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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 10^3 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.

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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

Abbreviations: CLA, caseous lymphadenitis; mPCR, multiplex PCR; PLD, phospholipase D.

The GenBank/EMBL/DDBJ accession number for the *rpoB* gene sequence of *Arcanobacterium pyogenes* is DQ680032.

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 102968^T) 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 37 °C for 30 min; 30 µl 30 % (w/v) sodium *N*-lauroylsarcosine (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/isoamyl 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 (Honoré-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.

Species	Source/strain designation
Arcanobacterium pyogenes	GUH HJ 26 BA224.11
A. pyogenes-like	GUH HJ 51 B
Corynebacterium amycolatum	GUH HJ 08 I4218 LV
C. bovis	GUH DL BOV25
C. diphtheriae	ATCC 13812
C. jeikeium	NCTC K411
C. pseudotuberculosis biovar *equi*	CIP 5297
C. pseudotuberculosis biovar *ovis* ($n=37$)*	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
C. pseudotuberculosis biovar *ovis*	UFBA T1
C. pseudotuberculosis biovar *ovis*	UFBA 1002
C. pseudotuberculosis biovar *ovis*	GUH HJ ULCC06.334,4
C. pseudotuberculosis biovar *ovis*	CIP 102968^T
C. renale	CIP 103421^T
C. ulcerans	GUH HJ ULCA3.675,1
C. ulcerans	GUH HJ 01 BM3796.3
C. ulcerans	GUH HJ 02 MN 675.3
C. ulcerans	GUH HJ ULCB12.735,2
Pasteurella multocida	IOC

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

(GenBank accession nos L16587 and L16585); 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 AJ490504 and AJ490503) 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 $MgCl_2$, 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)

Table 2. List of oligonucleotide primers used in this study

Target gene	Primers	Sequence (5′→3′)	Length of PCR products (bp)	Source/reference
16S rRNA gene	16S-F	ACCGCACTTTAGTGTGTGTG	816	Çetinkaya *et al.* (2002)
	16S-R	TCTCTACGCCGATCTTGTAT		
rpoB	C2700F	CGTATGAACATCGGCCAGGT	446	Khamis *et al.* (2004)
	C3130R	TCCATTTCGCCGAAGCGCTG		
pld	PLD-F	ATAAGCGTAAGCAGGGAGCA	203	This work
	PLD-R1	ATCAGCGGTGATTGTCTTCC		
	PLD-R2	ATCAGCGGTGATTGTCTTCCAGG		
12S rRNA gene	12S-F	CCAGCCACCGCGGTCATACG	274	This work
	12S-R	TGAGTTTCGGGCTGTTGCCG		

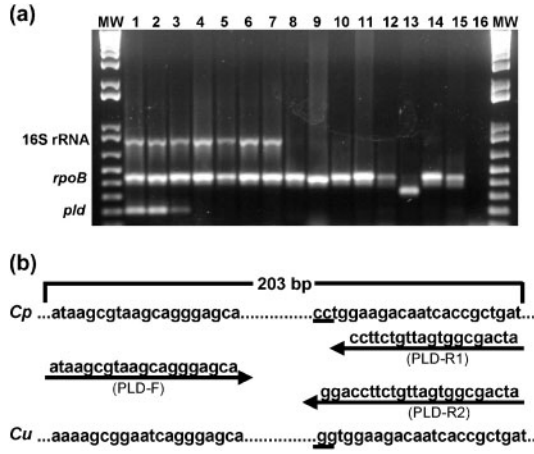

Fig. 1. Analytical specificity of the mPCR assay. (a) Amplification profiles of bacteria genetically or morphologically related to *C. pseudotuberculosis*. Lanes 1–15 show reactions with DNA from the following bacterial strains: 1 and 2, *C. pseudotuberculosis ovis* strains CIP 102968^T and 1002; 3, *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 amycolatum* HJ 08 I4218 LV; 10, *Corynebacterium renale* CIP 103421^T; 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 (Honoré-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) polyethylene glycol (Kusukawa *et al.*, 1990) and sequenced using the DYEnamic 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^T, 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 10^1–10^6 c.f.u. of *C. pseudotuberculosis*.

Statistical analysis. An analysis to evaluate differences in the sensitivity of the mPCR assay between samples from sheep versus goats was performed with a χ^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^T DNA. The mPCR method

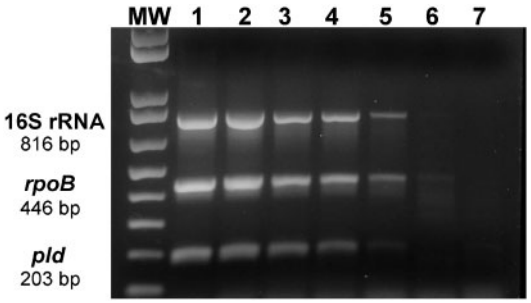

Fig. 2. Analytical sensitivity of the mPCR assay. Lanes 1–6, mPCR with serial tenfold dilutions of *C. pseudotuberculosis* CIP 102968^T genomic DNA, as follows: 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng. Lane 7, negative control (reaction without template DNA). MW, 1 kb Plus DNA Ladder (Invitrogen).

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 102968^T 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).

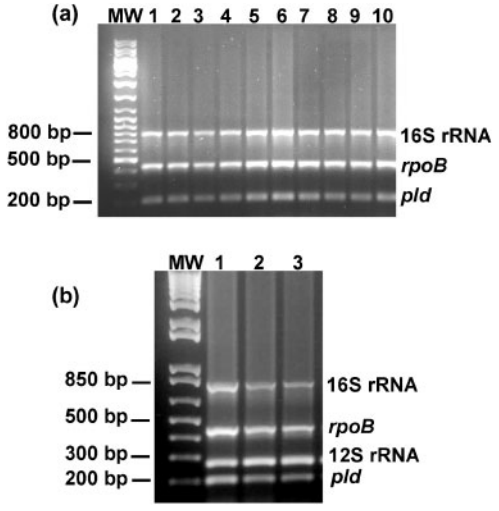

Fig. 3. Reproducibility of the mPCR assay. (a) Lanes 1–10, mPCR amplification profiles of 10 of the 40 field isolates of *C. pseudotuberculosis*. MW, GeneRuler DNA Ladder Mix (Fermentas). (b) Lanes 1–3, direct detection of *C. pseudotuberculosis* in pus samples of CLA-affected animals. MW, 1 kb Plus DNA Ladder (Invitrogen).

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

Pus samples*	mPCR result		Sensitivity (%)
	Positive	Negative	
Sheep ($n=12$)	11	1	91.7
Goats ($n=44$)	42	2	95.4
Total ($n=56$)	53	3	94.6

*Samples positive for *C. pseudotuberculosis* in bacteriological culture (gold standard).

Our mPCR assay was specific enough to differentiate *C. pseudotuberculosis* from *C. ulcerans* (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. ulcerans* 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. ulcerans* 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. ulcerans* (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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