Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples

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*Corynebacterium pseudotuberculosis* is the aetiological agent of caseous lymphadenitis (CLA), a debilitating disease of sheep and goats. Accurate diagnosis of CLA primarily relies on microbiological examination, followed by biochemical identification of isolates. In an effort to facilitate *C. pseudotuberculosis* detection, a multiplex PCR (mPCR) assay was developed targeting three genes of this bacterium: the 16S rRNA gene, *rpoB* and *pld*. This method allowed efficient identification of 40 isolates of this bacterium that had been identified previously by biochemical testing. Analysis of taxonomically related species did not generate the *C. pseudotuberculosis* mPCR amplification profile, thereby demonstrating the assay’s specificity. As little as 1 pg of *C. pseudotuberculosis* genomic DNA was detected by this mPCR assay, demonstrating the sensitivity of the method. The detection limit in clinical samples was estimated to be 10³ c.f.u. *C. pseudotuberculosis* could be detected directly in pus samples from infected sheep and goats (*n* = 56) with a high diagnostic sensitivity (94.6%). The developed assay significantly improves rapid *C. pseudotuberculosis* detection and could supersede bacteriological culture for microbiological and epidemiological diagnosis of CLA.

INTRODUCTION

*Corynebacterium pseudotuberculosis* is a mycolic acid-containing, facultative intracellular actinomycete associated with the development of abscesses in a variety of mammalian hosts (Songer et al., 1988; Peel et al., 1997; Dorella et al., 2006). This Gram-positive bacterium most commonly causes ulcerative lymphangitis in horses and caseous lymphadenitis (CLA) in small ruminants (Biberstein et al., 1971; Williamson, 2001). The latter is a chronic disease of sheep and goats, mainly characterized by the formation of suppurative abscesses in superficial and internal lymph nodes (Kuria et al., 2001; Williamson, 2001). CLA is distributed globally and causes important economic losses for ovine and caprine husbandries, due to reduced wool, meat and milk yields, culling of affected animals and condemnation of carcasses and skins in slaughterhouses (Williamson, 2001; Dorella et al., 2006).

Once established in a herd or flock, CLA eradication is problematic due to the inefficacy of antimicrobial therapy (Piontkowski & Shivvers, 1998; Stanford et al., 1998; Williamson, 2001). The most reliable control strategy for this disease involves vaccinating livestock and identifying and removing infected animals (Brown et al., 1986; Paton...
et al., 2003). However, this approach is hindered by limitations in current diagnostic techniques (Williamson, 2001; Menzies et al., 2004; Dorella et al., 2006).

Many serological tests have been proposed for CLA diagnosis, including a complement fixation test (Shigidi, 1979), synergistic haemolysis-inhibition test (Brown et al., 1987), microagglutination assay (Menzies & Muckle, 1989) and C. pseudotuberculosis phospholipase D (PLD) antigen-based ELISA (Dercksen et al., 2000). Although these tests may be of great value for the detection of subclinically infected animals, most present drawbacks, including low sensitivity, poor specificity and an inability to distinguish between previously exposed animals and those still harbouring the pathogen. Thus it is questionable whether such techniques should be employed to orient culling programmes (Williamson, 2001; Çetinkaya et al., 2002).

Accurate CLA diagnosis is based primarily upon clinical observations (external abscesses) and the identification of C. pseudotuberculosis by phenotypic and biochemical tests; this is important to differentiate this bacterium from other abscess-inducing pathogenic agents, such as Arcanobacterium pyogenes or Pasteurella multocida (Dercksen et al., 2000; Williamson, 2001; Dorella et al., 2006).

A 16S rRNA gene-based PCR assay has been developed to identify C. pseudotuberculosis isolates (Çetinkaya et al., 2002). Although this assay was useful for estimating the prevalence of CLA in the animals studied, it presented some limitations: (i) it was dependent on bacterial culture; and (ii) it was not specific enough to distinguish C. pseudotuberculosis from Corynebacterium ulcerans (Çetinkaya et al., 2002).

In order to improve C. pseudotuberculosis detection by PCR, we developed a multiplex PCR (mPCR) assay and adapted a protocol to extract bacterial genomic DNA directly from clinical samples. Amplification of multiple loci in a single reaction through mPCR is a powerful and widely used tool for the rapid and specific identification of pathogenic bacteria (Wadowsky et al., 1996; Halbert et al., 2005; O’Halloran & Cafferkey, 2005; Persson & Olsen, 2005). Our mPCR targeted three C. pseudotuberculosis genes: the 16S rRNA gene, the gene of choice for most microbial taxonomy studies (Çetinkaya et al., 2002; Khamis et al., 2005); rpoB, the RNA polymerase β-subunit gene, currently used to study phylogenetic relationships in the genera Corynebacterium and Mycobacterium (Khamis et al., 2004, 2005; Dorella et al., 2006); and pld, which encodes the exotoxin PLD, a sphingomyelinase implicated in the virulence of C. pseudotuberculosis, C. ulcerans and Arcanobacterium haemolyticum (McNamara et al., 1995). This mPCR enabled specific identification of C. pseudotuberculosis isolates in culture and direct detection in pus samples from CLA-affected animals.

**METHODS**

**Bacterial strains and culture conditions.** The bacterial strains used in this study are listed in Table 1. A total of 40 caprine isolates of C. pseudotuberculosis were obtained from different sources. One type strain of C. pseudotuberculosis biovar ovis (strain CIP 102968T) and 13 strains of genetically or morphologically related bacteria were also used in the experiments.

Bacteria were grown routinely in brain heart infusion broth (BHI; Oxoid) or on 1.5 % (w/v) BHI bacteriological agar plates at 37 °C for 48–72 h. Tween 80 was added to the medium at a final concentration of 1 % (v/v) for the Corynebacterium bovis strain.

**Clinical specimens.** Fifty-six pus samples were collected aseptically from abscessed lymph nodes of naturally infected sheep (n = 12) and goats (n = 44) found in two CLA-endemic areas of Brazil. Microbiological examination, followed by biochemical identification, was used as a gold standard to confirm infection with C. pseudotuberculosis. In brief, bacteriological cultures were made of pus specimens and the resultant C. pseudotuberculosis-resembling colonies that stained Gram-positive were tested further for biochemical properties (glucose fermentation, urease and catalase) (Smibert & Krieg, 1994; Dercksen et al., 2000). Synergistic haemolysis with Rhodococcus equi ATCC 33701 and inhibition of β-haemolysis by Staphylococcus aureus ATCC 25923 were also evaluated (Smibert & Krieg, 1994; Dercksen et al., 2000). Species identification was confirmed using the API Coryne battery (bioMérieux).

Blood samples were taken from confirmed C. pseudotuberculosis-infected animals (n = 19) and from healthy animals (n = 20) using the Vacutainer blood collection system (Becton Dickinson).

**DNA isolation**

Two different protocols were adapted for extracting DNA from pure bacterial cultures and clinical samples.

(i) **Bacterial cultures.** Chromosomal DNA extraction was performed in the same manner for all bacterial strains. A 20 ml 48–72 h culture was centrifuged at 4 °C and 2000 g for 20 min. Cell pellets were resuspended in 1 ml Tris/EDTA/RNase [10 mM Tris/ HCl (pH 7.0), 10 mM EDTA (pH 8.0), 300 mM NaCl, 50 μg RNase A ml⁻¹] and centrifuged again under the same conditions. Supernatants were discarded and the pellets were resuspended in 1 ml TE/lysozyme [25 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0), 10 mM NaCl, 10 mg lysozyme ml⁻¹]. Samples were then incubated at 57 °C for 30 min; 30 μl 30 % (w/v) sodium N-lauroyl-sarcosine (sarcosyl) was added and the mixture was incubated for 20 min at 65 °C, followed by incubation for 5 min at 4 °C. DNA was purified using phenol/chloroform/isooamyl alcohol and precipitated with ethanol (Sambrook et al., 1989). DNA concentrations were determined spectrophotometrically.

(ii) **Clinical samples.** A DNA extraction protocol previously described for clinical samples from tuberculosis patients (Honore-Bouakline et al., 2003) was adapted for use in this study. Briefly, 100 mg pus or the pellet of a 2 ml blood sample was resuspended in 1 ml TE/lysozyme. Samples were incubated for 1 h at 37 °C, 20 μl proteinase K (20 mg ml⁻¹; Invitrogen) was added, followed by incubation for 2 h at 56 °C. Samples were divided into two aliquots of 500 μl and 25 μl 30 % (w/v) sarcosyl was added to each; mixtures were incubated for 20 min at 65 °C and then for 5 min at 4 °C. DNA was purified and precipitated as described above.

**Primers and mPCR conditions.** The oligonucleotide primers used in this study are listed in Table 2. Primers targeting the 16S rRNA and rpoB genes of C. pseudotuberculosis were obtained from previously published work (Çetinkaya et al., 2002; Khamis et al., 2004). Primers targeting the pld gene were designed by aligning the pld nucleotide sequences of C. pseudotuberculosis and C. ulcerans.
Table 1. Bacterial strains and field isolates of C. pseudotuberculosis used in this study

GUH, Ghent University Hospital, Belgium (DL and HJ: initials of donators Devrise Luc and Hommez Joseph); UFBA, Collection of Micro-organisms of Universidade Federal da Bahia, Brazil; NCTC, National Collection of Type Cultures, UK; IOC, Collection of Micro-organisms of Instituto Oswaldo Cruz, Brazil; CIP, Collection of the Institut Pasteur, France; ATCC, American Type Culture Collection, USA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source/strain designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arcanobacterium pyogenes</td>
<td>GUH HJ 26 BA224.11</td>
</tr>
<tr>
<td>A. pyogenes-like</td>
<td>GUH HJ 51 B</td>
</tr>
<tr>
<td>Corynebacterium amycolatum</td>
<td>GUH HJ 08 I4218 LV</td>
</tr>
<tr>
<td>C. bovis</td>
<td>GUH DL BOV25</td>
</tr>
<tr>
<td>C. diphtheriae</td>
<td>ATCC 13812</td>
</tr>
<tr>
<td>C. jeikeium</td>
<td>NCTC K411</td>
</tr>
<tr>
<td>C. pseudotuberculosis biovar equi</td>
<td>CIP 5297</td>
</tr>
<tr>
<td>C. pseudotuberculosis biovar ovis</td>
<td>VD21; VD23; VD27; VD28; VD33; VD36; VD37; VD38; VD41; VD42; VD43; VD44; VD45; VD46; VD48; VD50; VD52; VD53; VD54; VD55; VD56; VD57; VD58; VD59; VD60; VD61; VD62; VD63; VD99; 01; 05; 08; 09; 11; 12; 21; 217</td>
</tr>
<tr>
<td>C. pseudotuberculosis biovar ovis (n = 37)*</td>
<td>UFBA T1</td>
</tr>
<tr>
<td>C. pseudotuberculosis biovar ovis</td>
<td>UFBA 1002</td>
</tr>
<tr>
<td>C. pseudotuberculosis biovar ovis</td>
<td>GUH HJ ULCC06.334,4</td>
</tr>
<tr>
<td>C. pseudotuberculosis biovar ovis</td>
<td>CIP 102968\textsuperscript{T}</td>
</tr>
<tr>
<td>C. renale</td>
<td>CIP 103421\textsuperscript{T}</td>
</tr>
<tr>
<td>C. ulcerans</td>
<td>GUH HJ ULCA3.675,1</td>
</tr>
<tr>
<td>C. ulcerans</td>
<td>GUH HJ 01 BM3796.3</td>
</tr>
<tr>
<td>C. ulcerans</td>
<td>GUH HJ 02 MN 675.3</td>
</tr>
<tr>
<td>C. ulcerans</td>
<td>GUH HJ ULCB12.735,2</td>
</tr>
<tr>
<td>Pasteurella multocida</td>
<td>IOC</td>
</tr>
</tbody>
</table>

*Caprine field isolates; obtained from Universidade Federal da Bahia, Brazil.

The reverse primer PLD-R1 was used in association with the forward primer PLD-F to amplify the pld genes of both bacteria, whilst primer PLD-R2 excluded C. ulcerans (see Fig. 1b). A primer pair targeting the mitochondrial 12S rRNA genes of Capra hircus and Ovis aries (GenBank accession nos A1490504 and A1490503) was designed to function as an internal amplification control for the mPCRs performed with DNA extracted directly from clinical samples of CLA. This amplification control allowed identification of samples that possessed factors inhibitory for the mPCR assay (Hoorfar et al., 2004).

Multiplex PCRs were performed in a final reaction volume of 10 μl containing 1.5 U AccuPrime Taq DNA polymerase (Invitrogen), 1 × PCR Buffer II [200 mM Tris/HCl, 500 mM KCl, 15 mM MgCl₂, 2 mM dNTPs, AccuPrime protein (Invitrogen), 10% glycerol] and 2 μM of each of the primers 16S-F/16S-R, C2700F/C3130R and PLD-F/PLD-R2. For mPCRs performed with DNA extracted from clinical samples, primers 12S-F/12S-R were added to the reaction mixture at 0.2 μM each. The template concentration was approximately 10 ng DNA extracted from cultured C. pseudotuberculosis or from clinical specimens. Where necessary, serial twofold dilutions (1:2, 1:4 and 1:8) were performed.

Table 2. List of oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Sequence (5’→3’)</th>
<th>Length of PCR products (bp)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene</td>
<td>16S-F</td>
<td>ACCGCACCTTTAGTGTGTGTG</td>
<td>816</td>
<td>Çetinkaya et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>16S-R</td>
<td>TCITCITACCGCGGATCTTGTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rpoB</td>
<td>C2700F</td>
<td>CGTATGAAACATCGCCAGGTT</td>
<td>446</td>
<td>Khamis et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>C3130R</td>
<td>TCCATTTCGCGGAGGCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pld</td>
<td>PLD-F</td>
<td>ATAAAGCGTAAAGCGGGAGCA</td>
<td>203</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>PLD-R1</td>
<td>ATCAGCGGTAGTTGTCTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLD-R2</td>
<td>ATCAGCGGTAGTTGTCTTCCAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12S rRNA gene</td>
<td>12S-F</td>
<td>CCAGCCACCGCGGGCTACAG</td>
<td>274</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>12S-R</td>
<td>TGAGTTTCGGCGGCTTTGGCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
agarose gels and visualized by ethidium bromide staining. amplified products were resolved by electrophoresis on 1.0 % (w/v)
dermatation at 95
°C (PTC-100; MJ Research) under the following conditions: initial
40 s and 68
°C for 1 min 30 s; final extension at 68
°C for 7 min. The
Fig. 1. Analytical specificity of the mPCR assay. (a) Amplification
profiles of bacteria genetically or morphologically related to C.
pseudotuberculosis. Lanes 1–15 show reactions with DNA from
the following bacterial strains: 1 and 2, C. pseudotuberculosis
ovis strains CIP 102968\(^7\) and 1002; 3, C. pseudotuberculosis
equi CIP 5297; 4–7, C. ulcerans strains ULCA3.675.1, HJ 01
BM3796.3, HJ 02 MN 675.3 and ULCB12.735.2; 8, C. diphtheriae
ATCC 13812; 9, Corynebacterium amyclatum HJ 08 14218
LV; 10, Corynebacterium renale CIP 103421\(^1\); 11, C. bovis DL
BOV25; 12, Corynebacterium jeikeium K411; 13, Arcanobacterium
pyogenes HJ 26 BA224.11; 14, A. pyogenes-like HJ 51
B; 15, Pasteurella multocida. Lane 16, negative control (reaction
without template DNA). MW, 1 kb Plus DNA Ladder (Invitrogen).
(b) Schematic representation of primer-annealing sites in the
pld
genes of C. pseudotuberculosis (Cp) and C. ulcerans (Cu). The
underlined nucleotides indicate positions of mismatch between
the two aligned gene sequences (see text for details).

were performed on samples to dilute inhibitory components (Honré-
Bouakline et al., 2003). Reactions were carried out in a thermal cycler
(PTC-100; MJ Research) under the following conditions: initial
denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 1 min, 58 °C for
40 s and 68 °C for 1 min 30 s; final extension at 68 °C for 7 min. The
amplified products were resolved by electrophoresis on 1.0 % (w/v)
agarose gels and visualized by ethidium bromide staining.

Sequencing of singleplex PCR products. In order to confirm
the mPCR results, ten randomly chosen isolates were tested further
in singleplex PCR assays with the three C. pseudotuberculosis-specific
primer pairs. PCR products were precipitated with 15 % (w/v)
poly
ethylene glycol (Kusukawa et al., 1990) and sequenced using the
DYE
namic ET Dye Terminator kit (Amersham Biosciences), according
to the manufacturer’s instructions. The sequences were compared
with previously published 16S rRNA (GenBank accession nos
X81916, X81907 and X84255), rpoB (GenBank accession no.
AY492239) and pld (GenBank accession nos L16586 and L16587) C.
pseudotuberculosis gene sequences, by searching for similarity using
BLAST-N (Altschul et al., 1990).

Analytical specificity and sensitivity. To evaluate the specificity
of the mPCR assay, reactions were performed with genomic DNA
extracted from bacterial strains taxonomically similar to C.
pseudotuberculosis biovar ovis (Table 1). Serial tenfold dilutions of DNA from
C. pseudotuberculosis type strain CIP 102968\(^7\), ranging from 10 to
0.0001 ng DNA per reaction, were used to test the sensitivity of the
method.

To evaluate the assay’s detection limit, blood samples from goats that
did not present clinical symptoms of CLA, and which were negative in
ELISA tests (performed according to Paule et al., 2003), were seeded
with 10\(^{-6}\)–10\(^{-10}\) c.f.u. of C. pseudotuberculosis.

Statistical analysis. An analysis to evaluate differences in the sen-
tivity of the mPCR assay between samples from sheep versus goats
was performed with a \( \chi^2 \) test, using the STATISTICA software package
(StatSoft).

RESULTS

Specificity and sensitivity of the mPCR assay

Purified genomic DNAs were used to evaluate the sensitivity
and specificity of the mPCR assay. When tested with DNA from
bacterial strains taxonomically related to C. pseudotuberculosis
biovar ovis (Table 1), only C. pseudotuberculosis biovar equi CIP 5297 yielded a similar mPCR profile, with the 816, 446 and 203 bp amplicons corresponding to the 16S
rRNA, rpoB and pld genes, respectively (Fig. 1a). As in a
previous study (Khamis et al., 2004), all of the corynebacteria
species that we tested generated an amplicon of
~446 bp, corresponding to the rpoB gene (Fig. 1a). As
expected, the pld gene product was not detected in any of the
four C. ulcerans strains (Fig. 1a, lanes 4–7), illustrating the
ability of the mPCR assay to differentiate this bacterium
from C. pseudotuberculosis. Moreover, two other pathogenic
bacteria associated with the formation of abscesses in small
ruminants, A. pyogenes and P. multocida, were clearly
distinguished (Fig. 1a). The ~300 bp product generated by
amplification of A. pyogenes genomic DNA was sequenced
and assigned as the rpoB gene following a search for
homology in GenBank using BLAST-N (data not shown).

To determine the sensitivity of the assay, reactions were
performed with serial tenfold dilutions of purified C.
pseudotuberculosis CIP 102968\(^7\) DNA. The mPCR method

Fig. 2. Analytical sensitivity of the mPCR assay. Lanes 1–6,
mPCR with serial tenfold dilutions of C. pseudotuberculosis
CIP 102968\(^7\) genomic DNA, as follows: 10, 1, 0.1, 0.01,
0.001 and 0.0001 ng. Lane 7, negative control (reaction with-
out template DNA). MW, 1 kb Plus DNA Ladder (Invitrogen).
was able to detect bacterial DNA at concentrations ranging from 10 to 0.001 ng per reaction (Fig. 2).

To assess the detection limit of the assay in clinical specimens, blood samples of healthy animals were spiked with tenfold dilutions of a pure culture of C. pseudotuberculosis CIP 102968T and the mixtures were processed using the entire DNA extraction protocol. Amplification products were detected in reactions containing $10^3$–$10^6$ c.f.u. C. pseudotuberculosis (ml sample)$^{-1}$ (data not shown).

**Identification of bacterial isolates**

The characteristic mPCR amplification profile was obtained for all 40 C. pseudotuberculosis biovar ovis field isolates tested (Table 1). Fig. 3(a) shows the mPCR profiles of ten randomly chosen C. pseudotuberculosis biovar ovis field isolates.

Identification of the ten isolates was confirmed by sequencing each of the three amplicons generated, followed by a search for similar sequences using BLAST-N (data not shown).

**Direct detection of C. pseudotuberculosis in clinical samples by mPCR**

Pus samples were collected from the lymph nodes of CLA-affected sheep and goats and DNA was extracted directly from these specimens using our protocol (see Methods). A bacteriological culture was prepared from the samples as a gold standard to confirm infection by C. pseudotuberculosis. The mPCR protocol was performed as described for purified bacterial DNA; however, a non-competitive internal amplification control (12S rDNA; see Methods) was included in these reactions in order to allow differentiation between inhibited reactions, i.e. those in which there was no amplification of the internal control, and reactions in which the mPCR yielded a negative result (Hoorfar et al., 2004). Typical mPCR profiles of clinical samples are shown in Fig. 3(b).

Most of the undiluted samples were inhibitory for the mPCR assay, but more than 52% yielded detectable results at a 1:2 dilution and ~ 45% showed amplification at a 1:4 dilution, whilst only 3% showed amplification at a 1:8 dilution. The proportions of samples positive in the mPCR assay were not significantly different when comparing samples of sheep versus goats ($P=0.60$). Table 3 summarizes the results obtained for pus samples.

The ability of the mPCR assay to detect C. pseudotuberculosis in blood samples from confirmed CLA-affected animals was also evaluated; however, none of the 19 blood samples that we tested yielded a positive result (data not shown).

**DISCUSSION**

CLA remains a cause of concern for sheep and goat producers worldwide (Baird, 1997; Williamson, 2001; Paton et al., 2003; Dorella et al., 2006). Due to the high transmission rate of its aetiological agent, C. pseudotuberculosis, within a herd or flock, some authors suggest the culling of any animal from which this bacterium has been cultured as a means of controlling spread of the disease (Baird, 1997; Williamson, 2001). As microbiological and biochemical methods are not always straightforward, the development of a rapid and specific diagnostic tool is imperative for the control of CLA (Çetinkaya et al., 2002).

The only previously reported molecular method employed for specific identification of C. pseudotuberculosis isolated from pus samples of CLA-affected animals, based on the amplification of a 16S rRNA gene fragment (Çetinkaya et al., 2002), has two major drawbacks: (i) dependence on bacterial culture; and (ii) an inability to distinguish the bacterium C. ulcerans.

Table 3. Results of direct detection of C. pseudotuberculosis in pus samples of CLA-affected animals using the mPCR assay

<table>
<thead>
<tr>
<th>Pus samples*</th>
<th>mPCR result</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Sheep ($n=12$)</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Goats ($n=44$)</td>
<td>42</td>
<td>2</td>
</tr>
<tr>
<td>Total ($n=56$)</td>
<td>53</td>
<td>3</td>
</tr>
</tbody>
</table>

*Samples positive for C. pseudotuberculosis in bacteriological culture (gold standard).
Our mPCR assay was specific enough to differentiate C. pseudotuberculosis from C. ulcers (Fig. 1a). These two bacteria have been reported to possess 99.7% similarity between their 16S rRNA genes and 93.6% between their rpoB genes (Riegel et al., 1995; Khamis et al., 2004); most of their biochemical properties are similar and both may eventually produce diphtheric toxin (Riegel et al., 1995; DeWinter et al., 2005). The high genomic similarity may explain the inability of a previously published study to differentiate these two bacteria by PCR (Çetinkaya et al., 2002). As C. ulcers is also a PLD producer, the reverse primer used to amplify the pld gene in our study was designed so that it possessed a mismatch in its 3’ end (two Gs instead of two Cs) (Fig. 1b), allowing differentiation from C. pseudotuberculosis due to the absence of the 203 bp amplicon.

Although C. ulcers is primarily recognized as a veterinary pathogen, there has been a marked increase in the number of human infections (DeWinter et al., 2005; De Zoysa et al., 2005) and it appears that domestic cats and dogs can be potential reservoirs (De Zoysa et al., 2005). In this context, our mPCR would be able to identify bacteria collected from the nasal discharge of domestic animals and could differentiate bacteria cultured from samples from diphtheria patients by using the primer pair PLD-F/PLD-R1, which allows amplification of the pld gene of C. ulcers (see Fig. 1b).

Çetinkaya et al. (2002) reported a weaker amplification of the 816 bp 16S rRNA gene amplicon in biovar equi of C. pseudotuberculosis when compared with that of biovar ovis. This result was attributed to a mismatch (an A instead of a G) five bases from the 5’ end of the forward primer used (16S-F; Table 2). However, this result was not reproducible under the conditions used in our study. We propose that amplification of the 16S rRNA gene using primers 16S-F/16S-R is not sufficient to differentiate the two biovars of C. pseudotuberculosis. It is interesting that infection of small ruminants with biovar equi is not known to occur (Spier et al., 2004).

The mPCR assay was able to confirm the identification of 40 C. pseudotuberculosis field isolates and reliably detected this bacterium in a reaction mixture containing as little as 0.001 ng of genomic DNA, illustrating its reproducibility and high sensitivity.

A DNA extraction protocol was adapted to recover bacterial genomic DNA directly from clinical samples of CLA-affected animals. This strategy offers advantages over bacteriological culture, including reduced time of analysis and the ability to detect unviable bacteria. The detection limit of the goat blood spiking experiment was $10^3$ c.f.u. C. pseudotuberculosis (ml sample)$^{-1}$. However, this value may underestimate the number of viable organisms, as intracellular bacteria such as C. pseudotuberculosis and R. equi have a tendency to clump when cultured (Harrington et al., 2005). A detection limit above $10^3$ c.f.u. (ml sample)$^{-1}$ would help explain the inability of the mPCR assay to detect C. pseudotuberculosis in blood samples from infected sheep and goats.

On the other hand, the mPCR efficiently detected this bacterium in a high proportion (94.6%) of confirmed CLA-infected pus samples. In contrast to serological CLA testing (Menzies & Muckle, 1989; Dercksen et al., 2000), the mPCR sensitivity was the same for both sheep and goats (Table 3).

As our mPCR assay provides an efficient, accurate, rapid and reproducible method for the identification of cultured C. pseudotuberculosis and its direct detection in pus samples, we propose that it may be used instead of bacteriological culture as a confirmatory CLA test.

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