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Short communication

Host associated polymorphisms in the *Corynebacterium pseudotuberculosis rpoB* gene sequence

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ABSTRACT

Corynebacterium pseudotuberculosis is a widespread facultative intracellular pathogen that causes caseous lymphadenitis disease in sheep and goats, and generates cutaneous abscesses and granulomas in horses and cattle. Although some genes have been studied for diagnostic and phylogenetic analysis within the genus *Corynebacterium*, at subspecies level the pathogen has been poorly analyzed. The aim of this study was to characterize *C. pseudotuberculosis* strains isolated from domestic animals, through the sequencing of a hypervariable *rpoB* gene segment. As result, there were identified host associated *rpoB* polymorphisms in strains infecting sheep, goats and horses from Chile. These differences suggest the existence of bacterial genotypes, in which the nucleotide similarity values were ranging from 98.8 to 99.8%. In conclusion, the analysis of polymorphisms in the partial *rpoB* sequence can be used as a diagnostic tool that differentiates *C. pseudotuberculosis* strains at subspecies level.

1. Introduction

Corynebacterium pseudotuberculosis is a cosmopolitan agent that causes the disease named caseous lymphadenitis (CLA) in sheep and goats. Additionally, the bacterium produces pectoral abscesses in horses and ulcerative cutaneous granulomas in cattle (Baird and Fontaine, 2007). Although CLA could generate important economic losses (Dorella et al., 2006), the virulence mechanisms of the agent have been scarcely characterized and the epidemiology of the infection is still poorly understood in many countries. The infection is endemic in Chile and its diagnosis is usually presumptive and achieved by clinical signs (Gädicke et al., 2008; Tadich et al., 2005).

Biochemical properties of *C. pseudotuberculosis* strains have been used to group isolates into biovars *ovis* and *equi*, which infect small ruminants and horses, respectively (Baird and Fontaine, 2007; Dorella et al., 2006; Songer et al., 1988). However, these tests have shown variability among strains belonging to the same animal host and require additional tools for accurate bacterial diagnosis (Cetinkaya et al., 2002; Songer et al., 1988).

The 16S RNA gene sequence has been the most frequently used molecular marker for bacterial identification and phylogeny. However, when analyzing *Corynebacterium* strains it has been determined that an internal region of the RNA polymerase beta subunit-encoding gene (*rpoB*) is a more suitable sequence than 16S RNA because of its higher nucleotide polymorphism (Khamis et al., 2004, 2005).

In this study we have characterized *C. pseudotuberculosis* strains isolated from sheep, goats and horses in Chile. Through the sequencing of a hypervariable *rpoB* gene segment we identified polymorphisms suggestive of three circulating genotypes among strains infecting domestic animals.

2. Materials and methods

2.1. Samples

Abscesses were sampled from sheep, goats and horses living in three different geographic regions of Chile: central

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Table 1
Characterization of *Corynebacterium pseudotuberculosis* strains isolated in this study.

Strain	Source[a]	Biochemical tests[b]							Hemolysis tests		PCR
		Glu	Lac	Thr	Sal	Gel	Nit	Cat	CAMP	R-CAMP[c]	*pld*[d]
CPO2	Sheep-P	+	–	–	–	–	–	+	+	+	+
CPO3	Sheep-P	+	–	–	–	–	–	+	+	+	+
CPO4	Sheep-P	+	–	–	–	–	–	+	+	+	+
CPO6	Sheep-P	+	–	–	–	–	–	+	+	+	+
CPO8	Sheep-P	+	–	–	–	–	–	+	+	+	+
CPO10	Sheep-P	+	–	–	–	–	+	+	+	+	+
CPO11	Sheep-P	+	–	–	–	–	+	+	+	+	+
CPO12	Sheep-P	+	–	–	–	–	–	+	+	+	+
CPO14	Sheep-P	+	–	+	–	–	–	+	+	+	+
CPO15	Sheep-C	+	–	–	–	–	–	+	+	+	+
CPO20	Sheep-C	+	–	–	–	–	+	+	+	+	+
CPO22	Sheep-C	+	–	–	–	–	+	+	+	+	+
CPO29	Sheep-C	+	–	–	–	–	–	+	+	+	+
CPO65	Sheep-C	+	–	–	–	–	–	+	+	+	+
CPO66	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO67	Sheep-S	+	–	+	–	–	–	+	+	+	+
CPO68	Sheep-S	+	–	–	–	–	+	+	+	+	+
CPO69	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO70	Sheep-S	+	–	–	–	–	+	+	+	+	+
CPO71	Sheep-S	+	–	+	–	–	+	+	+	+	+
CPO72	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO73	Sheep-S	+	–	+	–	–	–	+	+	+	+
CPO74	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO75	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO76	Sheep-S	+	–	+	–	–	–	+	+	+	+
CPO77	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO78	Sheep-S	+	–	–	–	–	–	+	+	+	+
CPO79	Sheep-S	+	–	–	–	–	+	+	+	+	+
CPO80	Sheep-S	+	–	–	–	–	+	+	+	+	+
CPC1	Goat-C	+	–	+	–	–	–	+	+	+	+
CPC2	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC3	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC4	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC7	Goat-C	+	–	+	–	–	–	+	+	+	+
CPC8	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC11	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC12	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC13	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC14	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC15	Goat-C	+	–	+	–	–	–	+	+	+	+
CPC16	Goat-C	+	–	+	–	–	–	+	+	+	+
CPC17	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC18	Goat-C	+	–	–	–	–	–	+	+	+	+
CPC19	Goat-C	+	–	–	–	–	–	+	+	+	+
CPE1	Horse-C	+	–	–	–	–	+	+	+	+	+
CPE3	Horse-C	+	–	–	–	–	+	+	+	+	+
CPE8	Horse-C	+	–	–	–	–	+	+	+	+	+
CPE17	Horse-C	+	–	+	–	–	+	+	+	+	+

[a] P, Patagonia; C, central region; S, south region.
[b] Glu, glucose; Lac, lactose; Thr, trehalose; Sal, salicin; Gel, gelatin hydrolysis; Nit, nitrate reduction; Cat, catalase.
[c] R-CAMP, reverse CAMP.
[d] Phospholipase D gene.

(Metropolitan and O'Higgins regions, near Santiago, the country capital), south (Bio-Bio region, 500 km south of Santiago) and Patagonia (Magallanes region, 3000 km south of Santiago) (Table 1).

2.2. Bacterial strains

After a clinical inspection or post mortem examination, cutaneous or internal lymph nodes with caseous abscesses were sampled using sterile swabs immersed in transport medium (Copan®, Cary-Blair cotton). Once at the lab, swabs were seeded onto trypticase soy agar (Becton Dickinson) plates supplemented with 5% (v/v) sheep blood and incubated at 37 °C for 48 h. Presumptive Gram-positive bacteria were identified by their biochemical properties (Dorella et al., 2006), CAMP and CAMP-inhibition tests (Barksdale et al., 1981) and with a PCR assay to amplify the phospholipase D (PLD) encoding gene using primers PLD-F (5′-ataagcgtaagcagggagca-3′) and PLD-R3 (5′-tcagcggt-gattgtcttcca-3′) as described previously (Pacheco et al., 2007).

2.3. Amplification and sequencing of the rpoB gene

Bacterial cells were disrupted with zirconia–silica beads under agitation in a Mini Bead Beater® instrument (Cole-Parmer) at 4600 rpm for 90 s. DNA was extracted using the DNazol® reagent (Invitrogen) following recommendations of the manufacturer.

The partial sequence of the *rpoB* gene from *Corynebacterium* strains was amplified using primers C2700F (5′-cgtatgaacatcggccaggt-3′) and C3130R (5′-tccatttcgccgaagcgctg-3′), as described previously (Khamis et al., 2004). DNA products were purified using the Wizard® SV clean-up system (Promega) and automated sequencing was performed by Macrogen DNA Sequencing Inc. (Seoul, Korea), using the same primers.

2.4. Analysis of rpoB gene sequences

Multiple-sequence alignments and percent similarities of *rpoB* sequences were obtained with the CLUSTAL W program (Thompson et al., 1994), and the phylogenetic tree was constructed using the neighbor-joining method with the software MEGA (Tamura et al., 2007).

Bootstrap values were obtained from 1000 randomly generated trees, and the *C. ulcerans rpoB* sequence (Genbank accession: AY492271) was included as the outgroup. In addition, nucleotide *rpoB* sequences belonging to *C. pseudotuberculosis* C231 and CIP 102968 strains isolated from sheep, already published with Genbank accession numbers CP001829 and AY492239, respectively, were also included in this analysis.

3. Results and discussion

The study included samples from 75 sheep, 25 goats and 10 horses, whose *C. pseudotuberculosis* infection rates were 39%, 60% and 40%, respectively.

Firstly, it was observed variability among *C. pseudotuberculosis* strains in the trehalose and nitrate reduction biochemical tests (Table 1). This finding has been attributed to the existence of biovars, which are distinguished mainly by the reduction of nitrate through positive reactions with biovar *equi* and negative reactions with biovar *ovis* strains (Dorella et al., 2006; Songer et al., 1988; Thompson et al., 1983). Although in this study the strains obtained from goats and horses were in accordance with these traits, it was determined that 31% (9/29) of strains isolated from sheep were able to reduce nitrate (Table 1), contrasting with previous reports which have shown values ranging from 0 to 2% of the analyzed biovar *ovis* strains (Connor et al., 2007; Costa et al., 1998; Songer et al., 1988). Considering that other authors have also reported unexpected results in this test (Connor et al., 2000) and that fermenting trehalose strains were also more frequent than previously reported data (Cetinkaya et al., 2002; Songer et al., 1988), we suggest that these biochemical tests have limited usefulness in the *C. pseudotuberculosis* characterization, especially when phenotypic intra-species variation is higher than the variation reported elsewhere, as could be the case with Chilean strains.

Table 2
Percentages (%) of nucleotide similarity among partial *rpoB* gene sequences of *C. pseudotuberculosis* ovine, caprine and equine genotypes and *C. ulcerans*.

	Ovine	Caprine	Equine	*C. ulcerans*
Ovine	100	99.8	98.8	90.4
Caprine		100	99.0	90.7
Equine			100	90.2
C. ulcerans				100

On the other hand, the *rpoB* gene is constituted by alternating conserved and variable regions, from which the hypervariable region, located between positions 2300 and 3300, has demonstrated to be the most suitable gene segment for the inference of phylogenetic relationships and bacterial identification (Adekambi et al., 2009). Since this situation has also been described for species of the genus *Corynebacterium* (Khamis et al., 2004, 2005), in this study the *C. pseudotuberculosis rpoB* hypervariable region was analyzed, allowing the identification of three different host-associated sequences, suggesting the existence of distinct ovine, caprine and equine genotypes (Genbank

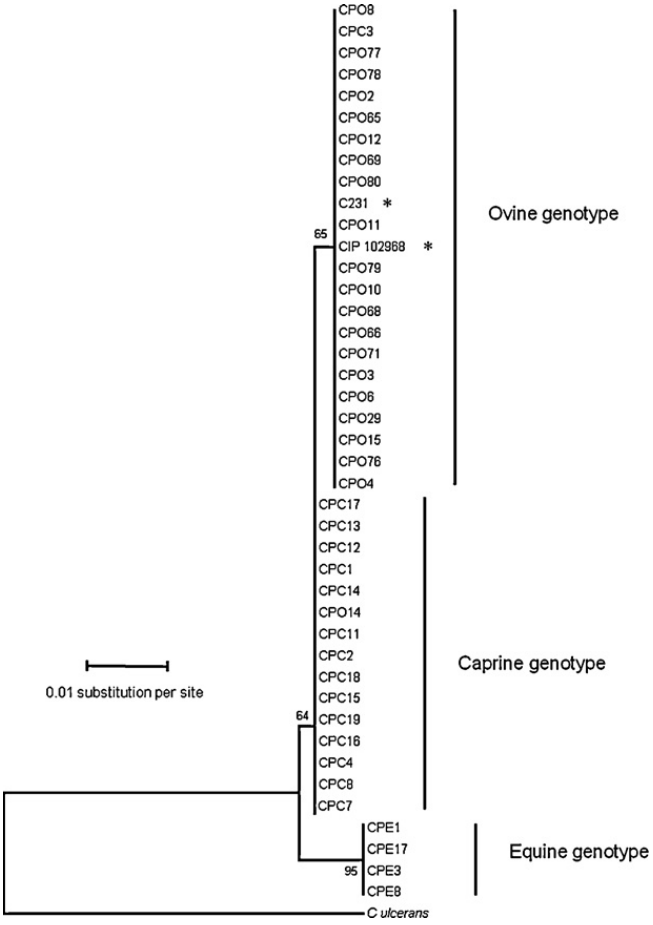

Fig. 1. Phylogenetic tree representing relationships of *C. pseudotuberculosis* strains obtained by the neighbor-joining method. The tree was derived from alignment of partial *rpoB* sequences, and includes *C. ulcerans* as the outgroup. A bootstrap value of 1000 was used, and the strength of each branch is indicated in the respective node (percentage). Nucleotide *rpoB* sequences which belong to *C. pseudotuberculosis* C231 and CIP 102968 strains are highlighted by asterisks.

accessions: HQ401568, HQ401569 and HQ401570, respectively), with variable nucleotide similarity values among them (Table 2). Furthermore, previously published *rpoB* sequences of *C. pseudotuberculosis* C231 and CIP102968 strains, isolated from sheep in Brazil and Australia respectively, were identical to the sequence of Chilean strains belonging to the same host (Fig. 1), suggesting that these results might also be found in other countries.

The closest phylogenetic relationship was observed between ovine and caprine genotypes (Fig. 1), which were differentiated by just one polymorphic site. In addition, the strains belonging to them may be shared between sheep and goats, since in this study one strain isolated from a sheep contains the *rpoB* sequence of the caprine genotype (CPO14) and on the contrary, one strain isolated from a goat contains the sequence of the ovine genotype (CPC3) (Fig. 1). If a mutation is not the cause, the most probable explanation for this finding is a previous inter-species transmission event, although we were unable to get the corresponding epidemiological evidence. In relation to strains isolated from horses, it seems that they are not shared with small ruminants, probably because of the higher genetic distance of the genotype they belong to (Fig. 1). However, a larger number of strains should be analyzed to confirm this hypothesis.

These mentioned nucleotide polymorphisms determine two missense mutations in the amino acid *RpoB* sequence. The first differentiates between sheep derived strains and the other strains (Gln57Glu), and the second differentiates between small ruminants and equine derived strains (Thr63Ala). Whether these changes have effects in the bacterial virulence or host preferences, are questions that also remain to be elucidated.

Finally, it has been reported that when the genus *Corynebacterium* is analyzed through its partial *rpoB* gene sequence, the cut-off value which determines strains belonging to the same species is 95% similarity (Khamis et al., 2005). Our results agree with this statement, confirming that the *rpoB* hypervariable fragment is also able to clearly differentiate *C. pseudotuberculosis* strains from its closest related specie, *C. ulcerans* (Table 2).

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