Detection of Corynebacterium pseudotuberculosis from sheep lymph nodes by PCR

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SUMMARY

Corynebacterium pseudotuberculosis is a facultative intracellular bacterium that causes caseous lymphadenitis in sheep and goats. This study was designed to evaluate the validity of PCR assay protocol for the direct detection of C. pseudotuberculosis in 147 samples of lymph nodes (prescapular and mediastinal) from carcasses of naturally infected sheep and to compare its performance with the traditional bacteriological culture technique. C. pseudotuberculosis was isolated in 81 samples mainly from prescapular nodes and a specific 203 bp PCR amplified pld gene DNA fragment was detected in 85 samples. The total agreement score between the 2 methods was 95.92%, the relative PCR sensitivity and specificity to culture being 98.76% and 92.42%, respectively. The positive and negative predictive probabilities given by PCR were 98.4% and 94.1%, respectively. The C. pseudotuberculosis detection limit given by PCR was 2.1x10^4 CFU/mL in sheep lymph tissue samples. In conclusion, the PCR assay proved to be a sensitive and rapid method for the detection of C. pseudotuberculosis in lymph node samples from naturally infected sheep.

Keywords: Corynebacterium pseudotuberculosis, caseous lymphadenitis, sheep, PCR, bacteriological culture.

RÉSUMÉ

Détection par PCR de Corynebacterium pseudotuberculosis dans les nœuds lymphatiques de moutons

Corynebacterium pseudotuberculosis est une bactérie intracellulaire facultative responsable de la lymphadénite caseuse des moutons et des chèvres. Cette étude a été réalisée afin d’évaluer la validité d’une méthode de détection directe de C. pseudotuberculosis par PCR dans 147 nœuds lymphatiques (précapsulaires et médiastinaux) prélévés sur des carcasses de moutons naturellement infectés et de comparer les performances de ce test avec celles de la culture bactériologique classiquement utilisée. C. pseudotuberculosis a été isolé à partir de 81 prélèvements, principalement de nœuds lymphatiques précapsulaires et un fragment d’ADN amplifié par PCR de 203 bp correspondant au gène pld de la bactérie a été mis en évidence dans 85 échantillons. L’agrément total entre les 2 méthodes a été de 95.92%, la sensibilité et la spécificité de la PCR par rapport à la culture bactériologique ont été respectivement de 98.76% et de 92.42% et les valeurs prédicitives positive et négative ont été de 98.4% et 94.1%. La limite de détection de C. pseudotuberculosis dans les échantillons de nœuds lymphatiques donnée par la PCR a été de 2.1x10^4 CFU/mL. En conclusion, la PCR apparait être une méthode sensible et rapide pour la détection de C. pseudotuberculosis dans des échantillons de nœuds lymphatiques provenant de moutons naturellement infectés.

Mots-clés : Corynebacterium pseudotuberculosis, lymphadénite caséeuse, mouton, PCR, culture bactériologique.

Introduction

Corynebacterium pseudotuberculosis is a Gram-positive, rod-shaped, non-spore-forming, facultative intracellular bacterium that causes caseous lymphadenitis (CLA) in sheep and goats [8, 20, 24]. It is also the causative agent of ulcerative lymphangitis in horses, and sporadic suppurative disease in other animal species, including humans [2, 3, 23]. A protein exotoxin, commonly known as phospholipase D (PLD) and lipids of the bacterial cell wall (mycolic and meso-diaminopimelic acids) have been identified as possible virulence factors of C. pseudotuberculosis [2, 3, 8, 23, 24].

CLA is a chronic disease in adult small ruminants and is characterized in the external form by fibrous encapsulated abscesses in the peripheral lymph nodes; in the internal form, abscesses sometimes form in the lungs and other visceral organs. These encapsulated abscesses contain concentric layers of yellow-green granular pus [3, 8, 29]. Enlargement of lymph nodes and the development of abscesses can rupture and contaminate the milk, lambs, kids, other animals and environment [2, 8, 26]. CLA is a highly prevalent disease in sheep and goat population’s worldwide [2, 6, 8, 26]. The prevalence rate is high in most countries; for example, 42.4% of 4,089 culled sheep originating in five regions of the western United States were positive [26] and 53.7% of 4,574 adult ewes slaughtered at a western Australian abattoir exhibited the disease [6]. Major economic losses are incurred due to decreased milk production, condemnation of portions of or entire carcasses, reproductive inefficiency, and devaluation of hides; and to a lesser extent deaths [2, 8, 9]. C. pseudotuberculosis has also public health significance, causing human lymphadenitis. Moreover, it is likely that sheep are the source of infection in humans [19] and the possible public health risk also contributes to economic losses [2, 19].
The diagnosis of CLA poses many problems. Palpation of affected lymph nodes is unreliable; it is not specific and does not detect early cases or cases in which only deep-seated lymph nodes and organs are involved [25]. The most commonly used diagnostic methods for the detection of C. pseudotuberculosis in animals are bacteriological culture techniques and serological assays. C. pseudotuberculosis grows relatively slowly on bacteriological culture media, so conventional bacteriological methods for the recovery and identification of this agent from different clinical samples can take three to five days and can only detect living organisms [10, 15, 21, 23]. Several serological tests have been used with variable results. Serum antibodies to C. pseudotuberculosis can be detected by haemolysis inhibition [9], anti-haemolysin inhibition [25], indirect haemagglutination [25], complement fixation [21], gel-diffusion precipitation [25], slide-and-tube agglutination tests [25], and several ELISA techniques [13, 27]. Although serological tests are faster, these tests are not enough specific or sensitive; furthermore, some tests are difficult to standardize, especially those based on haemolysis [13, 25, 27].

The lack of clinical features of CLA combined with the drawbacks of bacteriological and serological detection methods emphasize the need for reliable alternative diagnostic methods to detect C. pseudotuberculosis [10, 27] in clinical materials such as lymph nodes. Polymerase chain reaction (PCR)-based diagnostic techniques have the potential to meet the need for better diagnostic tools for infectious diseases caused by fastidious or slow-growing bacteria. There are a few studies on the detection of C. pseudotuberculosis DNA from pure culture [10, 12, 18] or pus samples [18]. However, according to the available data, direct PCR detection of C. pseudotuberculosis in lymph nodes has not been reported. In this study, it was aimed to validate and standardize a PCR protocol to detect C. pseudotuberculosis DNA in lymph node samples from naturally infected sheep and to compare its performance with the conventional bacteriological culture method.

**Materials and methods**

**ANIMALS AND SAMPLES**

The carcasses of 3-6 years old sheep, not previously immunized against CLA, slaughtered for human consumption as food were examined for the presence of CLA lesions in the Van Branch Slaughterhouse, Meat and Fish Institute, Eastern Turkey; a total of 1,913 and 2,382 sheep carcasses were examined in the winter and summer seasons, respectively. Abscessed lymph nodes (prescapular and mediastinal) were taken from 147 carcasses under strict aseptic precautions, with a sterile scalpel and placed in individual sterile containers. The samples were immediately transported to the laboratory and processed for culture, and stored at -70°C before PCR processing.

**BACTERIOLOGICAL ANALYSES**

Swabs from the prescapular and mediastinal lymph nodes were inoculated onto blood-agar base (Merck, Darmstad, Germany) containing 5% (v/v) deﬁbrinated sheep blood. The plates were incubated at 37°C for five days and were periodically checked for growth. Dry, yellowish-white, opaque, and crumbly colonies were selected and transferred onto brain-heart-infusion agar (BHI agar) (Oxoid, Basingstoke, England) for pure culture. Identification of C. pseudotuberculosis strains was performed using standard classification tests: colony morphology; haemolytic activity on blood agar; Gram stain; catalase and urease activity; fermentation of glucose, maltose, galactose, mannose, trehalose, lactose, arabinose, esculin, salicin, inositol and xylose; methyl red; motility; and nitrate reduction. CAMP (Christie, Atkins, Munch-Peterson) tests with *Staphylococcus aureus* and *Rhodococcus equi* were also performed [8, 15, 17, 21].

**POLYMERASE CHAIN REACTION (PCR) ASSAY**

To avoid possible contamination, the extraction, amplification and electrophoresis stages of the PCR were performed in a separate cabinet. All plastic ware used was DNase and RNase free, disposable and not re-used throughout the experiment. Different sets of micropipettes were used at each step of sample processing. DNA extraction, PCR-mix preparation and electrophoresis.

**DNA extraction from lymph nodes:**

Immediately prior to DNA extraction, samples were thawed at room temperature and any fat, pus or caseous mass present was removed using a sterile scalpel. A 4 g portion of lymph node sample (pooled prescapular and mediastinal lymph nodes) was placed in a stomacher™ (Seward Ltd., West Sussex, UK) with 8 mL of sterile phosphate-buffered saline (PBS, pH 7.2) and homogenized for 10 minutes. An aliquot of the homogenate (300 μL) was placed in a microtube for DNA extraction. Next, the same volume of lysis solution (10 mM Tris-HCl, 1% SDS, 100 mM NaCl, 2% Triton-X100, pH 8.0) and 15 μL of proteinase K (20 mg/mL) (Qiagen, Hilden, Germany) were added to the samples, and the contents were mixed thoroughly. Following 1 hour of boiling, saturated phenol (liquid phenol containing 0.1% 8-hydroxyquinoline, stabilized with 100 mM Tris-HCl, pH 8.0, and 0.2% 2-mercaptoethanol, 300 μL) was added; the contents were mixed vigorously for 5 minutes and then centrifuged at 11,600 g for 10 minutes at room temperature. An equal volume of chloroform-isoamyl alcohol (24:1) (Applichem, Darmstadt, Germany) was added to the aqueous layer and after mixing thoroughly for 5 minutes, tubes were centrifuged as before. Then, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol were added to the upper layer and mixed thoroughly. After incubation overnight at -20°C to precipitate the DNA and centrifugation
at 13,000 g for 10 minutes at 0°C, the supernatants were discarded and the pellets were washed sequentially with 95% and 70% ethanol. The samples were then vortexed and centrifuged at 11,600 g for 5 minutes at 4°C. The pellets were dried and suspended again in 50 µL of TE (Tris-EDTA) buffer (Applichem). In addition, for standardization of the extraction method, a commercial DNA-extraction kit (DNeasy Blood&Tissue Kit) (Qiagen) was also used according to the manufacturer’s recommendations. For this purpose, 25 µg of the pooled lymph tissue samples were used for the initial extraction material. DNA concentrations were determined spectrophotometrically (GBC, Dandenong, Australia) by reading absorbance at 260 and 280 nm. Samples were stored at -20°C until used as templates for amplification.

**Primers, amplification conditions and agarose gel electrophoresis:**

The oligonucleotide primers used for PCR were from the pld gene of *C. pseudotuberculosis*. Their sequences were described by PACHECO et al. [18] and were presented in Table I.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
<th>PCR product</th>
</tr>
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<tbody>
<tr>
<td>PLD-F</td>
<td>ATA AGC GTA AGC AGG GAG CA</td>
<td>203 bp</td>
</tr>
<tr>
<td>PLD-R2</td>
<td>ATC AGC GGT GAT TGT CTT CCA GG</td>
<td></td>
</tr>
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Table I: Primers used for PCR analyses of *Corynebacterium pseudotuberculosis* from lymph nodes in sheep [18].

The amplified products were analyzed by electrophoresis on a 2% (w/v) agarose gel run at 80-90 volts for 1.5-2 hours and then stained with ethidium bromide (0.3 mg/L). Amplified products were visualized using a computerized image-analysis system (Spectronics Co., Westburg, NY, USA). PCR products with a molecular size of 203 bp were considered positive for *C. pseudotuberculosis*.

**PCR limit of detection in lymph tissue samples:**

*C. pseudotuberculosis* Pl 18 strain was grown on BHI agar at 37°C for 48 hours. A single colony was removed from BHI agar, placed in 25 mL of BHI broth (Oxoid) containing 0.05% Tween 80 and incubated at 37°C for 48 hours. The culture was then held at 4°C for 12 hours and centrifuged at 4,500 g for 20 minutes. The supernatant was washed three times with sterile PBS (pH 7.2). Next, the pellet was suspended again in 10 mL of sterile PBS and 10-fold dilutions (from 10⁻¹ to 10⁻⁶) were made. From each of these dilutions, 0.1 mL was inoculated onto duplicate BHI agar plates and incubated at 37°C for 48 hours; following incubation, the colonies present were enumerated. Sterile distilled water (Applichem) served as the negative control and no growth was detected after incubation [16]. The number of organisms in the dilutions was estimated spectrophotometrically at 630 nm, and the concentration of the original *C. pseudotuberculosis* suspension was estimated at 2.1 x 10⁶ colony-forming units (CFU)/mL.

To assess the PCR detection limit, prescapular and mediastinal lymph node samples were collected from 14 slaughtered sheep that were considered to be free of CLA (i.e., no history of CLA and no positive results from bacteriological culture samples). These lymph nodes (pooled prescapular and mediastinal lymph nodes) were then artificially contaminated with known concentrations of *C. pseudotuberculosis* Pl 18 strain ranging from 2.1 x 10⁷ to 2.1 x 10⁴ CFU/mL at final concentrations. *C. pseudotuberculosis* DNA was extracted from all dilutions of lymph tissue samples and processed by PCR as described earlier. All experiments were done in triplicate.

**STATISTICAL ANALYSIS**

Eighty-one sheep tested positive according to the bacteriological culture and were accepted as true positives (gold standard) in the statistical analyses for the calculation of sensitivity and specificity. The chi-squared test was used to evaluate statistical significance and p < 0.05 was accepted as significant [28]. The level of agreement between the
methods (PCR assay and bacteriological culture method) was analyzed (with a 95% confidence interval) using the one proportion method in Minitab (http://www.minitab.com, released version 16; accessed 20 August 2011).

**Results**

**MICROBIOLOGICAL FINDINGS**

Smears of colonies revealed Gram-positive coryneform microorganisms. Isolates were identified as *C. pseudotuberculosis* with positive catalase, urease, glucose, galactose, maltose, mannose and methyl red tests and negative trehalose, arabinose, lactose, esculin, xylose, inositol, salicin, nitrate-reduction and motility tests. Isolates also inhibited the activity of *S. aureus* β-haemolysin and enhanced the haemolysis of *R. equi*.

*C. pseudotuberculosis* was isolated from 55.1% (81/147) of abscessed lymph nodes collected in 147 animals, with 39.5% (32/81) isolated in winter and 60.4% (49/81) in the summer. In addition, 61.7% (50/81) isolates were cultured from prescapular lymph nodes, 14.8% (12/81) from mediastinal lymph nodes, and 23.4% (19/81) were present in both lymph nodes (Table II). *C. pseudotuberculosis* was not isolated from the remaining 44.8% (66/147) samples, but 50.0% (33/66) of them were positive for *Staphylococcus* spp., 25.8% (17/66) for *Streptococcus* spp., and 21.2% (14/66) for *Escherichia coli*.

**MOLECULAR DETECTION OF C. PSEUDOTUBERCULOSIS**

A PCR amplified DNA fragment of 203 bp specific for the *pld* gene of *C. pseudotuberculosis* was evidenced in 85 lymph node samples (57.8%) (figure 1). Positive PCR results were detected in different aliquots containing a *C. pseudotuberculosis* density of at least 2.1 x 10^4 - 2.1 x 10^3 CFU/mL in lymph tissue samples (figure 2).

When the culture and PCR results were compared, 80 samples were positive (positive agreement score: 93.02% (80/86)) and 61 samples were negative (negative agreement score: 91.04% (61/66)) with the 2 methods, leading to a high total agreement score of 95.92% (141/147). However, 3.40% (5/147) samples tested negative by culture were positive by PCR and 0.68% (1/147) sample was positive by bacteriological culture but was negative by PCR (Table III). Consequently, the sensitivity (frequency of true positive samples among positive cultures) and specificity (frequency of true negative
samples among negative cultures) of PCR related to the bacteriological culture were 98.76% and 92.42%, respectively, leading to 98.4% as positive predictive probability and 94.1% as negative predictive probability (with a 95% confidence interval). In addition, differences in positive rates by the PCR and culture methods were not statistically significant.

**Discussion**

The results obtained in previous studies showed that the distribution of CLA lesions varies according to the anatomical localization [10, 15, 26]. Precapular lymph nodes were the most common site of superficial lesion development in the external form of the disease; the mediastinal and bronchial lymph nodes were the most frequently affected thoracic lymph nodes in the internal form [24]. Accordingly, prescapular and mediastinal lymph node samples were analysed in this study as representatives of the external and internal forms of CLA, respectively. *C. pseudotuberculosis* was isolated from 85.19% of the prescapular lymph node samples and 38.27% of the mediastinal lymph node samples. This result indicates that prescapular lymph nodes are more suitable for culture than mediastinal lymph nodes.

In a previous study, 41 abscessed lymph nodes from sheep were examined by the bacteriological culture method [15]: 85.4% of them were microbiologically positive, and *C. pseudotuberculosis* was isolated in 46.3% of cases. Additionally, *Micrococcus* spp. were cultured from 19.5% of the samples, *S. aureus* and *Pseudomonas aeruginosa* from 7.3%, and *S. epidermidis* from 4.8% [15]. In another study, *C. pseudotuberculosis* was isolated from 78.8% of 118 abscessed lymph nodes (89 from sheep and 29 from goats) [10]. Moreover, *C. pseudotuberculosis* was isolated from all pus samples collected from small ruminants [18]. In the current study, *C. pseudotuberculosis* was isolated from 55.1% of the lymph node samples, 39.5% of positive samples were found in winter and 60.4% in summer. In addition, *Staphylococcus* spp. was prominently identified in the other tissue samples negative for *C. pseudotuberculosis* following *Streptococcus* spp. and *E. coli*. These results indicate that several bacterial organisms are present in CLA lesions; however, *C. pseudotuberculosis* has been implicated as the major pathogen in CLA [24]. In this study, the isolation rate of *C. pseudotuberculosis* was higher in the summer season than in winter. This could be because infections in sheep are often introduced through superficial skin wounds; these wounds can commonly occur during shearing, castration, tagging and docking, procedures that all take place in the summer. The fact that the abattoir materials in the region of Van mostly represent unproductive and unhealthy sheep might have also contributed to this finding. The breeders are eager to slaughter the animals in a short period of time, meaning the animals may be slaughtered during the summer rather than at the beginning of winter.

Researches performed on naturally infected sheep materials to determine the diagnostic sensitivity and specificity of the PCR assay in comparison to bacteriological culture methods are scarce. PACHECO et al. [18] analysing pus samples collected from abscessed lymph nodes of 12 naturally infected sheep and 44 goats by bacteriological culture and multiplex PCR (mPCR) reported that the relative mPCR sensitivity was 91.7% in sheep, 95.4% in goats and 94.6% in both species. In the present study, 81 (55.1%) and 85 (57.8%) of the lymph-node samples from sheep were positive by culture and PCR, respectively. Comparing these techniques, 5 (3.40%) of the samples were PCR positive but culture negative and would be considered as false positive results, and only one sample (0.68%) was PCR negative but culture positive and would be considered as false negative result. Consequently, the relative PCR sensitivity and specificity compared to cultures in the present study were 98.76% and 92.42% respectively, leading to a positive predictive probability of 98.4% and to a negative predictive probability of 94.1%. The diagnostic gain obtained here was higher than previously reported by PACHECO et al. [18]. Several factors may influence the sensitivity, specificity and efficiency of PCR assays, including the nucleic acid extraction.
method, physic and chemical conditions of the reaction, total reaction volume and the concentration of target DNA in the samples [5]. Indeed, different samples and different input volumes of the extraction materials were used and different extraction protocols were applied in the two studies. Another plausible explanation for these differences could be the presence of various inhibitory substances such as haemoglobin or blood in the samples. PACHECO et al. [18] used pus samples that may have included more inhibitory substances than the lymph-tissue samples used in this study. Several methods have been attempted to decrease the effect of inhibitors, including sample dilution, the addition of cationic surfactants, or other sample facilitators such as BSA or polyethylene glycol [11, 14]. Differing from PACHECO et al. [18], BSA was added to the PCR reaction mixtures in this study, which may enhance the sensitivity of PCR assays. These findings may indicate that the analysis of lymph tissue samples by PCR is a better option for the detection of *C. pseudotuberculosis* DNA than the analysis of pus samples from post-mortem sheep.

Studies concerning the limits of detection for genomic DNA from *C. pseudotuberculosis* by PCR assays are also limited. Blood samples from healthy goats were seeded with 10^1-10^6 CFU/mL of *C. pseudotuberculosis* and then tested by mPCR. The amplification products were detected in reactions containing 10^6-10^7 CFU/mL of *C. pseudotuberculosis* [18]. In the present study, when the lymph-node samples from healthy sheep were artificially contaminated, the detection limit of the PCR assay was 2.1x10^3-2.1x10^4 CFU/mL of *C. pseudotuberculosis*. This detection limit is lower than those previously reported [18]. This may be due to several reasons. First, the spiked samples used were different in the two studies. Second, other inhibitory substances, such as such as collagen, lactoferrin and PCR conditions may affect the detection limit of the PCR assay [1, 22]. This detection limit does not pose a problem in testing samples for CLA as very large numbers of the microorganisms are present in CLA-infected sheep [7, 15, 27].

The cell-wall content of Corynebacteria is somewhat unique and is very high in lipid content, the most notable being mycolic and meso-diaminopimelic acids. On the basis of this lipid content, the Corynebacteria genus is often grouped with Mycobacteria, Nocardia and Rhodococci to form the “CMNR” group [4, 8]. Pathogen members of the CMNR group cause chronic and suppurative infections in animals [8, 21, 26]. This PCR protocol might be applied for the diagnosis of CMNR-group infections, but further studies in a variety of animal species will be required.

Bacteriological culture and serological assays have traditionally been used for the diagnosis of CLA, but these techniques have a number of drawbacks. Thus, an alternative, reliable method is needed to detect *C. pseudotuberculosis* in clinical materials. This study presents a standardized PCR protocol using lymph node samples from sheep; the positive and negative predictive probabilities of this assay were determined to be 98.4% and 94.1%, respectively. As a conclusion, data from the current study suggests that, due to its high sensitivity, relatively high specificity and rapidity, the PCR protocol could be utilized as an alternative to traditional bacteriological culture methods for the detection of *C. pseudotuberculosis* in lymph node samples from naturally infected sheep. The results of the present study are particularly important in aiding the understanding of the epidemiology of CLA and control of the disease.

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**References**


