotic resistance of *C. pseudotuberculosis* on a broader geographic scale.

5. Conclusion

In summary, our findings indicate that *C. pseudotuberculosis* remains a significant threat to the health and productivity of small ruminants in Razavi Khorasan province. The high prevalence of virulent, multidrug-resistant strains poses a formidable challenge to veterinary public health. It is therefore crucial to implement regular monitoring, enforce rational antibiotic use, strengthen farm biosecurity, and promote effective vaccination

strategies to limit the spread of CLA and its economic impact on the region's livestock industry.

Effective CLA control will require coordinated efforts: rigorous antimicrobial stewardship, regular epidemiological surveillance, improved farm biosecurity, and optimized vaccination strategies to reduce the burden and economic impact of this disease.

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Ethics

Not Applicable

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests statement

The authors declare that they have no conflict of interest.

Authors' contribution

AMAK Conducted data collection and drafted the manuscript. HRF Conceptualized and designed the initial study. ACHN and LM Provided consultation and guidance in drafting the manuscript. MJ Performed data analysis and interpretation of results. All authors reviewed and approved the final version of the manuscript.

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Data Availability

The datasets generated and analysed during the present study are not publicly accessible. They can be obtained only in coordination with the corresponding author, who will provide them upon reasonable request.

AI usage statement

"The authors used ChatGPT (OpenAI, GPT-4, March 2024 release) solely to assist with reference formatting and minor English-language editing. The tool was not employed for data generation, analysis, interpretation or scientific content creation, and all AI-suggested changes were reviewed and approved by the authors."

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