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ORIGINAL ARTICLE

Prevalence of Virulence-Related Genetic Determinants in *Corynebacterium pseudotuberculosis* Isolated from Suspected Ovine Caseous Lymphadenitis in Kerman Province, Iran

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Abstract

Caseous lymphadenitis is a chronic and contagious disease affecting sheep and goats worldwide, caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*). The pathogenicity of *C. pseudotuberculosis* is associated with several virulence genes, including *pld*, *hsp60*, *fagA*, *fagB*, *fagC*, *fagD*, *spaC*, *nanH*, and *sodC*. This study aimed to isolate *C. pseudotuberculosis* from lymph node samples of sheep suspected of caseous lymphadenitis (CLA) in Kerman Province, Iran, and to detect key virulence-associated genes using polymerase chain reaction (PCR). A total of 150 lymph node samples were collected from sheep with suspected CLA. After bacterial culture and purification, identification was supported by biochemical assays such as catalase production, nitrate reduction, urease activity, and the reverse CAMP test with *Staphylococcus aureus*. Isolates identified as *C. pseudotuberculosis* were further analyzed by PCR for the presence of *pld*, *hsp60*, *fagA*, *fagB*, *fagC*, *fagD*, *spaC*, *nanH*, and *sodC* virulence genes. Out of 150 samples, 17 were found to be infected with *C. pseudotuberculosis*. PCR results showed that among the 17 isolates, all isolates (100%) possessed the *hsp60*, *fagA*, *fagB*, *fagC*, *spaC*, *nanH*, and *sodC* genes; 15 isolates (88.23%) harbored the *fagD* gene and 14 (82.35%) carried the *pld* gene;. The high prevalence of multiple virulence genes among the *C. pseudotuberculosis* isolates highlights their potential role in the pathogenicity of CLA. These gene profiles may also inform future strategies for vaccine development and disease control.

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Introduction

Caseous lymphadenitis (CLA), caused by *C. pseudotuberculosis*, is a chronic contagious infectious disease primarily affecting sheep and goats. It results in considerable economic losses in small ruminant industries worldwide, especially in regions where sheep and goat farming are prevalent (1, 2). CLA negatively impacts wool, meat, and milk production, carcass quality, hide integrity, and herd fertility (3). Clinically, CLA typically manifests as abscesses in both superficial and internal lymphatic tissues, often leading to chronic emaciation in affected animals (4). CLA occurs in two clinical forms: external and internal. The external form affects superficial lymph nodes such as auricular, preauricular, submandibular, submental, and anterior cervical nodes. These nodes often become thick-walled, containing greenish, caseous pus (5). The internal form involves deeper lymph nodes and organs and may present with signs such as mastitis, respiratory distress, persistent coughing, and neurological symptoms, depending on the organ system involved (3, 5). The bacterium typically gains entry through skin abrasions caused by shearing, insect bites, or contaminated equipment, as well as via mucous membranes, inhalation, and ingestion (5, 6). Although the mortality rate is generally low, the disease has a high infection rate and a chronic course. In addition to sheep and goats, *C. pseudotuberculosis* has been isolated from cattle, horses, camels, deer, and even humans, indicating its zoonotic potential (2). Human infection can occur through direct contact with infected animals, contaminated environments, or ingestion of infectious materials (7).

The virulence of *C. pseudotuberculosis* is attributed to several well-characterized genes, including *pld*, *hsp60*, *fagA*, *fagB*, *fagC*, *fagD*, *spaC*, *nanH*, and *sodC*. These genes contribute to tissue invasion, immune evasion, and survival within host macrophages (8-10).

One of the key virulence factors of *C. pseudotuberculosis* is phospholipase D (PLD), a protein exotoxin that hydrolyses sphingomyelin in mammalian cell membranes. This activity increases vascular permeability and facilitates bacterial dissemination. PLD also contributes to endothelial cell damage, red blood cell lysis, phagocyte death, skin necrosis, and immune evasion by complement inactivation (10, 11). This gene is a major exotoxin that facilitates bacterial dissemination by hydrolysing sphingomyelin in host cell membranes, contributing to tissue necrosis and inflammation (1, 12). Another important virulence component is heat shock protein 60 (Hsp60), which induces an immune response by recruiting leukocytes to the infection site (8). *Hsp60* plays a crucial role in stress response and has immunogenic properties that contribute to immune modulation (8, 13).

Although the bacterium exhibits anti-phagocytic properties via its lipid coat, once internalized, it escapes the phagosome, replicates, and eventually causes phagocyte death, contributing to the formation of the characteristic abscesses of CLA (13). In addition, several genes located within the fag operon including *fagA*, *fagB*, *fagC*, and *fagD* encode proteins involved in iron acquisition, a critical factor for bacterial survival and virulence. These include an integral membrane protein (FagA), an iron enterobactin transporter (FagB), an ATP-binding membrane protein (FagC), and a siderophore-binding protein (FagD). The operon is situated downstream of *pld* and is upregulated under iron-limiting conditions, such as those encountered within host tissues. Although poorly expressed in vitro, these genes are significantly upregulated in vivo and enhance pathogenic potential. The *fag* gene cluster is essential for iron acquisition in the iron-limited environment of host tissues and is widely conserved in pathogenic isolates (9).

Other virulence determinants include *spaC*, which encodes the pilus-associated Spa protein. SpaC acts as a tip adhesin, mediating initial interactions between bacterial pili and host cell receptors, thus facilitating adhesion and intracellular invasion. The presence of *spaC*, which encodes a surface adhesion protein, supports the bacteria's ability to adhere to and colonize host cells (13).

The *nanH* gene encodes neuraminidase (sialidase), an enzyme that promotes host cell recognition and colonization. NanH, a neuraminidase, facilitates tissue invasion by cleaving sialic acid residues from host glycoproteins (14). The *sodC* gene encodes a membrane-bound, zinc-dependent superoxide dismutase that protects the bacterium against oxidative stress by neutralizing reactive oxygen species produced by host immune cells (15).

CLA is particularly challenging to control due to the intracellular survival of the bacterium, the formation of abscesses that limit antibiotic efficacy, and the chronic, recurrent nature of infection (11).

The present study aimed to detect the presence of the virulence-associated genes *pld*, *hsp60*, *fagA*, *fagB*, *fagC*, *fagD*, *spaC*, *nanH*, and *sodC* in *C. pseudotuberculosis* isolates obtained from lymph node samples of sheep suspected of having caseous lymphadenitis. Given the role of these genes in virulence and host-pathogen interaction, they represent promising targets for vaccine development and diagnostic tool refinement. The rationale for selecting

the sampling site was the development of a vaccine for sheep in that region.

Material and Methods

Sample Collection and Bacterial Culture

The sample size was estimated based on the expected prevalence of virulence-associated genes reported in previous studies (approximately 80%) using the standard prevalence estimation formula, $n = Z^2P(1-P)/d^2$, with a 95% confidence level. A total of 150 lymph node samples were collected from sheep suspected of cases of CLA from various slaughterhouses in Kerman Province, Iran. Animals showing clinical signs such as enlarged superficial lymph nodes, abscess formation, purulent discharge, or characteristic caseous lesions were included in the study. The specimens were maintained on ice during transportation to the Microbiology Laboratory of Shahrekord University. Upon arrival, the surface of each lymph node was disinfected, followed by an incision to extract purulent material. The purulent material from the lymph nodes was streaked onto sheep blood agar plates and incubated at 37°C for 48 hours. Colony purification was performed by repeated subculture of morphologically typical colonies. Resulting colonies were evaluated based on morphological characteristics and haemolytic activity. Gram staining was subsequently performed on smears prepared from the colonies. Suspected isolates were then sub-cultured for biochemical and molecular identification (16).

Biochemical Identification

To confirm the identity of *C. pseudotuberculosis*, a series of biochemical tests was conducted, including catalase activity, nitrate reduction, urease production, and the reverse CAMP (Christie–Atkins–Munch-Peterson) test using *Staphylococcus aureus* (ATCC 25923) as a reference strain (16).

DNA Extraction and PCR

For the molecular identification of virulence-associated genes, genomic DNA was extracted from pure bacterial colonies using the boiling method. Briefly, 3–4 colonies were suspended in 100 µL of distilled water and subjected to heat treatment at 100°C for 10 minutes. The lysate was centrifuged at 7000 × g for 10 minutes, and the supernatant, containing bacterial DNA, was used for PCR (17). The concentration and purity of the extracted DNA were measured at 260/280 nm using a NanoDrop spectrophotometer (Eppendorf, Germany), with a purity ratio of <1.8 considered acceptable.

PCR amplification was performed to detect nine virulence-related genes using specific primers. Gradient PCR was initially conducted to determine optimal annealing temperatures, and the final thermal cycling conditions for each gene are summarized in Table 1. Each 25 µL PCR reaction contained 12.5 µL of 2X PCR Master Mix (GeneDireX, Inc., Taiwan), 1 µL (10 pmol) of each forward and reverse primer (Metabion, Germany), 3 µL (10 pg) of template DNA, and 7.5 µL of nuclease-free water (18, 19). In each PCR assay, a reference strain of *C. pseudotuberculosis* (ATCC 19410) was used as the positive control, whereas sterile nuclease-free water served as the negative control.

The PCR products were electrophoresed on a 1.5% agarose gel containing a safe stain (Yekta Tajhiz Azma, Iran), and the bands were visualized using a gel documentation system (Uvitec, England). Distilled water was used instead of DNA as the negative control.

Statistical Analysis

All data were entered and analyzed using Microsoft Excel (Microsoft Corporation, 2018). Descriptive statistics were employed to determine the frequency and percentage of *C. pseudotuberculosis* isolation from lymph node samples, as well as the distribution of each virulence-associated gene (*pld*, *hsp60*, *fagA*, *fagB*, *fagC*, *fagD*, *spaC*, *nanH*, and *sodC*) among the confirmed isolates using the following formula:

Gene frequency (%) = (Number of positive isolates/Total number of isolates) ×100

The 95% confidence interval (CI) for the proportion was calculated using the following binomial distribution formula:

$$95\%\ CI = P \pm 1.96\sqrt{P(1-P)/n}$$

p = proportion of positive isolates
n = total number of isolates
1.96 = Z value for 95% confidence level

Table 1. Primer sequences and thermal cycling conditions used for the detection of *Corynebacterium pseudotuberculosis* virulence genes by PCR.

Gene	Sequence 5` to 3`	Thermal cycles	PCR product size (bp)	References
hsp60	F: 5'GATGGCAAAGCTGATTGCA'3 R: 5' TTAGTGGTGGTGATGGTG'3	95°C, 5 min → 40× (95°C, 30 s; 50°C, 45 s; 72°C, 2 min) → 72°C, 10 min	1621	19
plD	F: 5'ATGAGGGAGAAAGTTGTTTTA'3 R: 5'TCACCACGGGTTATCCGC'3	95°C, 5 min → 35× (95°C, 30 s; 60°C, 30 s; 72°C, 60 s) → 72°C, 10 min	924	18
*fag*A	F: 5'AGCAAGACCAAGAGACATGC'3 R: 5'AGTCTCAGCCCAACGTACAG'3	95°C, 5 min → 35× (95°C, 40 s; 58°C, 40 s; 72°C, 40 s) → 72°C, 10 min	245	
*fag*B	F: 5'GTGAGAAGAACCCCGGTATAAG'3 R: 5'TACCGCACTTATTCTGACACTG'3	95°C, 5 min → 38× (95°C, 40 s; 58°C, 40 s; 72°C, 40 s) → 72°C, 10 min	291	
*fag*C	F: 5'GTTTGGCTATCTCCTTGGTATG'3 R: 5'CGACCTTAGTGTTGACATACCC'3	95°C, 5 min → 35× (95°C, 40 s; 61°C, 40 s; 72°C, 40 s) → 72°C, 10 min	173	
*fag*D	F: 5'GAGACTATCGACCAGGCAGA'3 R: 5'ACTTCTTGGGGAGCAGTTCT'3		225	
nanH	F: 5'TGGCTAAGGCTATTGAAGACGCGA'3 R:5'AATCGTAGCTCCCTCAGCTGCTTT'3	95°C, 5 min → 35× (95°C, 40 s; 62°C, 40 s; 72°C, 40 s) → 72°C, 10 min	215	19
spaC	F: 5'AAGCCGTCAACCGACTACCGTTTA'3 R:5'AATTCAAACCGGTAGGAGCCAGGA'3		243	
sodC	F: 5'TTGTCGCGGCCTTCATGAACAATC'3 R: 5'CCATGGCTTCCACGTTCATGACAA'3		243	

Results

Following the cultivation of 150 lymph node samples, 17 produced white, convex, opaque, and brittle colonies (Figure 1). Gram staining revealed gram-positive, pleomorphic bacteria. Catalase, nitrate reduction, and urease tests were positive, and inhibition of hemolysis was observed in the reverse CAMP test with *Staphylococcus aureus* (Figure 1).

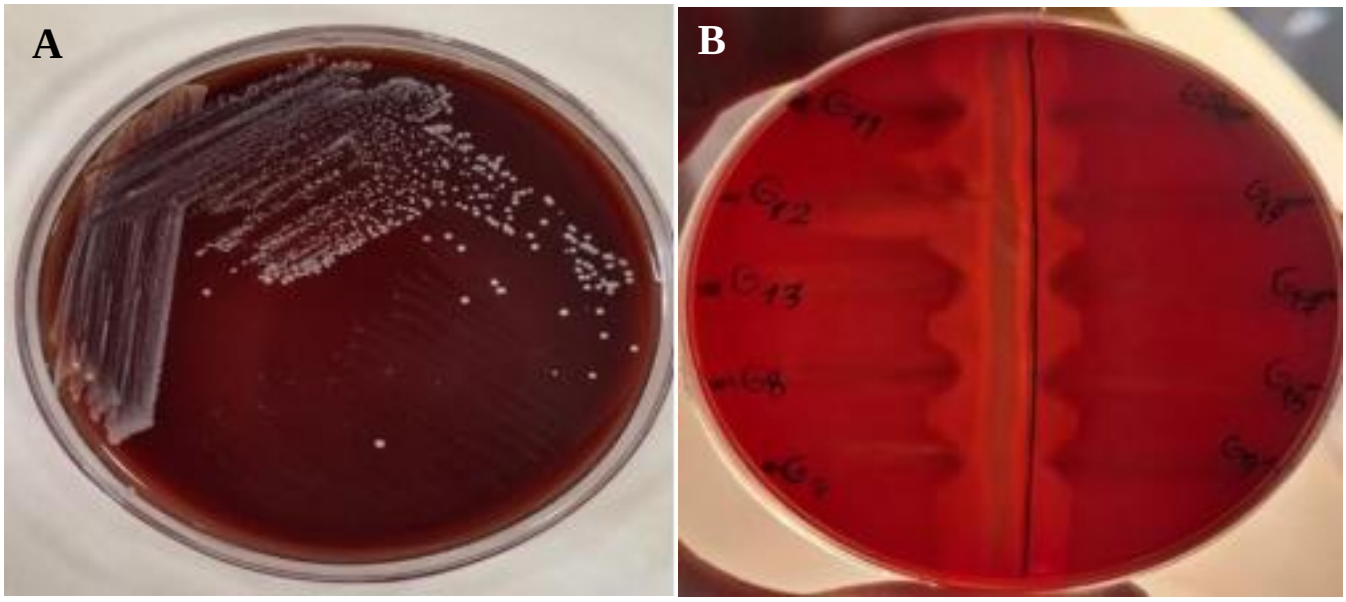

Figure 1. White, convex, opaque, and brittle colonies of *Corynebacterium pseudotuberculosis* on blood agar media (A). Reverse CAMP test. Inhibition of *Corynebacterium pseudotuberculosis* hemolysis by *S. aureus*. *S. aureus* has been cultured in the center of the plate, and suspicious samples have been cultured perpendicular to it (B).

Seventeen morphologically and biochemically confirmed isolates were subjected to PCR for virulence gene detection. The results showed that all isolates (100%) (95% CI: 80.5–100) were positive for *hsp60*, *fagA*, *fagB*, *fagC*, *nanH*, *spaC*, and *sodC* genes, 15 isolates (88.23%) (95% CI: 63.6–98.5) possessed the *fagD* gene and 14 isolates (82.35%) (95% CI: 56.6–96.2) carried the *pld* gene gene (Table 2 and Figures 2–4).

Among the 17 isolates, 12 were positive for all the virulence-associated genes analyzed in this study. Three

isolates were positive for all genes except *pld*, indicating possible genetic variation or deletion of this key virulence factor. Additionally, two isolates were positive for all genes except *fagD*, suggesting slight variability within the fag operon among local strains. These findings highlight both the overall conservation of virulence genes in the population and the presence of minor variations that may influence pathogenic potential.

Table 2. Prevalence of virulence-related genes in 17 isolates of *Corynebacterium pseudotuberculosis* from suspected ovine caseous lymphadenitis in Kerman Province, Iran.

Genes	Number of positive isolates	Prevalence (%)	Lower 95% confidence interval	Upper 95% confidence interval
hsp60	17	100.00	80.5	100
fagA	17	100.00	80.5	100
fagB	17	100.00	80.5	100
fagC	17	100.00	80.5	100
nanH	17	100.00	80.5	100
spaC	17	100.00	80.5	100
sodC	17	100.00	80.5	100
fagD	15	88.23	63.6	98.5
pld	14	82.35	56.6	96.2

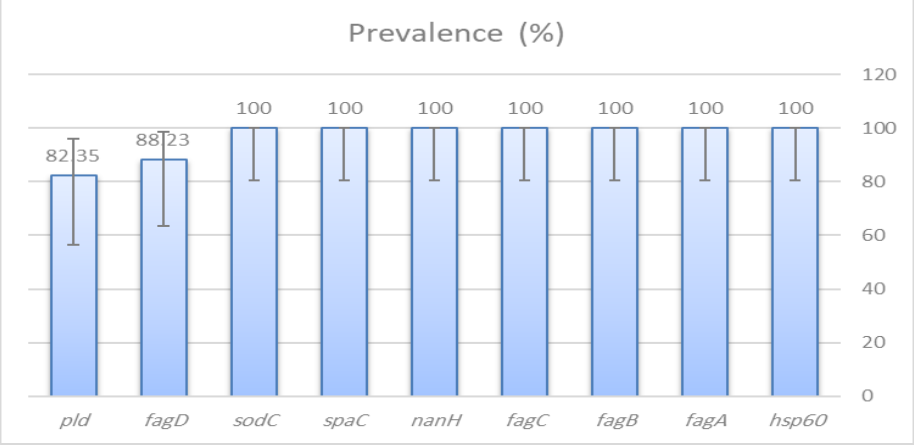

Figure 2. Distribution of virulence-associated genes among *Corynebacterium pseudotuberculosis* isolates recovered from ovine caseous lymphadenitis in Kerman Province, Iran. Error bars indicate 95% confidence intervals.

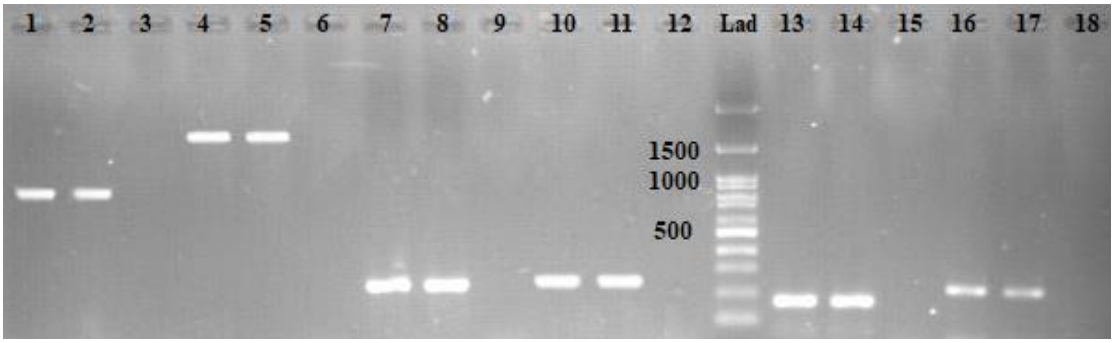

Figure 3. Agarose gel electrophoresis of PCR-amplified products for the *pld* (lanes 1–3, 924 bp), *hsp60* (lanes 4–6, 1621 bp), *fagA* (lanes 7–9, 245 bp), *fagB* (lanes 10–12, 291 bp), *fagC* (lanes 13–15, 173 bp), and *fagD* (lanes 16–18, 225 bp) genes.
Lad: 100-bp DNA ladder; Lanes 1, 4, 7, 10, 13, and 16: DNA from isolates of *Corynebacterium pseudotuberculosis*; Lanes 2, 5, 8, 11, 14, and 17: positive controls; Lanes 3, 6, 9, 12, 15, and 18: negative controls for the corresponding

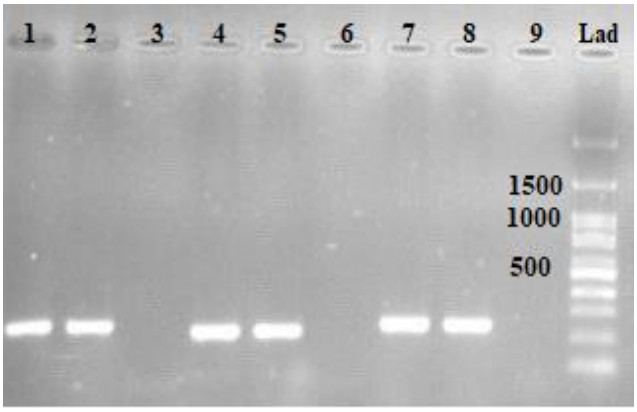

Figure 4. Agarose gel electrophoresis of PCR-amplified products for the *spaC* (lanes 1–3, 243 bp), *nanH* (lanes 4–6, 215 bp), and *sodC* (lanes 7–9, 243 bp) genes.
Lad: 100-bp DNA ladder; Lanes 1, 4, and 7: DNA from isolates of *Corynebacterium pseudotuberculosis*; Lanes 2, 5 and 8: positive controls; Lanes 3, 6, and 9: negative controls for the corresponding genes.

Discussion

In this study, *C. pseudotuberculosis* was successfully isolated from 17 out of 150 lymph node samples collected from sheep with suspected CLA in Kerman Province, Iran. The isolation rate (11.3%) aligns with earlier findings in endemic areas, indicating a high circulation of this pathogen in the region (20). This moderate prevalence suggests that CLA remains a persistent challenge in local flocks, potentially influenced by husbandry practices, animal movement, and environmental factors that favor bacterial transmission. All isolates were confirmed via both biochemical testing and PCR-based detection of virulence-associated genes, supporting the reliability of the identification process. The use of multiple confirmation methods strengthens confidence in the reported prevalence and ensures that both classical and molecular diagnostic criteria are met.

The *pld* gene, encoding phospholipase D, was detected in 14 out of 17 isolates (82.35%). While many studies have reported 100% prevalence of this gene among isolates (18, 21), the slightly lower frequency observed here might reflect strain variability, local epidemiological differences, or PCR sensitivity limitations. The presence of pld in the majority of isolates emphasizes its central role in tissue necrosis, lymph node dissemination, and virulence, confirming its importance as a key marker for pathogenic potential.

All isolates were positive for *hsp60*, *fagA*, *fagB*, *fagC*, *spaC*, *nanH*, and *sodC*, emphasizing their critical roles in bacterial survival and pathogenesis. Notably, *fagD* was detected in 15 of 17 isolates (88.23%), showing slight variability consistent with previous reports, such as Guerrero et al. (2018), who found *fagD* in 93% of isolates (22). While the core fag operon (A–C) is highly conserved, the partial presence of *fagD* could indicate deletions, point mutations, or regulatory differences in local strains (22). These variations may influence iron acquisition efficiency, intracellular survival, and overall pathogenicity.

The consistent detection of *sodC* in all isolates is particularly noteworthy, as this gene encodes a copper-zinc superoxide dismutase that neutralizes reactive oxygen species produced by host macrophages (23). This function is crucial for intracellular survival and persistence of *C. pseudotuberculosis* in chronic infections, highlighting *sodC* as a potential target for therapeutic interventions.

Comparison with global studies reinforces the significance of our findings. For example, Pathirana et al. (2022) identified 19 virulence genes in *C. pseudotuberculosis* isolates from goats in Sri Lanka, reporting universal presence of *pld*, *fagA–D*, *spaC*, *nanH*, and *sodC* (21). Similarly, Sá et al. (2013) reported 100% prevalence of *pld*, *fagA*, and *fagB* in sheep and goat isolates in Brazil, with slightly lower frequencies for *fagC* and *fagD* (18). These similarities suggest a conserved virulence gene profile among highly pathogenic strains worldwide, despite geographical separation.

The prevalence of multiple virulence genes in our isolates not only serves as an indicator of pathogenic potential but also has practical implications. Several studies have explored the development of recombinant or subunit vaccines containing *pld*, *fagA*, *sodC*, or *spaC*, which demonstrated promising protective immunity in animal models (24-27). Therefore, the high prevalence of these genes in the current study supports their use as vaccine candidates in regional control programs. Additionally, the conserved nature of these genes makes them suitable targets for molecular diagnostics, enabling rapid and accurate identification of CLA in flocks.

Finally, our findings highlight the need for continued surveillance of CLA in Iran. Regional variations in virulence gene profiles and prevalence rates underscore the importance of tailored control strategies, including vaccination, management practices, and early detection. Future studies should also explore genomic characterization of local strains to identify potential novel virulence factors, antimicrobial resistance patterns, and evolutionary dynamics, further informing disease control efforts.

Conclusion

This study highlights the high prevalence of virulence-associated genes in *C. pseudotuberculosis* strains isolated

from sheep in Kerman Province. The gene profile supports their key role in the pathogenicity of CLA and affirms their value in guiding vaccine strategies and molecular diagnostic tool development. Future studies involving whole-genome sequencing, gene expression profiling, and immunological assessments will further enhance our understanding of the pathogen's behaviour and inform regional disease management.

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Authors' Contributions

Hossein Ghaderpoori: Investigation, Formal Analysis, Data Curation, Writing – Original Draft, Writing – Review & Editing. **Masoud Ghorbanpoor:** Conceptualization, Project Administration, Methodology, Supervision, Investigation, Formal Analysis, Data Curation, Writing – Original Draft, Writing – Review & Editing. **Azam Mokhtari:** Investigation, Formal Analysis, Data Curation, Writing – Original Draft, Writing – Review & Editing. **Mohammad Mehdi Namavari:** Investigation, Formal Analysis, Data Curation, Writing – Original Draft, Writing – Review & Editing. All authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this published manuscript.

Ethical Approval

The present study was conducted in accordance with ethical principles and approved by the Ethics Committee of Shahrekord University (Ethics Code: IR.SKUMS.REC.1404.006).

Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

Not applicable.

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