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Mycobacterium tuberculosis subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain

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Isolates from the *Mycobacterium tuberculosis* complex cultured from caprine pathological tissue samples were biochemically and genetically characterized. The isolates were negative for nitrate reduction and niacin accumulation, they weakly hydrolysed Tween 80, were sensitive to pyrazinamide (50 μg ml^{-1}) and were resistant to 1 and 2 μg tiophene-2-carboxylic acid hydrazide ml^{-1} but not to 5 or 10 μg tiophene-2-carboxylic acid hydrazide ml^{-1}. Sequencing of the *pncA* gene revealed a polymorphism characteristic of *M. tuberculosis*, whereas *oxyR*, *katG* and *gyrA* sequences were characteristic of *Mycobacterium bovis*. The fingerprinting patterns obtained with IS*6110*, direct repeats and polymorphic G+C-rich sequence-associated RFLP and direct variable repeat-spacer oligonucelotide typing (spoligotyping) segregated these isolates from the other members of the complex. The results of this testing, together with the repeated association of this micro-organism with goats, suggest that a new member of this taxonomic complex not matching any of the classical species had been identified. This unusual mycobacterium may play a role in the epidemiology of animal and human tuberculosis in Spain. The name *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov. is proposed for these isolates. The type strain of *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov. is gM-1^T (= CIP 105776^T).

Keywords: *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov., *Mycobacterium tuberculosis* complex, tuberculosis, goats

INTRODUCTION

The *Mycobacterium tuberculosis* complex consists of a highly related group of acid–alcohol-fast bacilli which are human and animal pathogens. It comprises five classical species. *M. tuberculosis* (*sensu stricto*) infects human and non-human primates, and has also been found in dogs and cats (Snider, 1971; Liu *et al.*, 1980; Clercx *et al.*, 1992; Aranaz *et al.*, 1996b), pigs (Kleeberg & Nel, 1969), birds (Hoop *et al.*, 1996) and some wild animals (Thoen, 1994; Michalak *et al.*, 1998). *Mycobacterium bovis* (Karlson & Lessel, 1970), the causative agent of bovine tuberculosis, infects a wide range of domestic and wild hosts (Collins & Grange, 1983; O'Reilly & Daborn, 1995). Bacillus Calmette–Guérin (BCG) is a vaccine strain derived from *M. bovis*. *Mycobacterium africanum*, described by Castets *et al.* (1969), is a rather heterogeneous group of strains responsible of human tuberculosis in Equatorial Africa, and has properties that are in-

Abbreviations: BCG, *Bacillus* Calmette–Guérin; DR, direct repeat; DVR, direct variable repeat; IS, insertion sequence; ITS, internal transcribed spacer; PGRS, polymorphic G+C-rich sequence; PZA, pyrazinamide; spoligotyping, spacer oligonucleotide typing; TCH, tiophene-2-carboxylic acid hydrazide.

The EMBL accession number for the 16S rRNA gene sequence of strain gM-1^T is AJ131120.

termediate between the aforementioned species; it also infects primates (Thorel, 1980), cattle and pigs (Alfredsen & Saxegaard, 1992). Finally, *Mycobacterium microti* (Reed, 1957) is the 'vole bacillus' described by Wells & Oxon (1937). It is found mainly in small rodents, but infection has also been recorded in cats, pigs (Huitema & Jaartsveld, 1967) and llamas (Pattyn *et al*., 1970).

Extensive studies based on DNA homology have reported a 95–100% DNA relatedness between members of the complex, suggesting that these organisms belong to the same species (Baess, 1979; Bradley, 1973; Imaeda, 1985). Sequencing of the 16S rRNA gene has shown that there are no sequence differences between the members of the complex (Böddinghaus *et al*., 1990; Rogall *et al*., 1990). Furthermore, sequencing of the more variable internal transcribed spacers (ITSs) between 16S and 23S rRNA has also led to the same conclusion, proving the existence of a close evolutionary relationship (Frothingham *et al*., 1994; Glennon *et al*., 1994). DNA sequence analysis of *rpoB*, *katG*, *rpsL* and *gyrA* genes have revealed a very strong identity among bacteria of the *M. tuberculosis* complex (van Soolingen *et al*., 1997). The nucleotide sequence of the *dnaJ* gene is identical throughout the species of the *M. tuberculosis* complex (Takewaki *et al*., 1993); this is also true of the fragment of the 65 kDa heat-shock protein used for PCR-restriction enzyme pattern analysis (Fiss *et al*., 1992; Plikaytis *et al*., 1992; Telenti *et al*., 1993). This 100% homology would support the theory that the *M. tuberculosis* complex represents a single species and there is a considerable debate concerning the classical classification. Several authors have stated that members of the complex should be grouped as varieties or subspecies of *M. tuberculosis* (Collins & Yates, 1982) and that the division of the tuberculosis complex into five species is an artifact of the great historical interest in this pathogen (Frothingham *et al*., 1994). However, to our knowledge, the reclassification of these species as a single species has not been proposed formally. Their significance in human and veterinary medicine, and the differences in their epidemiology, pathology and antibiotic response mean that the former classification is a useful one.

These micro-organisms can be differentiated by means of phenetic characteristics, but, with numerical taxonomy, *M. tuberculosis* and *M. bovis* are connected to each other at the 97% level (Tsukamura, 1976). Identification of the *M. tuberculosis* complex has been traditionally based on growth characteristics (pigment production, colony morphology, growth rate) and biochemical tests. The most commonly used tests for speciation within the complex are niacin accumulation, nitratase activity, susceptibility to pyrazinamide and susceptibility to tiophene-2-carboxylic acid hydrazide (TCH). To complement the numerical studies, various genetic markers and molecular methods used in diagnosis and epidemiology have been used recently as taxonomic tools to classify different species of the *M. tuberculosis* complex (Sreevatsan *et al*., 1996; van Soolingen *et al*., 1997; Espinosa de los Monteros *et al*., 1998).

In this report we describe the results of a polyphasic taxonomy study of an unusual member of the *M. tuberculosis* complex, isolated from goats with disseminated lesions. These mycobacteria have clear phenetic, genetic and epidemiological traits that differentiate them from the classical species of this complex.

METHODS

Mycobacterial strains. A total of 121 isolates of mycobacteria were examined in this study. The isolates were obtained from tuberculosis-infected goats ($n = 119$), a sheep and a pig from herds located in several areas of Spain. Samples came from mediastinal, retropharyngeal and bronchial lymph nodes or from lungs with lesions. Strains and sources of mycobacteria are shown in Table 1.

Tissue samples were homogenized with sterile distilled water and decontaminated by the method of Tacquet & Tison (1961) or with hexadecylpyridinium chloride (Corner & Trajstman, 1988), centrifuged at 3500 r.p.m. (1068 ***g***) for 30 min (Sigma 3-10 centrifuge) and cultured on Coletsos (Coletsos, 1971) and 0·2% (w/v) pyruvate-enriched Löwenstein–Jensen media (Anonymous, 1954) at 36 °C.

Phenetic characterization. The isolates were subcultured on Coletsos medium and incubated at 36 °C for 4 weeks. The recommended biochemical tests for determining the systematics of the genus *Mycobacterium*, described for new slowly-growing mycobacterial species (Lévy-Frébault & Portaels, 1992), were applied.

Colonies were examined for acid–alcohol fastness by the Ziehl–Neelsen and auramine staining techniques. Type strain gM-1^T (CIP 105776^T) was tested for growth rate, for the ability to grow at various temperatures (25, 30, 36 and 43 °C), for pigment production in the dark and photoactivity, for tolerance of sodium chloride, picric acid, hydroxylamine, *p*-nitrobenzoic acid and oleic acid, for nitrate reduction, urease production, catalase activity (22 and 68 °C), arylsulphatase activity, Tween 80 hydrolysis, thiophen-2-carboxylic acid hydrazide susceptibility (growth inhibition in 1, 2, 5 and 10 μg TCH ml^{-1} incorporated into Middlebrook 7H10) and for pyrazinamidase production. These tests were performed according to standard procedures (Lutz, 1992). Niacin accumulation was determined according to the Konno method in Proskauer–Beck modified medium (Karlson *et al*., 1964). Other enzymic activities were determined using the API CORYNE and API ZYM systems (bioMérieux) after 24 h at 36 °C, testing in parallel clinical isolates of *M. bovis* and *M. tuberculosis*.

Susceptibility to first-line antituberculous drugs isoniazid (0·2 and 1 μg ml^{-1}), rifampim (1 μg ml^{-1}), ethambutol (5 and 10 μg ml^{-1}), pyrazinamide (50 μg ml^{-1}, at pH 5·5), streptomycin (2 and 10 μg ml^{-1}), *p*-aminosalicylic acid (2 and 10 μg ml^{-1}), ofloxacin (2·5 μg ml^{-1}), cycloserine (30 μg ml^{-1}) and thiacetazone (1 μg ml^{-1}) was determined using Middlebrook 7H10 medium according to standard procedures (Kent & Kubica, 1985).

GLC. Mycobacterial lipids were extracted and derivatized to

Table 1. Caprine mycobacterial strains used in this study

Geographic origin (Spain)	Herd and strain designation	Host species
Several villages of Catalunya (north-east)	Herd c-1: gC-1 to gC-11 Herd c-2: gC-12 to gC-26 Herd c-3: gC-27 and gC-28 Herd c-4: gC-29 to gC-31 Herd c-5: gC-32 and gC-33 Herd c-6: gC-34 to gC-38 Herd o-1: oC-1	Goat (38) and sheep (1)
Madrid (central)	Herd c-7: gM-1^T* Herd c-8: gM-2 to gM-33	Goat (33)
Ávila and Valladolid (entral west)	Herd c-9: gA-1 to gA-5 Herd c-10: gV-1 to gV-42	Goat (47)
Badajoz (south-west)	Herd c-11: gB-1 and pB-1	Goat (1) and pig (1)

* This strain was selected as the type strain (CIP 105776^T).

methyl esters according to a recommended method (Luquin *et al*., 1991). Bacteria (10 mg wet weight) were suspended in 1 ml of a mixture of methanol/toluene/sulphuric acid (30:15:1) and heated at 80 °C overnight. After cooling at room temperature, the sample was extracted with 2 ml hexane, transferred to a new tube and mixed with an equal volume of phosphate buffer (0·3 M) and sodium hydroxide (pH 12). The hexane upper layer was selected, transferred to a new vial and evaporated under a stream of nitrogen gas. The methylated esters, secondary alcohols and mycolic acid cleavage products were analysed with a Hewlett Packard 5890A gas chromatograph equipped with a flame-ionization detector. The identification of the eluted substances was performed by comparing the retention times with those of known standards on a fused silica capillary column (15 m long × 0·25 mm i.d.) with cross-linked methyl silicone as the stationary phase (SPB-1, Supelco). The column was programmed from 175 to 300 °C, increasing at 8 °C min^{-1} and was kept at 300 °C for 15 min. The temperatures for the injector and the detector were maintained at 275 and 315 °C, respectively. Helium was used as the carrier gas, with a flow rate of approximately 1 ml min^{-1}. The sample (1 µl) was loaded with a split ratio of 1:50. The chromatogram was interpreted using a Hewlett Packard 3390A electronic integrator.

Molecular identification. From each isolate, a single colony was suspended in sterile distilled water, boiled for 10 min and stored frozen at −20 °C until tested. The isolates were tested by PCR amplification of a genus-specific 16S rRNA fragment and MPB70 elements (Wilton & Cousins, 1992), IS*6110* (Hermans *et al*., 1990), IS*1081* and *mtp40* sequences (Liébana *et al*., 1996). Moreover, isolates were tested with the non-radioactive AccuProbe DNA probe (GenProbe) for the detection of *M. tuberculosis* complex according to the manufacturer's instructions.

The 5′ 1524 bp region of the 16S rRNA gene of type strain (gM-1^T) was amplified by PCR with universal primer 246 (positions 8–28; *Escherichia coli* numbering) (5′ AGA GTT TGA TCC TGG CTC AG 3′) and reverse pH (positions 1540–1521) (5′ AAG GAG GTG ATC CAG CCG CA 3′). The amplified 16S rRNA fragment was sequenced using primers aimed at the conserved regions of the rRNA.

Detection of gene polymorphisms. One isolate from each herd was tested with the allele-specific PCR for *pncA* that differentiates the nucleotide at position 169 (Espinosa de los Monteros *et al*., 1998). Briefly, each sample was subjected to two differential amplifications; both reactions were performed with the same forward primer, pncATB1.2 (5′-ATG CGG GCG TTG ATC ATC GTC-3′), and one of the two discriminator reverse primers, i.e. pncAMT-2 (5′-CGG TGT GCC GGA GAA GCG G-3′) or pncAMB-2 (5′-CGG TGT GCC GGA GAA GCC G-3′), according to the methods of Espinosa de los Monteros *et al*. (1998).

Several complete genes (or parts containing the expected polymorphism) of the type strain were characterized by DNA sequencing. The complete pyrazinamidase (*pncA*) gene was amplified with primers pncATB-1 (5′-AAA GAA TTC ATG CGG GCG TTG ATC ATC GT-3′) and pncATB-2 (5′-AAA GAA TTC TCA GGA GCT GCA AAC CAA CTC-3′) based on the sequence described (Scorpio & Zhang, 1996). The amplified product is a DNA fragment of 574 bp. A 620 bp portion of the catalase–peroxidase (*katG*) gene was amplified with forward primer katG904 (5′-AGC TCG TAT GGC ACC GGA AC-3′) and reverse primer katG1523 (5′-TTG ACC TCC CAC CCG ACT TG-3′) (Uhl *et al*., 1996). A 548 bp fragment of the *oxyR* homologue gene was amplified with forward primer (5′-GGT GAT ATA TCA CAC CAT A-3′) and reverse primer (5′-CTA TGC GAT CAG GCG TAC TTG-3′) (Sreevatsan *et al*., 1996). A 320 bp (78 to 397) fragment of the gene g*yrA* that encodes the subunit A of the DNA gyrase was amplified with primers gyrA1 (5′-CAG CTA CAT CGA CTA TGC G-3′) and gyrA2 (5′-GGG CTT CGG TGT ACC TCA T-3′) (Takiff *et al*., 1994).

Sequencing was performed with the DyeDeoxy (dRhodamine) Terminator Cycle Sequencing kit (Applied Biosystems) in an automatic ABI Prism 373 DNA sequencer with software provided by the manufacturer (Applied Biosystems) (C.I.B. and C.N.B. Sequencing Facilities,

Table 2. Source of the spoligotyping patterns of the *M. tuberculosis* complex strains included in the dendrogram (Fig. 1)

Data were compiled from published references and/or obtained in our laboratories.

No. in Fig. 1	Species/strain	Source	Country	Reference
	M. tuberculosis* subsp. *caprae			
1	spc-1 (gM-1^T)	Goat, sheep	Spain	Aranaz *et al.* (1996a)/*
2	spc-2 (gC-31)	Goat	Spain	Aranaz *et al.* (1996a)/*
3	spc-3 (gV-1)	Goat	Spain	Aranaz *et al.* (1996a)/*
4	spc-4 (gC-11)	Goat	Spain	Aranaz *et al.* (1996a)/*
	M. bovis			
5	spb-1	Cattle	Spain	Aranaz *et al.* (1996a)/*
6	spb-3	Cattle	Spain	Aranaz *et al.* (1996a)/*
7	spb-6	Cattle, wild boar	Spain	Aranaz *et al.* (1996a)/*
8	spb-7	Cattle, wild boar	Spain	Aranaz *et al.* (1996a)/*
9	spb-8	Cattle	Spain	Aranaz *et al.* (1996a)/*
10	spb-13	Cattle	Spain	Aranaz *et al.* (1996a)/*
11	spb-15	Cattle, deer	Spain	Aranaz *et al.* (1996a)/*
12	MDR outbreak†	HIV-infected human beings	Spain	Blázquez *et al* (1997)
13	sp-1	Cattle, buffalo, deer, goat, pig, badger	Australia, Ireland, Iran	Cousins *et al.* (1998)
14	sp-6	Human beings	Ireland	Cousins *et al.* (1998)
15	sp-7	Human beings	Iran	Cousins *et al.* (1998)
16	sp-8	Buffalo	Australia	Cousins *et al.* (1998)
17	sp-37	Cattle	UK	Cousins *et al.* (1998)
18	sp-42	AN5 reference strain, veal	–	Cousins *et al.* (1998)
19	sp-43	Human beings, cattle, elk, bison	Iran, Canada	Cousins *et al.* (1998)
20	sp-44	Eland	Canada	Cousins *et al.* (1998)
21	sp-46	Cattle	Iran	Cousins *et al.* (1998)
22	sp-47	Cattle, elk, deer, cougar	Canada	Cousins *et al.* (1998)
	BCG			
23	Pasteur	Vaccine strain	–	Goguet *et al.* (1997)/*
24	Russian	Vaccine strain	–	Goguet *et al.* (1997)/*
25	Japanese	Vaccine strain	–	Goguet *et al.* (1997)/*
	M. tuberculosis			
26	H37Rv	Reference strain	–	All references/*
27	Outbreak A	Human beings	France	Goguet *et al.* (1997)
28	Outbreak B	Human beings	France	Goguet *et al.* (1997)
29	Patient 1	Human being	France	Goguet *et al.* (1997)
30	Patient 2	Human being	France	Goguet *et al.* (1997)
31	E-1	Elephant	Spain	*
32	Dog 1	Pet dog	Spain	*
33	Dog 2	Pet dog	Spain	*
34	RyC 1	Human being	Spain	*
35	RyC 2	Human being	Spain	*
36	Cluster A	Human beings	Guadeloupe, French West Indies	Sola *et al.* (1998)
37	Cluster C	Human beings	Guadeloupe, French West Indies	Sola *et al.* (1998)
38	Cluster 1	Human beings	The Netherlands	Kamerbeek *et al.* (1997)
39	Cluster 2	Human beings	The Netherlands	Kamerbeek *et al.* (1997)
	M. africanum			
40	TMC 12	Reference strain, human being	–	*
41	TMC 3	Reference strain, human being	–	*
42	TMC 54	Reference strain, human being	–	*
43	Clinical isolate	Human being	Spain	*
	M. microti			
44	NCTC 08710^T	Reference strain, vole	–	*
45	RyC m	Laboratory isolate	Spain	*

* Obtained in our laboratories.

† MDR, Multidrug-resistant.

Madrid). The sequences generated were aligned with published mycobacterial sequences from the GenBank databases (accession nos U59967, Scorpio & Zhang, 1996; X83277, Heym *et al*., 1995; U18263, Sherman *et al*., 1995; and L27512, Takiff *et al*., 1994).

Genetic fingerprinting analysis. Procedures for the direct variable repeat (DVR) spacer oligonucleotide typing (DVR-spoligotyping) (Kamerbeek *et al*., 1997) and RFLP associated with the IS*6110* element, direct repeat (DR) and polymorphic G+C-rich sequences (PGRS) have been previously described (Aranaz *et al*., 1996a, 1998; Liébana *et al*., 1997). Briefly, isolates were grown in OADC (oleic acid, albumin, dextrose and catalase)-enriched Middlebrook 7H9 (Difco), and chromosomal DNA was extracted as previously described (Cousins *et al*., 1993). DNA was digested with restriction endonucleases *Pvu*II and *Alu*I (Boehringer Mannheim), Southern-blotted and hybridized with the strain-specific markers. The probe for the IS*6110* right-hand side of the *Pvu*II site was a 245 bp fragment amplified by PCR with primers INS-1 and INS-2 (Hermans *et al*., 1990) and labelled with digoxigenin-11-dUTP using the DIG High Prime kit (Boehringer Mannheim). Oligonucleotide probes IS*6110* (left-hand side: 5′-CGA TGA ACC GCC CCG GCA TGT CCG GAG ACT C-3′), DR (5′-GTG GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3′) and PGRS (5′-CCG CCG TTG CCG CCG TTG CCG CCG TTG CCG CCG-3′) were end-labelled with digoxigenin-11-dUTP using the DIG oligonucleotide tailing kit (Boehringer Mannheim). The hybridized probes were detected using the alkaline-phosphatase-conjugated antibody detection kit and chemiluminescent substrate for alkaline phosphatase (CSPD; Boehringer Mannheim).

Spoligotyping (Kamerbeek *et al*., 1997) was used to determine the presence or absence of spacer DNA sequences in the DR locus. Spacers were amplified by PCR with primers Dr-a and Dr-b (biotin-labelled) and subsequently detected by hybridization on to a membrane containing 37 oligonucleotides derived from *M. tuberculosis* and six from *M. bovis* BCG covalently linked. Labelled hybridized product was detected with streptavidin–peroxidase conjugate (Boehringer Mannheim) and the ECL system (Amersham). The analysis of the hybridization pattern was performed with the GELCOMPAR program, version 3.1 (Applied Maths). The patterns obtained were compared with a large database containing approximately 500 *M. bovis* strains from cattle, pets and wild animals (wild boar, red deer and fallow deer) from several Spanish regions; the database included strains isolated from humans.

The caprine isolates were included in a dendrogram generated with spoligotyping patterns of a representative number of strains of the classical species belonging to the *M. tuberculosis* complex that have been obtained at our laboratories or published in the literature (Table 2). The bands obtained in the spoligotyping were treated as discrete characters and recorded in a data matrix by scoring 1 for the presence and 0 for the absence of every spacer. Similarity between strains was determined with the software TAXAN 3.0 (Sea Grant College) using the band-sharing coefficient calculated by the formula of Jaccard ($N_{xy}/(N_x+N_y-N_{xy})$ where N_{xy} is the number of spacers common to strains x and y, and N_x and N_y are the number of spacers in strain x and y, respectively).

RESULTS

Morphology and growth

The organism was an acid–alcohol-fast, non-motile rod that grew slowly at 36 °C, forming smooth and non-chromogenic colonies. Visible growth in primary culture usually took 4–6 weeks incubation. Subculture growth was achieved in 3–4 weeks at 36 °C, but not after 8 weeks at 25, 30 or 43 °C. Colonies appeared faster and in greater numbers in Coletsos and Löwenstein–Jensen media with pyruvate (20 d) than in conventional Löwenstein–Jensen (26 d). The organisms gave a cording pattern in Proskauer medium, though poorly in comparison with *M. tuberculosis*.

Phenotypic characteristics

Growth on Middlebrook 7H10 medium in the presence of sodium chloride (5% w/v), picric acid (0·2%), hydroxylamine (500 μg ml^{-1}), *p*-nitrobenzoic acid (500 μg ml^{-1}) or oleic acid (250 μg ml^{-1}) was negative. The isolates were negative for nitrate reductase and niacin accumulation. Pyrazinamidase activity was not detected on Middlebrook 7H10 medium (4 and 14 d). They were PZA (50 μg ml^{-1})-sensitive and resistant to 1 or 2 μg TCH ml^{-1} (5 and 2–5% colonies, respectively) but not to 5 or 10 μg TCH ml^{-1}. They weakly hydrolysed Tween 80 (after 10 d) and did not hydrolyse arylsulphatase (after 3 and 14 d). The catalase test was positive at room temperature and negative at 68 °C. Urease activity (Christensen) was negative and urease activity (discs) was positive after 4 h. Acid phosphatase, alkaline phosphatase, β-glucosidase and esterase were positive as detected with the API CORYNE and API ZYM systems. The type strain was susceptible to all antibiotics tested [1% of colonies were resistant to pyrazinamide (PZA)].

GLC

The pattern obtained and the percentage of the constituents were characteristic of micro-organisms of the *M. tuberculosis* complex: there were large amounts of hexadecanoic ($C_{16:0}$), octadecanoic ($C_{18:0}$) and octadecenoic ($C_{18:1}$) acids, as well as tuberculostearic (10-methyloctadecanoic) acid. Hexacosanoic acid ($C_{26:0}$) was the primary mycolic acid cleavage product of the strain; secondary alcohols were not observed.

Molecular identification

A DNA fragment of 1030 bp was obtained in all isolates after PCR amplification of a 16S rRNA genus *Mycobacterium*-specific region. All isolates harboured the sequences IS*6110*, IS*1081* and MPB70 reported to

Table 3. Differential characteristics of the caprine mycobacterial isolate in comparison with other members of the *M. tuberculosis* complex

Data were taken from references cited in the text. Abbreviations: +, positive; −, negative; v, variable; S, sensitive; R, resistant.

Species	IS*6110*	IS*1081*	MPB70	*mtp40*	Niacin accumulation	Nitrate reduction	TCH	PZA	*pncA* c 57	*katG* c 463	*oxyR* n 285	*gyrA* c 95
M. tuberculosis												
Classical	+*	+	+	+*	+	+	R	S	CAC (His)	CGG (Arg), CTG (Leu)	G	AGC (Ser), ACC (Thr)
Asian	+*	+	+	+	+	+	S	S	CAC	CGG/CTG	G	–
M. africanum												
Type I	+	+	+	+*	v	−	S	S	CAC	CTG	G	ACC
Type II	+	+	+	+*	v	+	S	S	CAC	CTG	G	ACC
M. bovis	+	+	+	−	−	−	S	R	GAC (Asp)	CTG	A	ACC
M. bovis BCG	+	+	+	−	−	−	S	R	GAC	CTG	A	ACC
M. microti	+	+	+	−	+	−	S	S	CAC	CTG	G	ACC
M. caprae	**+**	**+**	**+**	**−**	**−**	**−**	**S†**	**S**	**CAC**	**CTG**	**A**	**ACC**

* Very occasionally, members of these species lack the genetic element (Liébana *et al.*, 1996).

† Resistant to 1 and 2 μg TCH ml^{-1}, but sensitive to 5 and 10 μg TCH ml^{-1}.

be specific for micro-organisms within the *M. tuberculosis* complex, but all of them lacked the *mtp40* element (as demonstrated by PCR amplification). The AccuProbe (GenProbe) system also classified these isolates within the *M. tuberculosis* complex.

The 5′ 1524 bp of the 16S rRNA sequence of the type strain was determined (EMBL accession no. AJ131120) and was identical to published 16S rRNA gene sequences of *M. tuberculosis* complex strains.

Gene polymorphisms

All isolates tested with the allele-specific PCR presented a *pncA* polymorphism (Scorpio *et al.*, 1997), characteristic of *M. tuberculosis*, considered as the wild-type sequence. This sequence should code for a functional enzyme. However, *M. bovis* and BCG have a single point mutation in the *pncA* gene, changing a C to a G at nucleotide position 169, resulting in a His to Asp substitution (Scorpio & Zhang, 1996). DNA sequencing of the complete *pncA* gene of the caprine type strain did not detect other mutations.

The *katG* sequence of the caprine isolate contains a thymine instead of a guanine at position 1388, thus codon 463 (CTG) gives a Leu. This polymorphism is found in *M. bovis*, *M. africanum*, *M. microti* and in some *M. tuberculosis* strains (Muser *et al.*, 1996; Sreevatsan *et al.*, 1997). The *oxyR* analysis showed that the mycobacterial isolates from goats had an adenine at position 285. This polymorphism differentiates *M. bovis* from other complex members that have a G residue (Sreevatsan *et al.*, 1996). Codon 95 of the *gyrA* gene in the goat isolate displayed an ACC (threonine), a fluoroquinolone-susceptible pattern found in some *M. tuberculosis* strains and in *M. bovis*, BCG and *M. africanum* (Takiff *et al.*, 1994). No additional sequence variations were found. Table 3 shows the results of the most representative tests cited above.

RFLP and spoligotyping analysis

After characterization by IS*6110*, DR and PGRS-associated RFLPs and DVR-spoligotyping, the fingerprinting patterns of the caprine strains formed a tight homogeneous group. When the caprine patterns were analysed in the context of results obtained with a wide group of *M. bovis* strains obtained from cattle, deer, wild boar and cats typed with the same genetic elements, the caprine group was clearly segregated by means of both DVR-spoligotyping and RFLPs (Aranaz *et al.*, 1998). The isolate obtained from the pig from herd c-11 had the same characteristics as the caprine mycobacterial isolates.

The dendrogram (Fig. 1) shows the relationships of the spoligotyping patterns of *M. tuberculosis* complex organisms. A major division, separated at a distance of 0·2, includes the *M. microti* reference strain NCTC 08710^T and a laboratory isolate. The broad group of strains is divided into two major clusters at a distance of 0·35. The first cluster comprises only the caprine mycobacterial isolates. The second cluster is subsequently divided at 0·58 into two branches: one of them includes strains of *M. bovis* and *M. africanum*; the second one includes the *M. tuberculosis* strains.

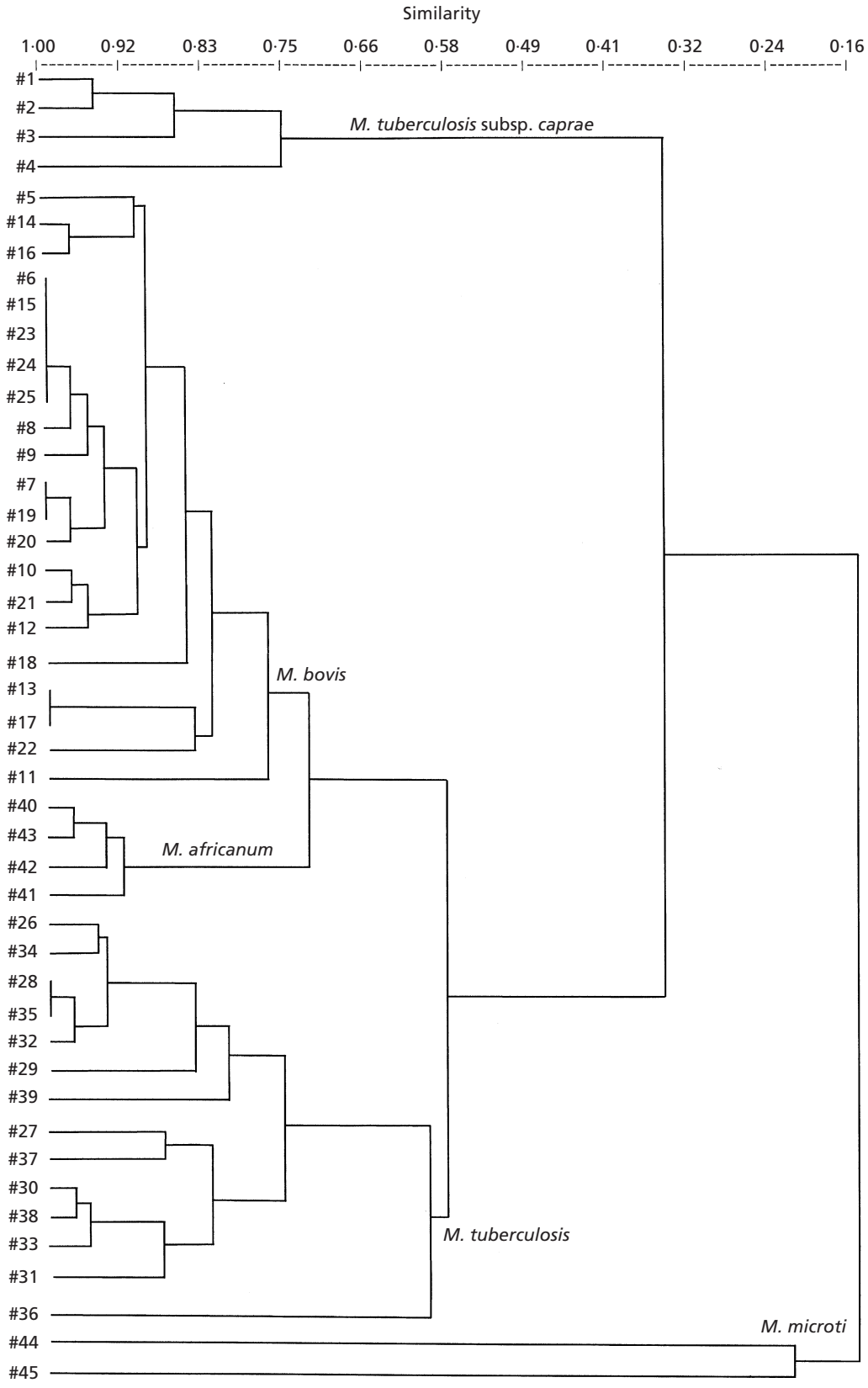

Fig. 1. Dendrogram constructed with the spoligotyping patterns of 45 *Mycobacterium tuberculosis* complex organisms, including representive reference strains and clinical isolates of all species (data in Table 2). Pattern similarities were analysed with the TAXAN software; the JACCARD algorithm was used for analysis.

DISCUSSION

In this report we describe the results of a polyphasic taxonomy study of an unusual mycobacterium that belongs to the *M. tuberculosis* complex. The isolates share the presence of DNA elements, such as IS*6110*, IS*1081*, MPB70 and DR, that are considered specific to the *M. tuberculosis* complex. The AccuProbe test, 16S rRNA gene sequencing and GLC confirm these results. The results of these tests, coupled with the animal origin of the isolates, initially led us to identify this group of strains as *M. bovis*. However, further study highlighted the presence of the special phenetic and genetic characteristics that indicated that these strains differed from the other members of the complex.

Firstly, the isolates were PZA sensitive, whereas *M. bovis* isolates are naturally resistant to PZA. Pyrazinamidase activity is a stable feature commonly used to distinguish *M. bovis* from the other members of the *M. tuberculosis* complex. The caprine isolates tested had the wild-type polymorphism found in *M. tuberculosis*, *M. microti* and *M. africanum* (Scorpio *et al.*, 1997; Sreevatsan *et al.*, 1997). These results agree with those published by Espinosa de los Monteros *et al.* (1998). No other mutations were found in the *pncA* gene sequence. Other enzymic activities that distinguish between the caprine isolates and the classical *M. bovis* are alkaline phosphatase, β-glucosidase and hydrolysis of Tween 80. Furthermore, the caprine isolates can be distinguished from *M. africanum* and *M. microti* by TCH susceptibility. Secondly, the special combination of the polymorphisms in the sequenced genes has not been found in the other members of the complex and could be considered ancestral in mycobacteria. We speculate, therefore, that goat isolates could be the ancestral condition suggested by Sreevatsan *et al.* (1997) and Espinosa de los Monteros *et al.* (1998). Thirdly, the fingerprinting patterns obtained with IS*6110*, DR and PGRS-associated RFLP and DVR-spoligotyping are very different from those obtained for other members of the complex examined in our laboratory or published by other authors (Aranaz *et al.*, 1996a, 1998; Blázquez *et al.*, 1997; Goguet *et al.*, 1997; Kamerbeek *et al.*, 1997; Cousins *et al.*, 1998; Sola *et al.*, 1998). All of the goat isolates tested were included in a common cluster of patterns for both RFLP and DVR-spoligotyping, and were clearly segregated from the bovine and wild-animal isolates. van Soolingen *et al.* (1997) reported the usefulness of large DNA fingerprint databases (such as those of IS*6110* and spoligotypes) in mycobacterial systematics. These results show that caprine strains are a genetically distinct cluster within the *M. tuberculosis* complex.

The great importance of *M. tuberculosis* as a pathogen of humans and animals has led workers to allot separate species names to variants that, when examined by criteria applied to other species, could be recognizable as intraspecific variants of a single species (Collins & Yates, 1982). As an example, the 16S rRNA gene sequence, considered specific to nearly every organism and used to establish phylogenetic relationships and identify micro-organisms (Stackebrandt & Woese, 1981), is the same in all members of the *M. tuberculosis* complex. However, this apparent lack of correlation between 16S rDNA sequences and species assignment also occurs with other species of the genus *Mycobacterium*, i.e. between *Mycobacterium malmoense* and *Mycobacterium szulgai*, or *Mycobacterium gastri* and *Mycobacterium kansasii* (Rogall *et al.*, 1990). The existence of a spectrum of variants could confirm the theory that we are looking at a single species (*M. tuberculosis*) with different variants owing to selection by the environment and host niches, thus suggesting a certain degree of host specificity. As a consequence of the existing controversy in the classification of the species within the *M. tuberculosis* complex, the biological meaning of such variations is unclear.

It is worth noting that the caprine strains were isolated from different geographical areas of Spain, and that in most cases the same typing pattern was found in all tuberculous goats of a herd. These points highlight the ecological and clinical relevance of this taxon. On the other hand, only a few cases of tuberculosis have been reported in goats in countries with a high incidence of *M. bovis* in cattle. We suggest the posssibility that this unusual strain is a variety more adapted to goats than is *M. bovis*, but this theory must be confirmed by experimental infections. It is tempting to speculate as to how long tuberculosis may have been endemic in goats and whether the close relationship between goats and this mycobacterial isolate is responsible for such limited genetic diversity.

As with the other members of the complex, a degree of host specificity does not preclude the possibility of infection of other species. In this study, the caprine mycobacterial isolate was also found in a sheep and in a pig, both of which were in close contact with goats. Surprisingly, despite the high incidence of tuberculosis in Spanish goat herds, the caprine type has not been found in cattle. While this work was in progress, Gutiérrez *et al.* (1997) reported three human isolates that displayed caprine spoligotyping patterns. When the origin of the strains was traced, it was found that one of the patients was resident in a rural area in which goat farming was common; the second patient worked in an abattoir and the third was a veterinary practitioner (Gutiérrez *et al.*, 1997). The public-health implications of these strains deserve further investigation.

The biochemical, genetic and epidemiological differences found between the classical members of the *M. tuberculosis* complex and the mycobacteria isolated from the goat herds suggest that the caprine isolates could be considered as belonging to a new member of the *M. tuberculosis* complex. These strains could be clasified as a new taxon in its own right, rather than a

sub-group of *M. bovis*. We propose the name *Mycobacterium tuberculosis* subsp. *caprae* for these strains.

Description of *Mycobacterium tuberculosis* subsp. *caprae* sp. nov.

Mycobacterium tuberculosis subsp. *caprae* (ca′p.rae. L. fem. gen. n. *caprae* referring to *capra*, the L. fem. n. for goat, the host animal from which the bacteria are isolated).

Strains can be isolated from the lymph nodes and lungs of tuberculous goats. These organisms are acid–alcohol-fast, non-spore forming, non-motile bacilli with weak cording formation. Growth is enhanced with pyruvate and usually occurs after 4–6 weeks incubation at 36 °C. Colonies are smooth and non-chromogenic. Strains are negative for niacin accumulation and nitrate reduction, sensitive to PZA (50 μg ml^{-1}), resistant to 1 and 2 μg TCH ml^{-1}, but sensitive to 5 and 10 μg TCH ml^{-1}. They weakly hydrolyse Tween 80 (10 d) and do not hydrolyse arylsulphatase (3 and 14 d). Other enzymic activities include acid and alkaline phosphatases and β-glucosidase. Catalase test is positive at room temperature and negative at 68 °C. The key biochemical features that separate this subspecies from the previously established taxa within the *M. tuberculosis* complex are as follows: niacin accumulation, nitrate reduction and TCH susceptibility differentiate the new taxon from *M. tuberculosis*; PZA susceptibility differentiates it from *M. bovis* and *M. bovis* BCG; resistance to 1 μg TCH ml^{-1} differentiates it from *M. africanum*; and niacin accumulation differentiates it from *M. microti*. The sequence of the 16S rRNA and the GLC profile are characteristic of all members of the *M. tuberculosis* complex. All isolates contain the sequences IS*6110*, IS*1081* and MPB70 and lack the *mtp40* element. The strains present a special combination of gene polymorphisms: the *pncA* gene contains CAC (His) at codon 57; codon 463 of the *katG* gene, CTG, gives a Leu; nucleotide 285 of the *oxyR* pseudogene is an adenine; and codon 95 of the *gyrA* gene displays an ACC (Thr). The DVR-spoligotyping and RFLP patterns are unique. The type strain is gM-1^T (= CIP 105776^T), which has the characteristics described for the taxon.

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