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Establishment and Application of a SYBR Green I qPCR Detection Method Based on the *CP40* Gene of *Corynebacterium pseudotuberculosis* Biovar Ovi

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Abstract

Caseous lymphadenitis (CLA), an infectious disease caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*), poses a significant economic burden to the global small ruminant industry. This study aimed to investigate genetic variations in the *CP40* gene of *C. pseudotuberculosis* and to develop a rapid detection assay for enhanced pathogen identification. Homology analysis was performed to compare the *CP40* gene sequence of the FJ-PN strain with other *Corynebacterium* species. Specific primers targeting *CP40* were designed, and a SYBR Green I-based real-time PCR protocol was optimized. The assay's specificity, sensitivity, and reproducibility were subsequently validated. The FJ-PN strain exhibited ≥99.65% nucleotide identity and ≥98.94% amino acid identity with *C. pseudotuberculosis* biovar ovi reference strains, showing 90.18–91.84% nucleotide identity and 88.63–90.77% amino acid identity with *C. pseudotuberculosis* biovar equi, and ≤82.71% nucleotide identity and ≤78.63% amino acid identity with other *Corynebacterium* species. The established qPCR assay demonstrated high specificity, the limit of detection was 52 copies/μL, and it demonstrated good reproducibility (intra- and inter-assay CV < 1.0%). Clinical sample testing revealed 18.8% positivity rates in nasal swabs, which was higher than that detected by conventional PCR (16.3%). These results indicate that the *CP40* gene is evolutionarily conserved and represents a specific molecular marker for the identification of *C. pseudotuberculosis* biovar ovis. The developed SYBR Green I real-time PCR assay enables the efficient detection of *C. pseudotuberculosis* and provides technical support for CLA surveillance and control.

Keywords: *Corynebacterium pseudotuberculosis biovar ovi;* CP40 *gene; SYBR Green I fluorescent* quantitative PCR

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1. Introduction

Corynebacterium pseudotuberculosis (*C. pseudotuberculosis*) is a Gram-positive bacterium classified within the class Actinobacteria, order Mycobacteriales, family Corynebacteriaceae, and genus *Corynebacterium*. It was predominantly detected in goat and sheep populations and poses substantial economic burdens to global small ruminant husbandry systems. This pathogen exhibits zoonotic potential with a broad host range encompassing camels, equines, bovines, and rodents, and is characterized by chronic infectious manifestations. Based on the nitrate reduction test, *C. pseudotuberculosis* can be divided into biovar ovis

and biovar equis. In small ruminants, *C. pseudotuberculosis* biovar ovis induces caseous lymphadenitis (CLA), which primarily presents as chronic or subclinical infection [1]. CLA pathology results in measurable reductions in carcass yield, lactation performance, and fiber production in affected animals. Clinically, CLA abscesses are categorized based on anatomical localization into superficial, visceral, and mixed types. Once inter-animal transmission is established within agricultural livestock populations, the eradication of *C. pseudotuberculosis* becomes substantially challenging. Infected hosts continuously shed viable pathogens into the environment [2]. Furthermore, the distinctive cell wall architecture of *C. pseudotuberculosis* confers prolonged environmental persistence, thereby facilitating recurrent infection cycles among animal populations [3]. Notwithstanding the low mortality incidence associated with CLA, clinical interventions remain inefficient due to the pathogen's intracellular survival strategies. Unchecked epidemic progression can culminate in herd-wide infection prevalence, thereby incurring substantial economic ramifications for livestock producers [4].

CLA is widespread in goat and sheep farms globally and exhibits a high prevalence in many regions [5,6], with reports from Europe, America, Australia, Africa, and Asia. The average incidence rate in Portugal is 34% [7], while the prevalence in adult sheep in Canada is approximately 36% [8], and in Western Australia, it exceeds 20% [9]. In Ethiopia, the prevalence rate of CLA was 10.68%, with an isolation rate of *C. pseudotuberculosis* reaching 72% [10]. In an epidemiological survey conducted on dairy goat farms in Shaanxi Province of China, the antibody level testing revealed a *C. pseudotuberculosis* seropositivity rate of 16–34% [11]. Currently, there are no effective treatments for CLA, and the diseased animals which were unaffected by treatment or converted from body surface to visceral type should be eliminated in time. Therefore, the development of robust diagnostic methodologies for the early detection and isolation of infected animals is urgently needed. It is worth mentioning that biochemical tests for identifying *C. pseudotuberculosis* are unreliable due to heterogeneity among some *Corynebacterium* species. In addition, variations in laboratory conditions, operating techniques, and the inherent limitations of biochemical assays may alter the biochemical test results of the same strain [12]; therefore, biochemical tests are rarely used to identify *Corynebacterium* at present. Established detection methods include serological and molecular biological approaches [13,14]; however, there is no report of a SYBR Green I qPCR detection method for *C. pseudotuberculosis*, which offers simplicity, high sensitivity, and cost-effectiveness. Meanwhile, a key challenge is to avoid nonspecific amplification of biovar equis on the qPCR platform and improve the accuracy of the method.

Corynebacterial protease 40 (CP40) is a secreted serine protease encoded by the *CP40* gene of *C. pseudotuberculosis* and has been shown to confer robust protective immunity against CLA in ovine vaccination models [15]. Recent phylogenetic classification places CP40 within the GH18 family of endoglycosidases [5], and it is among the most extensively characterized virulence factors of *C. pseudotuberculosis*.

Bioinformatic analysis of 90 complete genome sequences derived from goat and sheep *C. pseudotuberculosis* strains reveals universal conservation of the *CP40* gene locus with high sequence homology across isolates. In this study, the *CP40* gene was selected as the target sequence, and a conserved region was identified through phylogenetic analysis to establish a SYBR Green I-based qPCR assay for the specific detection of *C. pseudotuberculosis*, thereby providing a technical foundation for pathogen surveillance and epidemiological investigation.

2. Materials and Methods

2.1. Strain and Specimens

The standard strains were *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) biovar equis (CCUG43567) and *Staphylococcus aureus* (*S. aureus*) (ATCC25923). *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) biovar ovis FJ-PN strain, *Pasteurella multocida* (*P. multocida*), *Mannheimia haemolytica* (*M. haemolytica*), and *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) were all identified and preserved by the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agricultural Sciences [16]. The sample collection phase of the epidemiological investigation was conducted from March 2023 to December 2023 across a total of eight goat farms in Fujian Province. All biological specimens were transported under refrigerated conditions with ice packs to the veterinary research institute within an 8 h post-collection timeframe for subsequent laboratory processing. All experimental manipulations were conducted in the biosafety level-2 (BSL-2) laboratory.

2.2. Main Reagents and Consumables

The Real-Time Fluorescence Quantification Kit qPCR SuperMix Universal (item number 11762100) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The nucleic acid extraction kit EasyPure Bacteria Genomic DNA Kit (item number EE161-01), PCR amplification kit 2 × TransTaq-T PCR SuperMix (item number AS122), T cloning vector kit pMD19-T Vector Cloning Kit (item number 6013), gel recovery kit Quick Gel Extraction Kit (item number EG101), and plasmid small-scale extraction kit Plasma MiniPrep Kit (item number EM101) were obtained from Beijing Quanshijin Biotechnology Co., Ltd. (Beijing, China). The fluorescent quantitative eight-row tube (PCR-0208-C) was purchased from Axygen (New York, NY, USA). Conventional chemical reagents and consumables were acquired from Sangon Biotech Co., Ltd. (Shanghai, China).

2.3. Cloning of Target Genes

2.3.1. Primer Design

To clone the target gene, specific primers were designed based on the target sequence. This study referred to the *CP40* gene sequences of *C. pseudotuberculosis* strains 2J-L (OL347712) and MEX29 (CP016826) registered on Genbank, and a pair of specific primers were designed using Primer Premier 5.0 software. The primers were named CP-FJF1 and CP-FJR1, with sequences of 5′-CTTCGGCTATTACCGCACCT-3′ and 5′-AGTCTGTGTAGATTCGCCG-3′, respectively. The primers were synthesized by BioSune Biotechnology Co., Ltd. (Shanghai, China), and the expected fragment length was 926 bp.

2.3.2. Conventional PCR Amplification

Add sterile PBS (1:3 volume ratio) to the nasal swab specimens from goats and mix thoroughly. Freeze and thaw the mixture three times, then centrifuge at 1538× *g* (radius: 86 mm) for 20 min. Discard the supernatant and use the EasyPure Bacteria Genomic DNA Kit to extract DNA from the nasal swabs using the bacterial and viral nucleic acid extraction protocol. Conventional PCR reaction system: 2× TransTaq-T PCR SuperMix: 12.5 μL, primer (10 μM): CP-FJF1 1 μL (final concentration 0.5 μM) and CP-FJR1 1 μL (final concentration 0.5 μM), Template DNA: 2 μL, ddH_2O: add to 25 μL. The thermal cycling parameters were set as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. Extend at 72 °C for 10 min after the cycle ends. The PCR products were identified by 1.0% agarose gel electrophoresis. The PCR-amplified target fragments were excised from gels and purified utilizing a gel extraction kit, followed by cloning into the pMD19-T Simple Cloning vector system. Positive recombinant plasmids were selected via standard screening

protocols and subsequently submitted to Boshan Biotechnology Co., Ltd. (Shanghai, China) for DNA sequencing analysis.

2.3.3. Sequence Analysis

Sequence validation was performed using the BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 September 2025) algorithm on the NCBI database. Nucleotide homology analysis of the *CP40* gene sequences was conducted via Megalign software (Version 7.1.0), comparing the obtained sequences with reference *CP40* gene sequences of *C. pseudotuberculosis* strains retrieved from public databases.

2.3.4. Phylogenetic Analysis

Phylogenetic relationships among the strains were inferred using MEGA7.0 software (Version 7.0.14), and a neighbor-joining (NJ) phylogenetic tree was constructed with 1000 bootstrap replicates (bootstrap = 1000).

2.4. Establishment of Real-Time Fluorescent Quantitative PCR Detection Method

2.4.1. Real-Time Quantitative PCR Primer Design

Based on the characteristics of the *CP40* gene analyzed in Section 2.3.3, BioEdit software (Version 7.2.6) was used for multiple sequence alignment, with some results shown in Figure 1. Specific real-time fluorescence quantitative qPCR primers were designed using Oligo (v7.37). The primer sequences were 5′-TCCTCACCGCAGTAGGAGAA-3′ and 5′-TGGTGAAATCGTCTCCAT-3′, named CP-CP40F and CP-CP40R, respectively, with an expected fragment size of 139 bp. The primers were synthesized by Boshang Biotechnology Co., Ltd. (Shanghai, China).



Figure 1. Multiple sequence alignment was performed using BioEdit software (Version 7.2.6), with sequences retrieved from NCBI. The boxed regions denote primer binding sites, where black indicates complete primer–sequence complementarity (*Corynebacterium pseudotuberculosis* biovar ovi), red indicates downstream primer–sequence mismatches (*Corynebacterium pseudotuberculosis* biovar equi), and green indicates mismatches in both upstream and downstream primers (other *Corynebacterium* species). Note: Red letter: T base; blue letter: C base; green letter: A base; black letter: G base, identical bases are represented by color-coded dots.

2.4.2. Optimization of Reaction Conditions

The *CP40* gene plasmid (named PMD19T-CP40), which contains *C. pseudotuberculosis* identified and sequenced in Section 2.3.2, was used as the positive standard for this study. After measuring its concentration with a micro-nucleic acid analyzer, it was converted to a copy number (5.2×10^{10} copies/μL) and subjected to a 10-fold serial dilution for subsequent use. A total of 20 μL of the real-time fluorescent quantitative PCR reaction system was prepared strictly following the manufacturer's protocol for the fluorescent quantitative reagent kit. Reaction condition optimization was performed using a Roche Lightcycler 96 real-time fluorescent quantitative PCR instrument (Basel, Switzerland), with gradient adjustments of primer concentrations (200, 400, 600, 800, and 1000 nM) and annealing/extension temperatures (52, 54, 56, 58, and 60 °C), each maintained for 15 s. Post-amplification cycling, the optimal reaction parameters were determined based

on amplification curves and dissociation curves generated by the instrument-integrated software analysis module.

2.4.3. Establishment of Standard Curve

Six serial dilutions of the standard plasmid (concentration range: 5.2×10^6 to 5.2×10^1 copies/μL) were selected as templates for a real-time fluorescence quantitative PCR reaction. Amplification was performed under the optimized reaction conditions and obtained amplification curves. Plot the standard linear regression equation (standard curve) for the real-time fluorescence quantitative PCR reaction established in this study, with the commonly used logarithm of the starting copy number of the standard sample as the horizontal axis and the cycle threshold (Ct value) as the vertical axis.

2.4.4. Specific Test

The optimized qPCR reaction system was utilized to detect *C. pseudotuberculosis* and prevalent pathogenic strains in goats and sheep, including *P. multocida*, *M. haemolytica*, *S. aureus*, and *M. ovipneumoniae*, respectively. The genomic DNA of all tested bacteria was diluted to a final concentration of 1 ng/μL and subjected to qPCR amplification. The specificity of the established qPCR assay was evaluated based on the amplification results.

2.4.5. Sensitivity Test

Standard plasmids diluted to serial dilution (with a content of 5.2–5.2×10^3 copies/μL) as a template for real-time fluorescence quantitative PCR reaction were used. Under optimized qPCR reaction conditions, each dilution was tested in triplicate to determine the minimum reliably detectable copy number. The coefficient of variation (CV) of the Ct values was required to be <1.0%, and the lowest copy number that met this criterion was defined as the limit of detection (LOD). Concomitantly, bacterial cultures were propagated until reaching an optical density (OD) of 0.6, followed by 10-fold serial dilution for colony enumeration to determine the minimum bacterial load detectable by the established method.

2.4.6. Repeatability Test

The established real-time fluorescent quantitative PCR assay was employed to detect standard plasmids (with concentrations of 5.2×10^2 copies/μL, 5.2×10^4 copies/μL, and 5.2×10^6 copies/μL). Each concentration of standard plasmid was analyzed in triplicate, and the intra-group CV was subsequently calculated based on the resulting Ct values. For the inter-assay reproducibility assessment, the aforementioned standard samples were individually aliquoted and stored at −20 °C. Samples were retrieved at 7-day intervals and subjected to detection using the same qPCR protocol for a total of three independent trials. The inter-group coefficient of variation (CV) was then determined.

2.4.7. Clinical Sample Testing

A total of 80 nasal swab specimens were homogenized with sterile PBS at a 1:3 volume ratio. After vortex oscillation homogenization for 1 min and centrifugation at 1538× *g* (radius: 86 mm) for 20 min, the supernatant was discarded, and bacterial genomic DNA was isolated and purified utilizing the EasyPure Bacteria Genomic DNA Kit (Beijing Quanshijin Biotechnology Co., Ltd., Beijing, China) with the purification protocol, which included enzymatic lysis of bacterial cells, specific adsorption of DNA onto a silica gel membrane column, and sequential removal of proteinaceous impurities through washing steps. The purified DNA was finally eluted and stored at −20 °C until use. The established qPCR assay and conventional PCR method were simultaneously employed to detect the specimens, aiming to validate the consistency between the two detection protocols. Randomly selected

amplified products were cloned for sequencing according to the protocol described in Section 2.3.2, followed by bioinformatic alignment and comparative analysis of the obtained sequencing data.

3. Results and Analysis

3.1. Amplification Results of Target Genes

The *CP40* gene was amplified by PCR, and agarose gel electrophoresis revealed a single specific band at the expected size of 927 bp (Figure 2). The target fragment was gel-purified, cloned into a vector, and sequenced. After BLAST, the obtained sequence was consistent with the published *CP40* gene sequence in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/gene/, accessed on 15 September 2025).

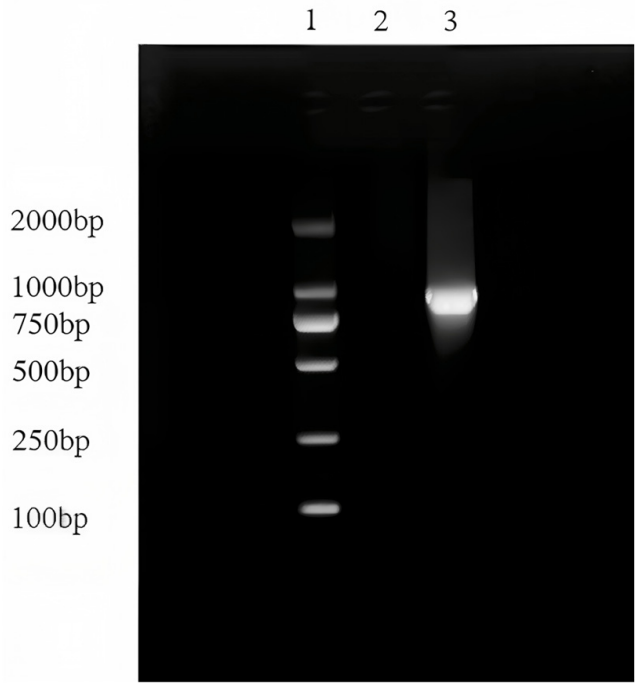


Figure 2. Amplification results of *CP40* gene of *C. pseudotuberculosis* biovar ovis FJ-PN strain. 1. DNA marker (DL2000). 2. Blank control. 3. Amplification product (927 bp).

3.2. CP40 Nucleotide Homology Analysis

Nucleotide homology analysis revealed that the FJ-PN strain shares 99.65–100% nucleotide identity and 98.94–100% amino acid identity with *C. pseudotuberculosis* biovar ovi proteins deposited in GenBank. In comparison to *C. pseudotuberculosis* biovar equi, the nucleotide homology ranges from 90.18 to 91.84% and amino acid identity ranges from 88.63 to 90.77%. Homology with other *Corynebacterium* species is less than 82.71% at the nucleotide level and 78.63% at the amino acid level.

3.3. CP40 Genetic Evolution Analysis

Phylogenetic analysis (Figure 3) demonstrated that the FJ-PN strain clusters within the same evolutionary clade as reference *C. pseudotuberculosis* strains published in GenBank, closer to the evolutionary branch of biovar ovis. Although *C. pseudotuberculosis* biovar ovis and *C. pseudotuberculosis* biovar equi exhibit certain differences, they both belong to the species *C. pseudotuberculosis* and form distinct evolutionary branches with other *Corynebacterium* species.

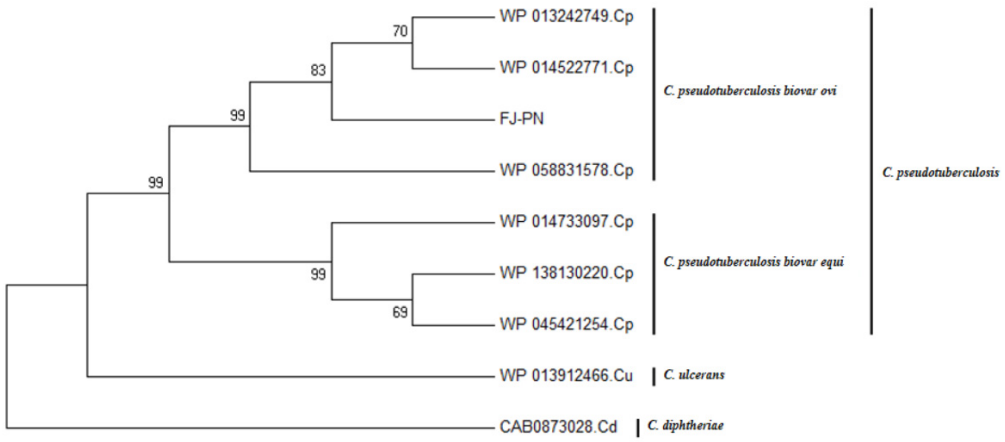


Figure 3. Phylogenetic analysis based on the *CP40* gene of *C. pseudotuberculosis*. A neighbor-joining (NJ) phylogenetic tree was constructed with 1000 bootstrap replicates.

3.4. Optimization of SYBR Green I qPCR Reaction System

The optimized reaction system consisted of 10 μL SYBR Green I Master Mix, 0.8 μL each of forward and reverse primers (final concentration, 1.6 μM), 2 μL of template DNA, and deionized water supplemented to 20 μL. The annealing temperature optimization results showed that the amplification efficiency was highest at 56 °C, higher than that at 58 °C and 60 °C. Melting curve analysis indicated that the Tm value of the product at 56 °C was (81.69 ± 0.22) °C, with a single peak, indicating good amplification specificity. In contrast, when the template concentration ≤52 copies/μL, a non-single peak appeared at 52 °C and 54 °C, suggesting non-specific amplification. The reaction program is set to pre-denature at 95 °C for 2 min. Subsequently, 40 cycles of amplification were performed (maintained at 95 °C for 10 s, annealed at 56 °C for 15 s). Fluorescence signals are collected at the extension stage of each cycle (default instrument parameters).

3.5. Establishment of Standard Curve and Analysis of Amplification Efficiency

A standard curve was generated using 10-fold serially diluted standard plasmids (5.2×10^1–5.2×10^6 copies/μL). Linear regression analysis yielded a correlation coefficient of $R^2 = 1.00$, with a slope of −3.285 and a Y-axis intercept of 36.04. Using the formula $E = 10^{-1/\text{slope}} - 1$, the amplification efficiency was calculated to be E = 101%, indicating satisfactory amplification efficiency of the reaction system (Figure 4).

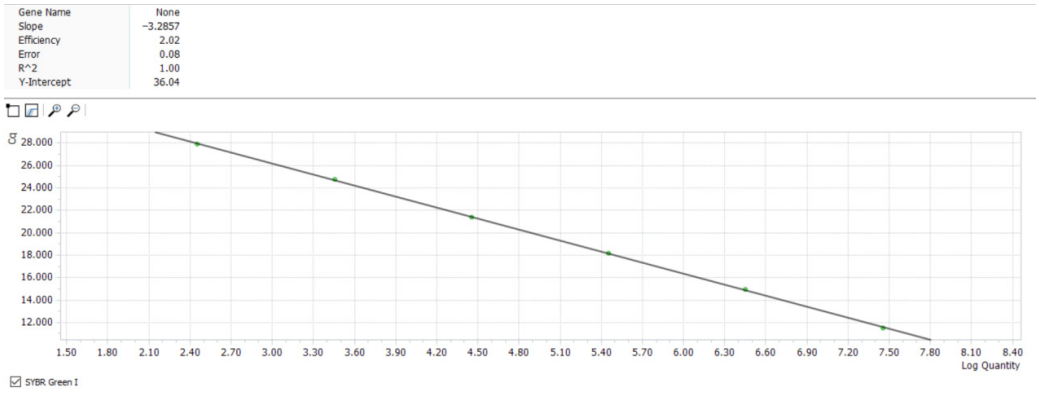


Figure 4. The standard curve for the SYBR Green I qPCR method. A robust linear correlation was demonstrated, with the standard curve for *C. pseudotuberculosis* biovar ovis exhibiting a coefficient of determination (R^2) of 1.00 when plotted on a linear scale. Ct values corresponding to the standard product (5.2×10^1–5.2×10^6 copies/μL) with green color dots.

3.6. Specific Verification

Specificity validation was conducted using *C. pseudotuberculosis* biovar ovis, *C. pseudotuberculosis* biovar equis, and common pathogens of goats and sheep (*P. multocida*, *M. haemolytica*, *S. aureus*, and *M. ovipneumoniae*). Amplification curves were exclusively observed in *C. pseudotuberculosis* biovar ovis samples, with Ct values ranging from 18.32 to 22.15, whereas no fluorescent signals were detected in other pathogens (Figure 5A). Melting curve (Figure 5B) analysis revealed a single dissociation peak for *C. pseudotuberculosis* biovar ovis at 81.69 ± 0.22 °C (Tm value). Combined analysis of amplification and melting curves confirmed the high specificity of the established qPCR assay.

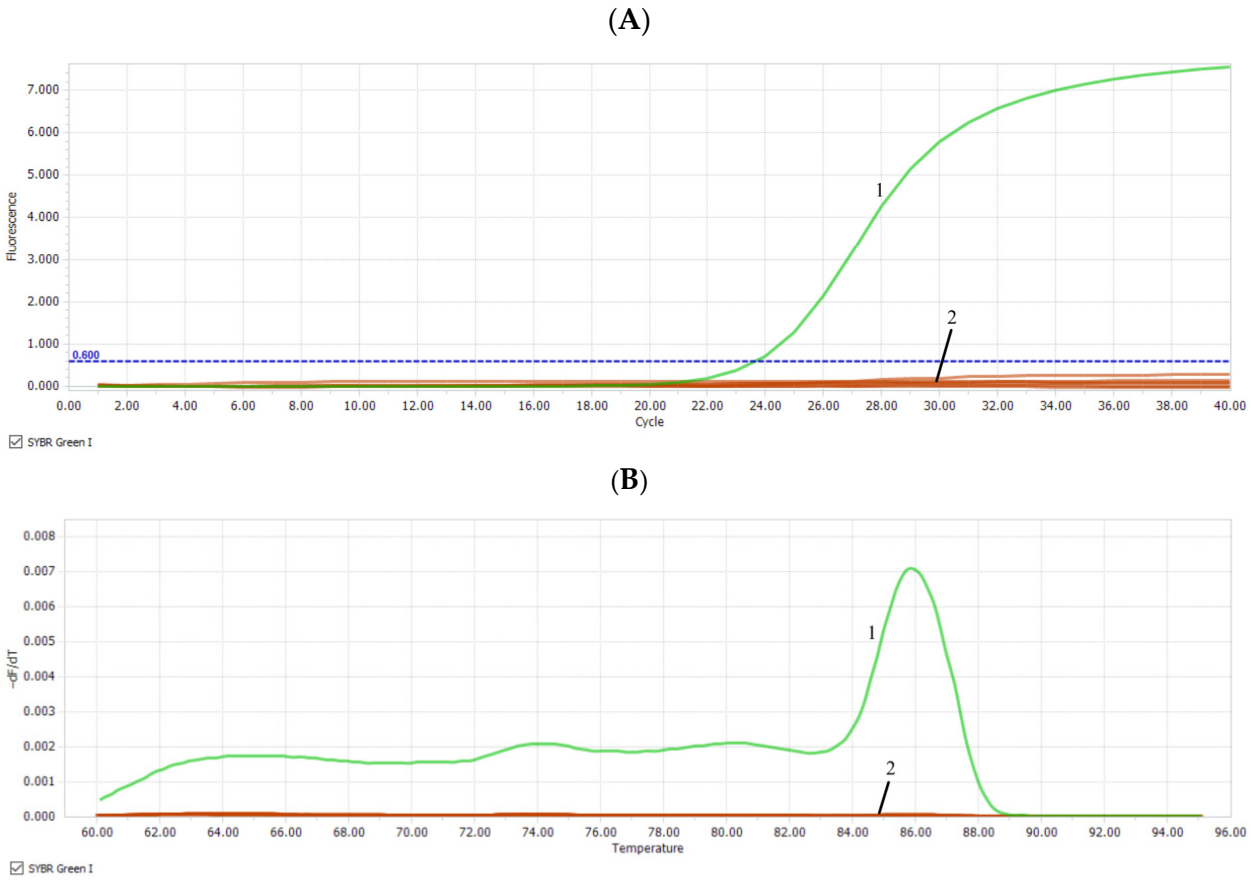


Figure 5. The specificity test for the SYBR Green I qPCR method. (**A**) Amplification curve. (**B**) Melting curves. 1: *C. pseudotuberculosis* biovar ovi. 2: *C. pseudotuberculosis* biovar equi, *P. multocida*, *M. haemolytica*, *S. aureus*, and *M. ovipneumoniae*.

3.7. Sensitivity Assay

The established method was applied to evaluate the analytical sensitivity using 10-fold serially diluted standard plasmids. As illustrated in Figure 6, at a standard plasmid concentration of 5.2 copies/μL (5.2×10^0 copies/μL), the Ct values exceeded 40, rendering it undetectable by the instrument; therefore, the minimum detection limit was determined to be 52 copies/μL with a Ct value of 34.89 ± 0.2. In this case, a Ct value ≤ 35, and simultaneously, a Ct value ≤ 5 at a bacterial concentration of 10^2 CFU/mL indicates stable detection; therefore, this method can detect a colony count of 10^2 CFU/mL.

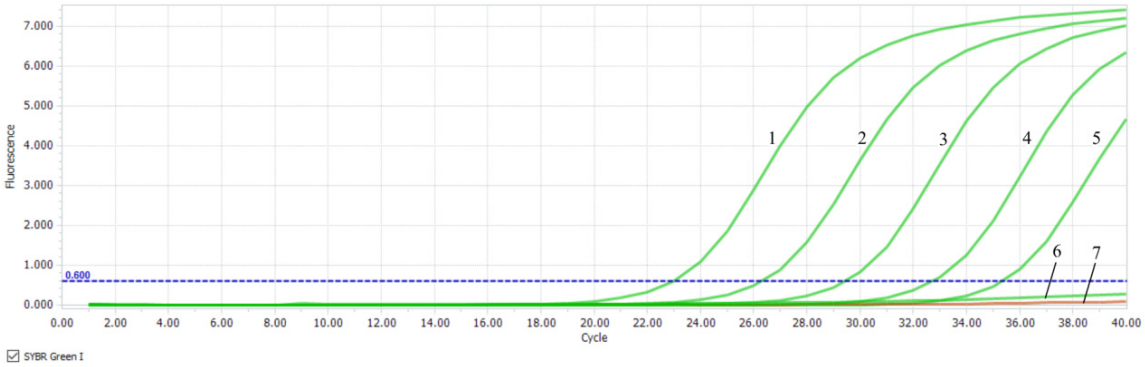


Figure 6. Sensitivity test for the SYBR Green I qPCR method. 1~6: Positive plasmids diluted from 10^5 to 10^0. 7: Negative control. The blue dashed line represents the fluorescence threshold.

3.8. Repeatability Assay

Intra-assay and inter-assay reproducibility were evaluated using PMD19T-CP40 standard plasmids at concentrations of 5.2×10^6, 5.2×10^4, and 5.2×10^2 copies/μL as templates. The established SYBR Green I qPCR assay demonstrated coefficients of variation (CV) below 1.0% for both intra-batch and inter-batch experiments (Table 1), confirming the high reproducibility of the developed method.

Table 1. Reproducibility analysis for the SYBR Green I qPCR method.

Concentration of Plasmid Standards (Copies/μL)	Intra-Assay Reproducibility		Inter-Assay Reproducibility	
	Ct ± SD	CV/%	Ct ± SD	CV/%
5.2×10^6	18.18 ± 0.03	0.19	18.12 ± 0.12	0.68
5.2×10^4	24.79 ± 0.06	0.24	24.71 ± 0.17	0.70
5.2×10^2	31.5 ± 0.15	0.48	31.49 ± 0.30	0.94

3.9. Clinical Specimen Validation

For clinical validation, a total of 80 nasal swab specimens were randomly collected, homogenized in sterile phosphate-buffered saline (PBS), and analyzed using the established SYBR Green I qPCR assay. The results are shown in Table 2. Diagnostic analysis identified a total of 15 positive nasal swab specimens (18.8% positivity rate). Parallel detection via the conventional PCR method yielded a total of 13 positive nasal swab specimens (16.3% positivity rate). Complete diagnostic concordance was observed between conventional PCR and the fluorescent quantitative PCR assay for all positive samples. The qPCR assay further identified two specimens that yielded negative results via conventional PCR, thereby demonstrating enhanced detection sensitivity. The CT values of qPCR for samples that showed negative results in conventional PCR were 33.85 and 34.16. A total of five positive specimens were randomly selected (including two specimens that yielded negative outcomes in conventional PCR assays) and identified as *C. pseudotuberculosis* biovar ovis after cloning and sequencing, indicating that the detection method established in this study can be used for the epidemiological investigation of the pathogen.

Table 2. Conventional PCR and SYBR Green I qPCR method detection of clinical samples.

	Nasal Swab		
Detection Method	**Positive Number**	**Negative Number**	**Positivity Rate**
Conventional PCR	13	67	16.3%
SYBR Green I qPCR	15	65	18.8%

4. Discussion

Caseous lymphadenitis (CLA) in goats and sheep, caused by *Corynebacterium pseudotuberculosis* biovar ovi (*C. pseudotuberculosis*), poses a significant economic threat to the global small ruminant industry. Among the various virulence factors of *C. pseudotuberculosis*, secreted protease CP40 has been identified as a key determinant through proteomic studies. Integrated proteomic and transcriptional analyses have experimentally validated the involvement of this protein in bacterial pathogenesis [17]. Experimental investigations have demonstrated that CP40 exhibits glycosidase activity toward IgG; it hydrolyzes biantennary glycans on both human and ovine IgGs [18]. This hydrolysis of IgG polysaccharides may help other bacteria, such as Streptococcus pyogenes, evade recognition by the immune system during colonization and infection in the body [19]. Research on CP40 has shown that the protein is indeed expressed in infected sheep, and the immune response against this protein can provide anti-infective protection [20]. Given the functional importance of CP40, understanding its molecular characteristics is crucial for developing effective countermeasures against *C. pseudotuberculosis* biovar ovis. This study analysis revealed that the *CP40* gene sequence of the FJ-PN strain shares 99.65–100% nucleotide identity and 98.94–100% amino acid identity with other *C. pseudotuberculosis* biovar ovis strains. In contrast, sequence identity with biovar equi strains was approximately 90% at both the nucleotide and amino acid levels, and identity with other *Corynebacterium* species was below 82.71% and 78.63%, respectively. This high conservation within biovar ovis and significant divergence from closely related organisms confirms the suitability of *CP40* gene as a specific molecular target for pathogen identification. Leveraging this specificity, we developed a SYBR Green I-based qPCR assay capable of accurately detecting *C. pseudotuberculosis* biovar ovis in goat nasal swab specimens. The assay showed no cross-reactivity with *C. pseudotuberculosis* biovar equi, *P. multitocida, M. haemolytica*, and other strains. Therefore, this method avoids false-positive results caused by non-specific interference of *C. pseudotuberculosis* biovar equi, thus accurately assessing the prevalence of CLA in goat and sheep herds. Its application enhances the accuracy of diagnostic outcomes, supporting timely intervention and improved herd health management.

The detection method based on SYBR Green I qPCR has the characteristics of being fast, specific, and sensitive, and is widely used. At present, the identification methods for *C. pseudotuberculosis* are only ordinary PCR [21] or require multiple pairs of primers [22,23]. There is currently no report on the SYBR Green I fluorescence quantitative PCR method for *C. pseudotuberculosis*, and no established method for diagnosing *C. pseudotuberculosis* biovar ovi. This study establishes the first SYBR Green-based qPCR detection method for *C. pseudotuberculosis* biovar ovi, addressing the technical gap in the molecular detection of this pathogen. Conventional PCR assays exhibit limited sensitivity (10^3–10^4 CFU/mL) and necessitate post-amplification electrophoresis, introducing risks of aerosol contamination. Existing multiplex qPCR protocols require multiple primer sets, complicating experimental design. The developed SYBR Green I qPCR assay demonstrates enhanced sensitivity (10^2 CFU/mL), employs a single primer pair, features streamlined operation, and enables detection within 60 min, rendering it superior for clinical diagnostics and epidemiological surveillance of *C. pseudotuberculosis* biovar ovi. In this study, a pair of specific primers were designed using the *CP40* gene fragment of *C. pseudotuberculosis*. Through condition optimization, a real-time qPCR method for detecting *C. pseudotuberculosis* was established. This method has strong specificity and only produces positive fluorescence signals for *C. pseudotuberculosis* detection. There is no cross-reactivity with *P.multocida*, *M.haemolytica*, *S.aureus*, and *M.ovipneumoniae*. Through sensitivity and repeatability verification, it has been shown that this method has high sensitivity and good repeatability, with a minimum detection limit of 52 copies/μL. Intra- and inter-batch replicate experiments consistently yielded

a low coefficient of variation, with all values below 1.0%. A threshold cycle (Ct) value ≤ 35 at a bacterial concentration of 10^2 CFU/mL indicates stable detection and confirms bacterial colonization. Ct values exceeding 35 require cautious interpretation, considering potential sample contamination or absence of host colonization. Triplicate technical replicates are mandatory to verify result consistency. Inconsistent amplification suggests sample contamination, whereas consistent positive results indicate either host colonization or environmental presence of *C. pseudotuberculosis* biovar ovi, necessitating implementation of isolation protocols or environmental decontamination measures. Concomitant bacterial isolation and serological assays should be performed to validate fluorescence quantification results, followed by the implementation of targeted isolation and therapeutic interventions as clinically indicated.

The common surface type of CLA can be observed with the naked eye as granulomas formed in the surface lymph nodes and subcutaneous tissue; however, the lack of obvious symptoms means the visceral type is easily overlooked [24]. Currently, diagnosis primarily relies on the pus from granulomas. For the visceral type, laboratory diagnosis is only possible after visceral abscesses are discovered post-mortem. Therefore, it is crucial to effectively identify asymptomatic animals for diagnosis, to ensure immediate treatment and isolation measures for contaminated animals [25]. Studies have shown that *C. pseudotuberculosis* can be isolated from the nasal fluid of diseased sheep [26]. Sheep with only lung abscess and no surface discharge disease are also important sources of transmission of *C. pseudotuberculosis* to the herd [27]. It is speculated that *C. pseudotuberculosis* has the potential to spread through respiratory secretions. In a survey in Spain, it was found that visceral lesions in sheep accounted for as high as 72.79%, of which 85.06% were located in the lungs [28]. Therefore, it is necessary to detect *C. pseudotuberculosis* through nasal fluid and isolate the affected animals in a timely manner. This study compared the detection rates of nasal fluid from diseased goats with *C. pseudotuberculosis* symptoms. The established qPCR method was applied to detect clinical samples. A total of 15 positive samples were detected from nasal fluid, with a positive rate of 18.8%. Using conventional PCR detection, a total of 13 samples were detected in nasal fluid with a positive rate of 16.3%. This indicates that the sensitivity of qPCR is higher than that of conventional PCR. The qPCR detection method established in this study demonstrates significant application potential in resource-limited settings. Although the initial investment in real-time fluorescent quantitative PCR (qPCR) instruments is relatively high, this method offers distinct advantages in terms of operational costs, detection throughput, and timeliness. For primary care settings, it is recommended that veterinarians collect nasal swab samples and transport them to regional testing centers for centralized analysis using foam boxes with ice packs to maintain low temperatures. The advantages of this model include the following: Sample stability: *C. pseudotuberculosis* exhibits strong environmental resistance and can survive for several months at low temperatures. Detection efficiency: A single run can process 96 samples, making it suitable for population screening. Cost-effectiveness: The reagent cost per sample is lower than that of probe-based methods or bacterial isolation and identification. Rapid turnaround: Results can be obtained within hours from sample receipt, demonstrating significantly higher efficiency compared to methods such as bacterial isolation and identification or ELISA. For CLA prevention and control, this method enables the early identification of infected animals, facilitating timely isolation and management, thereby effectively interrupting the transmission chain of pathogens within livestock populations.

5. Conclusions

In conclusion, the CP40 protein represents a critical virulence determinant of *C. pseudotuberculosis*, where its glycosidase activity facilitates bacterial evasion of host immune

surveillance. In addition, the *CP40* gene sequence exhibits high conservation among *C. pseudotuberculosis* biovar ovi strains while demonstrating low homology with other *Corynebacterium* species, thereby serving as a specific molecular marker for biovar ovi identification. The established SYBR Green I qPCR assay, leveraging the species-specific *CP40* gene, provides a novel tool for the rapid and accurate identification of the pathogen in nasal swab specimens, which holds significant implications for the early diagnosis and integrated control of CLA. This method exhibits advantages including visualization capability, high sensitivity, strong specificity, operational simplicity, rapid turnaround time, and relatively low cost. In summary, the established assay holds significant promise for improving the early diagnosis and integrated control of caseous lymphadenitis (CLA), thereby contributing to the mitigation of its global transmission.

Author Contributions: All listed authors have made substantial contributions to conception and design, data acquisition or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published. The corresponding author, Y.C., is responsible for ensuring that all aspects of the work are accurately represented and assumes full responsibility for the integrity of the data and the accuracy of the data analysis. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Experimental Animal Ethics Committee, Institute of Animal Husbandry and Medicine, Fujian Academy of Agricultural Sciences. Approval Code: 202307FJ009. Approval Date: 14 July 2023. All procedures involving laboratory animals were conducted in compliance with relevant ethical guidelines and regulations.

Informed Consent Statement: Written informed consent was obtained from the owners of the farm animals prior to the performance of all study-related procedures on the animals. The owners were fully informed of the study purposes, procedures, potential risks, and benefits, and voluntarily agreed to the participation of their animals. All operations involving farm animals complied with relevant veterinary ethical guidelines and regulatory requirements.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Unpublished data are not available.

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