

Mycobacterium caprae Infection in Livestock and Wildlife, Spain

Technical Appendix

Specific Characteristics

Mycobacterium caprae (1), formerly known as *M. tuberculosis* subsp. *caprae* (2), and *M. bovis* subsp. *caprae* (3) forms a genetically distinct cluster within the *M. tuberculosis* complex. The main features differentiating these isolates from the other members are a special combination of polymorphisms at pyrazinamidase (*pncA*), catalase (*katG*), and subunits A and B of the gyrase (*gyrA* and *gyrB*) genes (4,5); the pattern of regions of difference (presence of RD4 and absence of RD5 to 10) (6–8); and specific patterns obtained by direct variable repeat spacer oligonucleotide typing technique (spoligotyping); and restriction fragment length polymorphism associated with IS6110, polymorphic GC-rich sequences, and direct repeat elements (9,10).

Bacteriology

Tissue samples consisted usually of retropharyngeal, mediastinal, bronchial, and mesenteric lymph nodes, lung and liver. All samples were maintained at -20°C until culture. Samples from each animal were pooled, homogenized with sterile distilled water, decontaminated with 0.35% hexadecylpyridinium chloride for 30 min (11), centrifuged at $1,068 \times g$ for 30 min, and cultured on Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (bioMérieux España and Biomedics, Madrid, Spain) at 37°C for 3 mo. The isolates were identified as members of the *M. tuberculosis* complex by PCR amplification of *Mycobacterium* genus-specific 16S rRNA fragment (12) and MPB70 sequences (13) (primers used in the study are listed in the Table). All PCRs were performed on heat-killed cell suspensions.

Spoligotyping and Data Analysis

The spacer oligonucleotide typing (spoligotyping) method was performed as described by Kamerbeek et al. (14). The biotin-labelled amplified product was detected by hybridization onto a spoligotyping membrane (Isogen Bioscience BV, Maarsse, the Netherlands). Hybridized

product was detected with the streptavidin-peroxidase conjugate (Boehringer, Mannheim, Germany) and the electrochemical luminescence system (Amersham, Little Chalfont, UK) by exposing the radiograph film to the membrane. Purified sterile water and a clinical isolate of *M. tuberculosis* and *M. bovis* were included as controls in every batch of tests.

The spoligotyping results were enlisted in a Microsoft Office Access (Microsoft, Redmond, WA, USA) database along with the epidemiologic data (isolation date, animal species and geographical origin). The index of discrimination (D) described by Hunter and Gaston (15) was calculated to determine the discriminatory power of the spoligotyping at a national level. We used the website of the University of the Basque Country (www.insilico.ehu.es), filling in the number of unrelated strains for each spoligotype. For this purpose we only counted 1 spoligotype when isolates of the same herd or a precise geographical area shared identical patterns.

Detection of RD4 and Gene Polymorphisms

We used the 3-primer PCR described by Mostowy et al. (16). Purified sterile water and a clinical isolate of *M. bovis* were included as controls. The presence (545-bp gel band) or absence (210-bp gel band) of RD4 was detected by agarose gel electrophoresis.

The complete *pncA* gene (17) and a part of the *gyrB* (18) containing the expected polymorphism for *M. caprae* were amplified. The products were purified with the Qiaquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the DyeDeoxy (dRhodamine) Terminator Cycle Sequencing kit in an automatic ABI Prism 373 DNA sequencer (Applied Biosystems, C.I.B. Sequencing Facilities, Madrid, Spain). The sequences generated were aligned with published mycobacterial sequences from the GenBank database (www.ncbi.nlm.nih.gov/GenBank, accession nos. U59967 [17] and L27512 [18]). Sequencing of the *pncA* demonstrated a C at nucleotide 169, a common characteristic for *M. tuberculosis*, *M. africanum*, *M. microti*, and *M. caprae* that results in the functional wild-type *pncA* (17). The *gyrB* gene sequence polymorphisms analysis detected, as well the characteristic profile for *M. caprae* that consists of a G at nucleotide 1311 and a C at position 1410, are common to caprine strains and the other members of the complex, except *M. bovis* (5).

Variable Number Tandem Repeat Analysis

The PCR for each locus was carried out by using the HotStar Taq DNA polymerase kit (QIAGEN) in a Bio-Rad (Hercules, CA, USA) MyCycler Thermal Cycler. Genomic DNA from

M. bovis BCG Danish was used as a positive control, reaction mixtures lacking mycobacterial DNA were used as a negative control. The number of tandem repeats (alleles) was determined by estimating the amplicon size of the PCR product by electrophoresis on 2.5% agarose gel at 45V for 3 h with a 100-bp ladder (Biotools, B&M Labs, Madrid, Spain).

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Table. List of primers used in a study of *Mycobacterium caprae* infection in livestock and wildlife, Spain*

Target†	Primer	Sequence, 5' → 3'	Product, bp	Reference
16S rRNA	MYCGEN-F MYCGEN-R	AGAGTTTGATCCTGGCTCAG TGCACACAGGCCACAAGGGA	1,030	(12)
MPB70	TB1-F TB1-R	GAACAATCCGGAGTTGACAA AGCACGCTGTCAATCATGTA	372	(13)
DR spoligotyping	DR-a DR-b	GGTTTTGGGTCTGACGAC CCGAGAGGGGACGGAAAC	ladder	(14)
RD4	RD4-L RD4-R RD4-wtR	GAACGCGACGACCTCATATTCC CTAAGATATCCGGTACGCCCGC CTGTGGCTATGGGGCTCTAC	545/210 (presence/ absence)	(6,16)
<i>pncA</i>	pncATB-1 pncATB-2	ATGCGGGCGTTGATCATCGT TCAGGAGCTGCAAACCAACTC	574	(4,17)
<i>gyrB</i>	MTUBf MTUBr	TCGGACGCGTATGCGATATC ACATACAGTTCGGACTTGCG	1,020	(5,18)
VNTR2165 (ETR-A)	ETRA-F ETRA-R	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGGATTT	†	(19)
VNTR2461 (ETR-B)	ETRB-F ETRB-R	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG	†	(20)
VNTR580 (ETR-D, MIRU 4)	ETRD-F ETRD-R	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC	†	(19,21)
VNTR3192 (ETR E, MIRU 31)	MIRU31-F MIRU31-R	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT	†	(22)
VNTR2996 (MIRU 26)	MIRU26-F MIRU26-R	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCCAATAG	†	(21)
VNTR2163a (QUB11a)	QUB11a-F QUB11a-R	CCCATCCCCTTAGCACATTCGTA TTCAGGGGGGATCCGGGA	†	(23,24)
VNTR2163b (QUB11b)	QUB11b-F QUB11b-R	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT	†	(23,24)
VNTR3232 (QUB3232)	3232-F 3232-R	CGGCGATGGTGCCGCCATG CTTGGTGAAGGCCCGATG	†	(21)

*VNTR, variable number tandem repeat; MIRU, mycobacterial interspersed repetitive unit.

†According to respective allele calling tables.