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Identification of *Corynebacterium pseudotuberculosis* from sheep by PCR-restriction analysis using the RNA polymerase β-subunit gene (*rpoB*)

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ABSTRACT

Caseous lymphadenitis, caused by *Corynebacterium pseudotuberculosis*, has a high prevalence in many regions of the world, including Argentina and Brazil. A polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method for the identification of this microorganism was designed based on the hypervariable region of the polymorphic RNA polymerase β-subunit gene (*rpoB*). All available *Corynebacterium rpoB* sequences were analyzed by computer-assisted restriction analysis. The *rpoB* PCR–RFLP pattern predicted by using endonucleases *Mse*I and *Stu*I clearly differentiated *C. pseudotuberculosis* from sixty-one other *Corynebacterium* species. This method was successfully applied to identify twelve wild *C. pseudotuberculosis* ovine isolates and one caprine isolate. It was also used to differentiate *C. pseudotuberculosis* from *Arcanobacterium pyogenes*, an ovine pathogen with similar clinical characteristics. These results indicate that this new molecular method can be used for the reliable identification of the pathogen, essential for the timely detection of infected animals and for epidemiological studies.
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1. Introduction

Corynebacterium pseudotuberculosis is a Gram-positive, facultative anaerobe and facultative intracellular parasite with worldwide distribution. It is the etiological agent of caseous lymphadenitis (CLA), a chronic suppurative necrotizing inflammation of lymph nodes in small ruminants (Pekelder, 2000; Baird and Fontaine, 2007; Robles, 2007). This bacterium is also the causative agent of ulcerative lymphangitis in cattle and horses, and external and internal abscesses in horses (Aleman et al., 1996; Spier and Whitcomb, 2007). Infection due to *C. pseudotuberculosis* has also been reported in buffalos, camelids, equids and rarely in humans (Peel et al., 1997; Selim, 2001; Anderson et al., 2004; Braga et al., 2006; Join-Lambert et al., 2006). Once established, the infection is difficult to eradicate because of its chronic and often sub-clinical nature and its poor response to therapeutics (Williamson, 2001; Dorella et al., 2006; Baird and Fontaine, 2007). The prevalence of CLA is high in many regions of the world, including South America, leading to significant economic losses due to a decrease in wool production and carcass quality (Pekelder, 2000; Baird and Fontaine, 2007). In Patagonia, the southernmost region of Argentina, where most Merino and Corriedale sheep and Angora goats are bred, the disease is endemic and prevalences can be as high as 70% within individual flocks (Robles and Olaechea, 2001; Estevao Belchior et al., 2006). In Brazil, a recent study conducted in goats has reported a seroprevalence of 78% (Seyffert et al., 2010). Vaccines are used in some countries with good results, but they have not yet been introduced in Argentina (Robles and Olaechea, 2001).

Several molecular typing methods have been used to determine the degree of relatedness between many different corynebacterial species, including nucleic acid hybridization, 16S rRNA gene sequence analysis and 16S rRNA gene restriction fragment length polymorphism (RFLP) (Pascual et al., 1995; Riegel et al., 1995; Ruimy et al., 1995; Hou et al., 1997; Björkroth et al., 1999). Sequence comparison of a hypervariable region within the *rpoB* gene, encoding the RNA polymerase β-subunit, has been proposed to replace or complement the 16S rRNA gene analysis for phylogenetic studies or accurate identification of *Corynebacterium* species, as *rpoB* is significantly more polymorphic than the 16S rRNA gene for members of the *Corynebacterium* genus (Khamis et al., 2004). However, DNA sequencing involves high costs, technical complexity and a considerable amount of time, making it difficult to apply for routine diagnostic testing. Recently a multiplex PCR assay for the identification of *C. pseudotuberculosis* that uses the 16S rRNA gene, *rpoB* and *pld* (encoding the phospholipase D in *C. pseudotuberculosis*, *Corynebacterium ulcerans* and *Arcanobacterium haemolyticum*) has been described (Pacheco et al., 2007). The purpose of the present study was to develop a straightforward

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Table 1
Strains of *Corynebacterium pseudotuberculosis* biovar ovis used in this study.

Field isolates[a]	Host	Lymph node/ organ source	Province, date of isolation
Intabari-7506	Caprine	Testicle	Río Negro, 2006
Intabari-5807	Ovine	Portal (hepatic) l. n.	Tierra del Fuego, 2007
Intabari-5907	Ovine	Precrural l. n.	Tierra del Fuego, 2007
Intabari-6007	Ovine	Inguinal l. n.	Río Negro, 2007
Intabari-K107, Intabari-K407, Intabari-K507	Ovine	Prescapular l. n.	Chubut, 2007
Intabari-K809, Intabari-K909	Ovine	Prescapular l. n.	Chubut, 2008
Intabari-K1009	Ovine	Prescapular and precrural l. n.	Chubut, 2008
Intabari-K1109, Intabari-K1209, Intabari-K1309	Ovine	Prescapular l. n	Chubut, 2009

[a] The strains of *C. pseudotuberculosis* presented in this table are part of the collection of the Bacteriology Laboratory of the Animal Health Group, from INTA Bariloche, Argentina. l. n.: lymph node.

and reliable molecular method for the identification of *C. pseudotuberculosis* based solely on the polymorphism of the widely distributed *rpoB* gene, allowing a proper and rapid identification for the timely detection of infected animals.

2. Materials and methods

2.1. Bacterial strains

For the isolation of *C. pseudotuberculosis* from ovines and caprines with clinical CLA from Patagonia, Argentina, blood agar plates enriched with 7% fresh sheep blood were used. The cultures were incubated with 5% CO_2 for 48 h at 37 °C. The *C. pseudotuberculosis* strains were characterized by biochemical routine assays. All were positive for β-hemolysis, catalase, urease and glucose fermentation, and negative for motility test, oxidase test, lactose and trehalose fermentation, arginine hydrolysis, liquefaction of gelatin, growth in MacConkey agar, hydrogen sulfide production, gas production from D-glucose, indole production, methyl red reaction and Voges–Proskauer reaction. All strains were also negative for nitrate reduction, indicating that they belong to biovar ovis. All *C. pseudotuberculosis* strains were characterized by 16S rRNA gene analysis as previously described (Çetinkaya et al., 2002). Data corresponding to the strains under study, including animal source, location and date of isolation are listed in Table 1. Four field strains of *Arcanobacterium pyogenes*, isolated from sheep from the same region with peritoneal abscesses, keratoconjunctivitis, or rhinitis and encephalitis, were also studied.

2.2. Sequence analysis

All the *rpoB* partial sequences publicly available in the GenBank database belonging to 62 *Corynebacterium* species were aligned using ClustalW2 (Larkin et al., 2007). Enzyme restriction patterns for the *rpoB* internal region of each *Corynebacterium* species were generated by using the REBASE program (http://rebase.neb.com) (Roberts et al., 2010).

2.3. PCR–RFLP

A 446-bp internal fragment of *rpoB* was amplified using previously described oligonucleotide primers C2700F and C3130R (Khamis et al., 2004). Crude lysates used as templates for all PCR amplifications were obtained as follows: several colonies from each bacterial isolate were boiled in nuclease-free water at 98 °C for 10 min and centrifuged for 30 s at 9900 *g*. The PCR mixtures contained PCR buffer (20 mM Tris–HCl, 50 mM KCl), 200 μM of each deoxynucleotide triphosphate, 1 μM each primer, 2 mM $MgCl_2$, 1.5 U of Taq DNA polymerase (Invitrogen) and 5 μl of DNA template in a total volume of 50 μl. The template was replaced by nuclease-free water for the negative PCR control. PCR assays were carried out as follows: 94 °C for 5 min, followed by 35 cycles of denaturalization at 94 °C for 45 s, annealing at 57 °C for 45 s and extension at 72 °C for 60 s, with a final cycle at 72 °C for 5 min. Following the PCR, 12 μl (approx. 700 ng) of amplified products obtained using primers C2700F and C3130R were digested overnight with 2.5 U *Mse*I (Invitrogen) and 5 U *Stu*I (Invitrogen) at 37 °C in separate reactions, according to the supplier's instructions. The digests were electrophoresed along with the uncut *rpoB* amplicons on a 2.2% wt/vol agarose gel containing ethidium bromide, at 100 V for 50 min. A 100-bp DNA ladder (CienMarker, Biodynamics) or 1-Kb DNA ladder (Promega) were used as molecular size standards.

3. Results and discussion

3.1. Sequence analysis of rpoB from C. pseudotuberculosis and C. ulcerans

The endonuclease restriction pattern of the hypervariable region of *rpoB* from *C. pseudotuberculosis* was analyzed and compared to that of available sequences from other species of *Corynebacterium.* In a first approach, the predicted restriction patterns for *C. pseudotuberculosis* and *C. ulcerans*, its most genetically related species (Khamis et al., 2004, 2005), were obtained using the REBASE program for all enzymes present in the program database and compared. The *rpoB* hypervariable region from *C. ulcerans* did not contain restriction sites for endonucleases *Mse*I and *Stu*I, and that from *C. pseudotuberculosis* had restriction sites resulting in predicted fragments that differed in length enough to be differentiated in standard agarose gels (98 and 348 bp for *Mse*I, and 191 and 255 bp for *Stu*I), so they were chosen for further analysis. Fig. 1 shows a schematic representation of the *rpoB* amplicon for *C. pseudotuberculosis* and the restriction sites for endonucleases *Mse*I and *Stu*I, selected for RFLP analysis.

3.2. In silico evaluation of PCR–RFLP for species differentiation

The suitability of *Mse*I and *Stu*I for the discrimination between *C. pseudotuberculosis* and other species of *Corynebacterium* was then assessed screening all the *rpoB* partial sequences publicly available in the GenBank database for restriction sites for these enzymes using the REBASE program. The in silico analysis showed that *rpoB* partial sequences of the majority of the species of *Corynebacterium* did not contain restriction sites for *Mse*I and *Stu*I (Table 2). Eleven out of sixty-two sequences had sites for only one of these endonucleases, and two sequences apart from *C. pseudotuberculosis* had sites for both enzymes, but in these cases the restriction fragment sizes predicted for *Mse*I were different from those of *C. pseudotuberculosis*. *A. pyogenes*, a pyogenic bacterium that produces suppurative processes in ovines, was included in the analysis because the lesions it produces are similar to those caused by *C. pseudotuberculosis*. *Rhodococcus equi* and *Turicella otitidis* were included based on their phylogenetic relatedness to *C. pseudotuberculosis* (Khamis et al., 2004). The *rpoB* partial sequences of *A. pyogenes*, *R. equi* and *T. otitidis* did not contain restriction sites for *Mse*I or *Stu*I (Table 2).

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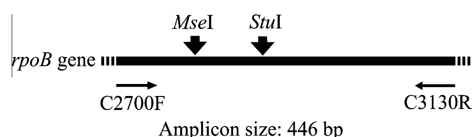

Fig. 1. Schematic representation of the *rpoB* gene showing the oligonucleotides used in the *rpoB* PCR assay. The size of the expected amplicon (bp, base pair) and the restriction sites of the endonucleases used in this work are shown for *C. pseudotuberculosis*.

When evaluating the reliability of the PCR–RFLP method, the existence of sequencing errors in each individual sequence deposited in the databases cannot be ruled out. In order to explore the possibility that a strain of a *Corynebacterium* species other than *C. pseudotuberculosis* could have the same restriction pattern observed for this species, five sequences of *Corynebacterium diphtheriae* and two of *C. ulcerans* available in the databases were compared. These two species are not sheep pathogens, but are the ones sharing the highest *rpoB* similarity with *C. pseudotuberculosis*. All sequences corresponding to *C. diphtheriae* were found to be identical at the sites analyzed, that is, none had restriction sites for *Stu*I and *Mse*I. The same results were obtained for *C. ulcerans*. It must be taken into account that several base changes would be required in order to change the restriction sites so that a strain from other *Corynebacterium* species acquired the pattern of *C. pseudotuberculosis*. For instance, two base changes would be necessary in *C. diphtheriae* (one in each restriction site) and three in *C. ulcerans* (one for the *Mse*I site and two for the *Stu*I site), to obtain the restriction pattern observed in *C. pseudotuberculosis*. On the other hand, it is possible that a strain of *C. pseudotuberculosis*, due to a single base change, could lose one of the restriction sites, changing its restriction pattern and leading to a misidentification. This possibility has been addressed by in silico analysis of a total of six *rpoB* sequences publicly available belonging to the *C. pseudotuberculosis* strains CIP102968^T, I19, 1002, C231, FRC41 and CD070 (D'Afonseca et al., 2010; Silva et al., 2011). These strains were isolated from several animal sources and from different geographical regions, and all their *rpoB* sequences had the exact same restriction pattern for *Mse*I and *Stu*I.

The in silico analysis indicated that using the *rpoB* PCR–RFLP approach, *C. pseudotuberculosis* can be readily distinguished from the other ovine pathogen *Corynebacterium* species: *C. camporealensis, C. capitovis* and *C. mastitidis*, none of which have restriction sites for *Stu*I or *Mse*I in the *rpoB* region analyzed. Furthermore, the technique proposed has the potential to differentiate *C. pseudotuberculosis* from the veterinary relevant species *C. auriscanis* (dogs), *C. bovis* (cows), *C. diphtheriae* (cows), *C. phocae* (seals), *C. ulcerans* (cows), *C. urealyticum* (dogs, cats), *C. cystitidis* (cows), *C. kutscheri* (mice, rats, guinea pigs), *C. pilosum* (cows) and *C. renale* (cows, pigs, goats).

3.3. rpoB PCR–RFLP of field strains

The identification method based on sequence analysis was experimentally tested using thirteen field isolates of *C. pseudotuberculosis* in order to verify that non-sequenced strains shared the predicted PCR-RFLP pattern. Twelve field strains of *C. pseudotuberculosis* were isolated from sheep and one from a goat. All animals were from Patagonia, Argentina, and had clinical CLA. These *C. pseudotuberculosis* strains can be considered representative of the outbreaks occurring in the region in the last five years (Table 1). Four field strains of *A. pyogenes*, isolated from sheep from the same region, were also studied. All strains were extensively characterized by biochemical routine assays (see Section 2). *C. pseudotuberculosis* strains were positive for the amplification of a 815-bp fragment from the 16S rRNA gene using oligonucleotides specific for *C. pseudotuberculosis* biovar ovis and biovar equi, that also amplify *C. ulcerans* (Çetinkaya et al., 2002; Pacheco et al., 2007).

A 446-bp internal fragment of *rpoB* was amplified using crude lysates of each bacterial strain as templates and the previously described oligonucleotide primers C2700F and C3130R (Khamis et al., 2004). These oligonucleotides generate amplicons not only for all *Corynebacterium* species but also for other bacteria such as *A. pyogenes, R. equi* and *T. otitidis* (Khamis et al., 2004; Pacheco et al., 2007). The *rpoB* PCR-RFLP analysis of the thirteen *C. pseudotubercu-*

Table 2
Predicted *Mse*I and *Stu*I restriction fragments of the *rpoB* amplicon in different *Corynebacterium* and other related species using the REBASE program.

Species and *rpoB* Genbank Acession No.	Predicted sizes of fragments (bp) after amplicon digestion with:	
	***Mse*I**	***Stu*I**
C. pseudotuberculosis AY492239	98, 348	191, 255
C. ulcerans AY492271	NR	NR
C. callunae AY492245	87, 123, 236	191, 255
C. glutamicum NC_003450	210, 236	191, 255
C. afermentans AY492265/ AY492266, *C. cystitidis* AY492251, *C. kutscheri* AY492257, *C. pilosum* AY492258, *C. renale* AY492240	NR	191, 255
C. macginleyi AY492276, *C. vitaeruminis* AY492273	98, 348	NR
C. kroppenstedtii AY492274	210, 245	NR
C. glucuronolyticum AY492256, *C. seminale* AY492263	123, 311	NR
C. striatum AY492267	123, 323	NR
Arcanobacterium pyogenes	NR	NR
Rhodococcus equi AY492285/ AY492286	NR	NR
Turicella otitidis AY492297	NR	NR

No restriction sites for these enzymes were found in the rpoB sequences of the following *Corynebacterium* species: *C. accolens* AY492242, *C. ammoniagenes* AY492243, *C. amycolatum* AY492241, *C. argentoratense* AY492249, *C. aurimucosum* AY492282, *C. auris* AY492234, *C. auriscanis* AY492244, *C. bovis* AY492236, *C. camporealensis* AY492246, *C. capitovis* AY492247, *C. casei* EU616817, *C. confusum* AY492248, *C. coyleae* AY492250, *C. diphtheriae* AY492230, *C. durum* AY492252, *C. efficiens* AP005215, *C. falsenii* AY492253, *C. felinum* AY492254, *C. flavescens* AY492255, *C. freneyi* AY492237, *C. genitalium* EU616818, *C. imitans* AY492259, *C. jeikeium* AY492231, *C. lipophiloflavum* AY492260, *C. mastitidis* AY492281, *C. matruchotii* AY492238, *C. minutissimum* AY492235, *C. mucifaciens* AY492261, *C. mycetoides* AY492262, *C. phocae* AY492277, *C. propinquum* AY492279, *C. pseudodiphtheriticum* AY492232, *C. pseudogenitalium* AY581868, *C. pyruviciproducens* FJ899747, *C. riegelii* AY492278, *C. simulans* AY492264, *C. singulare* AY492280, *C. spheniscorum* AY492283, *C. sundsvallense* AY492268, *C. terpenotabidum* AY492269, *C. testudinoris* AY492284, *C. thomssenii* AY492270, *C. tuberculostearicum* AY581869, *C. ulcerans* AY492271, *C. urealyticum* AY492275, *C. ureicelerivorans* FJ392022/ FJ392020/ FJ392018/ FJ392029/ FJ392021, *C. variabile* AY492272, *C. xerosis* AY492233

NR, not restricted.

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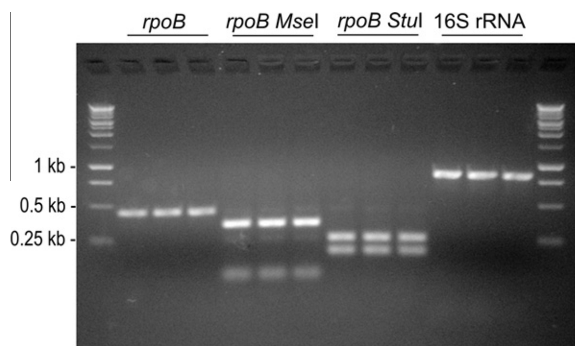

Fig. 2. Identification of three field isolates of *C. pseudotuberculosis* by PCR-RFLP. Lanes 2–4: *rpoB* gene amplification from Intabari K809, Intabari K909, Intabari K1009 strains. Lanes 5–7: *rpoB* gene *Mse*I restriction, strains in the same order. Lanes 8–10: *rpoB* gene *Stu*I restriction, same order. Lanes 11–13: 16S rRNA gene amplification, same order. Lanes 1 and 14: 1-Kb ladder.

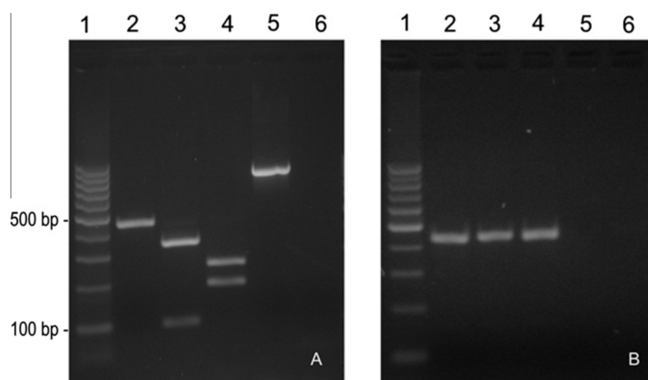

Fig. 3. *rpoB* PCR-RFLP pattern and 16S rRNA gene PCR obtained for *Corynebacterium pseudotuberculosis* biovar ovis (a) and *Arcanobacterium pyogenes* (b). *rpoB* PCR product (lanes 2) was digested with *Mse*I (lanes 3) or *Stu*I (lanes 4) and eletrophoresed on a 2.2% agarose gel. Lanes 5: 16S rRNA gene PCR product. Lanes 6: *rpoB* and 16S rRNA genes negative controls (both reactions were loaded in the same well). Lanes 1: 100-bp ladder.

losis isolates indicated that all of them exhibited exactly the same *Mse*I and *Stu*I restriction fragments, corresponding to the predicted pattern. Fig. 2 shows the *rpoB* PCR-RFLP pattern along with the 16S rRNA gene amplicon for three representative *C. pseudotuberculosis* field isolates.

The assay was also successfully applied to differentiate *C. pseudotuberculosis* from the four field isolates of *A. pyogenes*. Fig. 3 shows the *rpoB* PCR-RFLP pattern of *C. pseudotuberculosis* (Fig. 3A) and one of the *A. pyogenes* field strains (Fig. 3B), along with the 16S rRNA gene amplification fragment specific for *C. pseudotuberculosis*. An *rpoB* amplicon of similar size to that of *C. pseudotuberculosis* (450-bp) was observed for *A. pyogenes*, in contrast with results previously reported (Pacheco et al., 2007), but, as predicted, it was not digested with *Mse*I or *Stu*I. The different PCR–RFLP patterns for isolates of both species could be readily distinguished by simple visual comparison in agarose gels.

The present study proposes a molecular method involving the polymerase chain reaction-mediated amplification of an internal *rpoB* region using a simple boiling method for DNA extraction, followed by restriction fragment length polymorphism analysis. This method has been designed for the identification of *C. pseudotuberculosis*, distinguishing it from sixty-one other *Corynebacterium* species, including all members of this genus of importance in veterinary medicine, and from genetically related pathogens such as *R. equi* and *T. otitidis*. The PCR-RFLP technique described in this work has been experimentally tested to differentiate *C. pseudotuberculosis* from *A. pyogenes*, that produces similar lesions. This assay provides a rapid and simple diagnostic tool for the identification of *C. pseudotuberculosis* from sheep and goats and for the discrimination between this species and other related pathogenic bacteria.

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