Elevation of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz *et al.* 1999 to species rank as *Mycobacterium caprae* comb. nov., sp. nov.

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Mycobacterium tuberculosis complex isolates recovered from goats were originally classified as *Mycobacterium tuberculosis* subsp. *caprae*; however, this subspecies was recently reclassified as *Mycobacterium bovis* subsp. *caprae*. Besides biochemical (sensitivity to pyrazinamide) and epidemiological features, strains of this unusual member of the *M. tuberculosis* complex show a special combination of *pncA*, *oxyR*, *katG* and *gyrA* gene polymorphisms. Sequence analysis of the *gyrB* gene in these strains reveals special nucleotide substitutions not found in other members of the *M. tuberculosis* complex that can be used to differentiate caprine mycobacterial strains from *M. bovis* and other members of the *M. tuberculosis* complex. *M. tuberculosis* subsp. *caprae* now appears not to be restricted to Spanish goats, as strains of this organism have been isolated from cattle, wild boar and pigs. Its occurrence has also been reported in France, Austria and Germany. Two studies on the evolution of the *M. tuberculosis* complex based on the presence/absence of regions of difference have shown that the group of caprine isolates (or its ancestor) is older than *M. bovis* (or its ancestor). These findings reinforce the original suggestion that the caprine mycobacterial strains are a taxon of the *M. tuberculosis* complex, independent of *M. bovis*. Within the current context of the existing nomenclature of the *M. tuberculosis* complex, it is proposed that *M. tuberculosis* subsp. *caprae* be elevated to species status, as *Mycobacterium caprae* comb. nov., sp. nov.

Mycobacterium tuberculosis, *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti* are the classical members of the *M. tuberculosis* complex. These pathogens, which affect both humans and animals, are closely related from a taxonomic point of view. Some phenotypic characteristics have been used for speciation within the *M. tuberculosis* complex. The fact that this group of organisms is important as pathogens of worldwide significance has led to bias in the selection of tests for the classification of the mycobacteria, with an enrichment of those tests most likely to separate them. This led to a distortion of the numerical taxonomic matrix (Wayne, 1984). Numerical taxonomy places the members of the complex in a macrocluster, as they have a close relationship to *M. tuberculosis* (*sensu stricto*) and they should be reduced to subspecies thereof (Wayne & Kubica, 1986). DNA homology within the complex has raised some debate about the

convenience of the reorganization of the complex as a single species. However, the subtle phenotypic differences, the epidemiology (preferred host) and pathology, and the ease of use of the classical names have kept the traditional nomenclature.

In relatively recent times, because of the increased research on tuberculosis, and coupled with the availability of new molecular tools, other isolates with characteristics of the *M. tuberculosis* complex, but not matching any of the classical species, have been described, i.e. '*M. canettii*' (van Soolingen *et al.*, 1997) and the seal bacillus (Cousins *et al.*, 1990, 1993).

In 1999, we described the results of a polyphasic taxonomic study of an unusual member of the *M. tuberculosis* complex, and named this organism *Mycobacterium tuberculosis* subsp. *caprae* (Aranaz *et al.*, 1999), because it was first isolated from goats with disseminated tuberculous lesions. The main characteristics that differentiate isolates belonging to *M. tuberculosis* subsp. *caprae* from other members of the *M. tuberculosis* complex are a special combination of *pncA*, *katG* and *gyrA* gene polymorphisms that has not been found in other members of the complex, and specific

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Abbreviations: PZA, pyrazinamide; spoligotyping, spacer oligonucleotide typing.

fingerprinting patterns obtained from RFLP associated with IS6110, direct repeat and polymorphic GC-rich sequences, and direct variable repeat-spacer oligonucleotide typing (spoligotyping) that are very different to those obtained for other members.

Recently, Niemann *et al.* (2002) transferred *M. tuberculosis* subsp. *caprae* to the *M. bovis* group as *Mycobacterium bovis* subsp. *caprae*. The aim of this report is to present new genetic evidence for the taxon formerly named *M. tuberculosis* subsp. *caprae*, to refute this transfer. It should be noted that, although the name *M. bovis* subsp. *caprae* was validly published, according to Rule 27 (3) of the *Bacteriological Code* (Lapage *et al.*, 1992), *Mycobacterium bovis* subsp. *caprae* (Aranaz *et al.* 1999) Niemann *et al.* 2002 is not valid because, at the time of publication, the type strain of the species was not deposited in two publicly accessible service collections in different countries (De Vos & Trüper, 2000).

Phenotypic characteristics of the caprine mycobacterial isolates that differentiate them from *M. bovis* are a weak hydrolysis of Tween 80 after 10 days and sensitivity to pyrazinamide (PZA). Sensitivity to PZA has been used as a major criterion for distinguishing *M. bovis* from the other members of the *M. tuberculosis* complex. Growth of *M. bovis* strains is not inhibited by PZA, a key characteristic that was included by Karlson & Lessel (1970) in the validation of the name and description of the species, while *M. tuberculosis*, *M. africanum* and *M. microti* are susceptible to this anti-mycobacterial drug. However, a small percentage of *M. bovis* strains sensitive to PZA was cited by Collins & Yates (1981). Results of PZA susceptibility testing, depending on growth of the organisms when exposed to drugs, can be unreliable due to the technical difficulty in obtaining growth in the very acidic medium required for PZA activity (Davies *et al.*, 2000; Hewlett *et al.*, 1995). The test can vary depending on the method and media used, and can suffer from limited reproducibility and lack of reliability (Hannan *et al.*, 2001).

The sequencing of the pyrazinamidase gene (*pncA*) demonstrated a single C→G point mutation at nucleotide 169 that appears to be unique to *M. bovis* (Scorpio & Zhang, 1996), while *M. tuberculosis*, *M. africanum* and *M. microti* harbour the functional wild-type *pncA* gene. The 'defective' *pncA* gene of *M. bovis* is a stable feature that has been used for the design of genetic tests for differentiation of *M. bovis* from *M. tuberculosis* (Scorpio *et al.*, 1997; Espinosa de los Monteros *et al.*, 1998). The sequence of the *pncA* gene of the type strain of *M. tuberculosis* subsp. *caprae* (CIP 105776^T) and seven other caprine mycobacterial isolates of four different spoligotyping profiles, and from different hosts, reveals that all isolates have the wild-type *pncA* gene. This result shows that the allele polymorphism 169 C is a stable characteristic of the caprine isolates.

When the previously mentioned study of Collins & Yates (1981) was carried out, DNA-based techniques were not as accessible as they are today for most average laboratories;

thus, there are no data on the *pncA* and other gene sequences or on the spoligotyping profile of the strains used in their study, making it difficult to gather information on the real nature of the isolates examined by Collins & Yates (1981).

Further differentiation amongst caprine mycobacterial isolates and *M. bovis* is drawn from the *gyrB* gene sequence polymorphism analyses of several members of the *M. tuberculosis* complex from the studies of Kasai *et al.* (2000) and Niemann *et al.* (2000b). At nucleotide 1311, *M. bovis* and other members of the *M. tuberculosis* complex show a T, while caprine mycobacterial isolates show a G; at position 1410, *M. bovis* has a characteristic C→T substitution, which was proposed by Kasai *et al.* (2000) for differentiation of the species, while caprine strains and the other members of the *M. tuberculosis* complex have a C (Niemann *et al.*, 2000b). Sequencing of the *gyrB* gene from two Spanish caprine strains (the type strain of *M. tuberculosis* subsp. *caprae* plus an isolate from a domestic pig) and comparison to a sequence from the GenBank database (accession no. L27512; Kasai *et al.*, 2000) demonstrated that these strains also had the specific 'caprine' polymorphism. These results increase the combination of gene polymorphisms specific for this unusual member of the *M. tuberculosis* complex.

Caprine mycobacterial isolates have special features by genetic fingerprinting. By direct variable repeat-spoligotyping, performed with first generation membranes following the method of Kamerbeek *et al.* (1997), they form a homogeneous cluster easily recognizable by the absence of spacers 1, 3–16, 30–33 and 39–43. The lack of spacers 39–43 has also been described in *M. bovis* and *M. microti*. RFLP typing associated with IS6110, polymorphic GC-rich sequences and direct repeats also segregates caprine mycobacterial isolates from *M. bovis* isolates (Liébana *et al.*, 1997; Aranaz *et al.*, 1998).

Regarding its presence in other hosts, *M. tuberculosis* subsp. *caprae* was first isolated from goats, but it is not restricted to caprine herds. Predominant isolation of this pathogen from goats in our laboratory may reflect epidemiological facts (the main host species in Spain) and the high number of caprine herds we have studied. Until relatively recently, goat farming in Spain has remained as a traditional farming system with isolated populations, and herds have not been included in health schemes and eradication campaigns. We have also isolated caprine mycobacterial isolates from cattle, wild boar (*Sus scrofa*) and pigs under extensive management. As was commented on in the description of *M. tuberculosis* subsp. *caprae* (Aranaz *et al.*, 1999), isolates that displayed the caprine spoligotyping pattern have also been found in human patients, and these clinical cases have been linked with goat farming (Gutiérrez *et al.*, 1997).

Regarding the presence of *M. tuberculosis* subsp. *caprae* strains in other countries, the caprine spoligotype has also been identified in isolates from goats in France (Haddad *et al.*, 2001) and similar spoligotypes have been reported in cattle and free-living red deer (*Cervus elaphus*) in Austria

(Glawischnig *et al.*, 2000). We suggest that the clinical *M. bovis* isolates from cattle and humans described by Niemann *et al.* (2000a, b) are likely to be caprine isolates, because they share features such as susceptibility to PZA (they had the wild-type *pncA* sequence) and the sequence of the *gyrB* gene, and the spoligotyping patterns were defined by the typical absence of spacers.

Further evidence for the independence of the caprine mycobacterial isolates from *M. bovis* is derived from two recent studies that have examined the evolution of the *M. tuberculosis* complex (Brosch *et al.*, 2002; Mostowy *et al.*, 2002). Both these studies tested two different and representative sets of isolates of the *M. tuberculosis* complex ($n=100$ and $n=66$, respectively) from different hosts and countries. These isolates were tested for the presence/absence of regions of difference (RD), expected to represent unidirectional genetic events, and single nucleotide polymorphism analysis of the *pncA*, *gyrA* and *katG* genes was also done. Because the distribution of deletions suggests their order of occurrence during bacterial evolution, such studies propose an evolutionary scenario for the *M. tuberculosis* complex. In fact, the results of the RD analysis match those of the table of differential characteristics of the members

of the complex (Table 1). Results show that ancestral *M. tuberculosis* strains and '*M. canettii*' lack none of the studied deleted regions, and imply that the common ancestor for the *M. tuberculosis* complex would resemble *M. tuberculosis* or '*M. canettii*'. Other classical members of the *M. tuberculosis* complex form a separate lineage. The first branch would be represented by *M. africanum* (lacks RD9), then *M. microti*/the seal bacillus (lack RD9, and also RD7, RD8 and RD10) and caprine isolates (lack RD9, RD7, RD8, RD10, and RD5, RD6, RD12, RD13 and N-RD25). Classical *M. bovis* shows the greatest number of RD deletions relative to other members of the *M. tuberculosis* complex (apart of the aforementioned regions, it also lacks RD4) and would be the last link of this lineage. This fact would mean that the group of caprine mycobacterial isolates (or its ancestor) is older than *M. bovis* (or its ancestor).

These findings reinforce the original suggestion that the caprine mycobacterial strains are a taxon of the *M. tuberculosis* complex, independent of *M. bovis*. From a strict taxonomic point of view, the caprine isolates should be considered as a subspecies of *M. tuberculosis*. We believe that the nomenclature '*M. tuberculosis*' should prevail for the following reasons. First, there is compelling evidence

Table 1. Differential characteristics of the caprine mycobacterial isolates (*M. caprae*) in comparison with other members of the *M. tuberculosis* complex

Species: 1, *M. tuberculosis* (classical); 2, *M. tuberculosis* (Asian); 3, *M. africanum* (type I); 4, *M. africanum* (type II); 5, *M. microti*; 6, *M. caprae*; 7, *M. bovis*; 8, *M. bovis* BCG. IS6110, IS1081 and MPB70 are present in all members listed (very occasionally, *M. tuberculosis* strains lack the former). Data were taken from references cited in the text. +, Positive test result; –, negative test result; v, variable test result; ND, no data available or insufficient information; S, sensitive; R, resistant.

Characteristic	1	2	3	4	5	6	7	8
<i>mtp40</i>	+	+	+	+	–	–	–	–
Niacin accumulation	+	+	v	v	+	–	–	–
Nitrate reduction	+	+	–	+	–	–	–	–
TCH	R	S	S	S	S	S†	S	S
Pyrazinamide	S	S	S	S	S	S	R	R
<i>pncA</i> c 57	CAC (His)	CAC	CAC	CAC	CAC	CAC	GAC (Asp)	GAC
<i>katG</i> c 463	CGG (Arg) CTG (Leu)	CGG/CTG	ND	ND	CTG	CTG	CTG	CTG
<i>oxyR</i> n 285	G	G	G	G	G	A	A	A
<i>gyrA</i> c 95	AGC (Ser) ACC (Thr)	–	ACC	ACC	ACC	ACC	ACC	ACC
<i>gyrB</i> n 675/756/1311/ 1410/1450	C G T C G	ND	C G T C T	C G T C G	T G T C T	C A G C T	C A T T T	C A T T T
Spoligotyping (1st generation membranes)	At least one spacer 39–43: +	At least one spacer 39–43: +	At least one spacer 39–43: +	At least one spacer 39–43: +	Type vole, only 37–38: +. Other types described	1, 3–16, 30–33, 39–43: –	3, 9, 16, 39–43: –	1–2, 4–8, 10–15, 17–38: +. 3, 9, 16, 39–43: –

*Occasionally some isolates lack this element (Liébana *et al.*, 1996).

†Resistant to 1 and 2 µg 2-thiophenecarboxylic acid hydrazide (TCH) ml^{–1}, but sensitive to 5 and 10 µg TCH ml^{–1}.

that *M. tuberculosis* is the ancestor of the caprine mycobacterial taxon, or the member of the complex more related to it. Second, *M. tuberculosis* is the type species of the genus *Mycobacterium* and was the first member of the complex to be described. Third, *M. tuberculosis* is by far the most important pathogen of the complex in terms of the number of infected hosts and its public health implications. However, for an easy recognition of this unusual mycobacterial pathogen from goats by the health community, in terms of diagnosis and epidemiology, and within the current context of the existing nomenclature of the *M. tuberculosis* complex, we suggest the name *Mycobacterium caprae* for this organism.

Correct identification of mycobacteria is necessary for accurate diagnosis and appropriate treatment, and for epidemiological study in order to trace the index case or source of infection. Therefore, it is advisable that assignation to a 'species' or 'subspecies' should not be based exclusively on phenotypic characteristics when other tests can be performed with low cost and complexity. It is to be expected that additional techniques will become available in the future, and that they will help to achieve a more definitive classification of these important pathogens. Such techniques should lead us further in the taxonomy of the *M. tuberculosis* complex.

Description of *Mycobacterium caprae* comb. nov., sp. nov.

Mycobacterium caprae (ca'p.rae. L. fem. gen. n. *caprae* referring to *capra*, the L. fem. n. for goat, the host animal from which the bacterium was first isolated).

The description is the same as that given for *M. tuberculosis* subsp. *caprae* (Aranaz *et al.*, 1999), with the addition that isolates present a specific *gyrB* gene polymorphism: nucleotide 1311 is a G and nucleotide 1410 is a C.

The type strain is gM-1^T (=CIP 105776^T=ATCC BAA-824^T), which has the characteristics described for the taxon.

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References

Aranaz, A., Liébana, E., Mateos, A., Domínguez, L. & Cousins, D. (1998). Restriction fragment length polymorphism and spacer oligonucleotide typing: a comparative analysis of fingerprinting strategies for *Mycobacterium bovis*. *Vet Microbiol* **61**, 311–324.

Aranaz, A., Liébana, E., Gómez-Mampaso, E. & 8 other authors (1999). *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain. *Int J Syst Bacteriol* **49**, 1263–1273.

Brosch, R., Gordon, S. V., Marmiesse, M. & 12 other authors (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci U S A* **99**, 3684–3689.

Collins, C. H. & Yates, M. D. (1981). A study of bovine strains of *Mycobacterium tuberculosis* isolated from humans in South-East England, 1977–1979. *Tubercle* **62**, 113–116.

Cousins, D. V., Francis, B. R., Gow, B. L., Collins, D. M., McGlashan, C. H., Gregory, A. & MacKenzie, R. M. (1990). Tuberculosis in captive seals: bacteriological studies on an isolate belonging to the *Mycobacterium tuberculosis* complex. *Res Vet Sci* **48**, 196–200.

Cousins, D. V., Williams, S. N., Reuter, R., Forshaw, D., Chadwick, D., Coughran, D., Collins, P. & Gales, N. (1993). Tuberculosis in wild seals and characterisation of the seal bacillus. *Aust Vet J* **70**, 92–97.

Davies, A. P., Billington, O. J., McHugh, T. D., Mitchison, D. A. & Gillespie, S. H. (2000). Comparison of phenotypic and genotypic method for pyrazinamide susceptibility testing with *Mycobacterium tuberculosis*. *J Clin Microbiol* **38**, 3686–3688.

De Vos, P. & Trüper, H. G. (2000). Judicial Commission of the International Committee on Systematic Bacteriology. IXth International (IUMS) Congress of Bacteriology and Applied Microbiology. Minutes of the meetings, 14, 15 and 18 August 1999, Sydney, Australia. *Int J Syst Evol Microbiol* **50**, 2239–2244.

Espinosa de los Monteros, L. E., Galán, J. C., Gutiérrez, M. & 8 other authors (1998). Allele-specific PCR method based on *pncA* and *oxyR* sequences for distinguishing *Mycobacterium bovis* from *Mycobacterium tuberculosis*: intraspecific *M. bovis pncA* sequence polymorphism. *J Clin Microbiol* **36**, 239–242.

Glawischig, W., Prodinger, W. M. & Schöpf, K. (2000). Bovine tuberculosis in free-living Austrian red deer (*Cervus elaphus*): epidemiological investigation using DNA-fingerprinting. In *Abstracts of the 4th Meeting of the European Wildlife Disease Association, Zaragoza (Spain), 20–23 September, 2000*. Zaragoza: European Wildlife Disease Association.

Gutiérrez, M., Samper, S., Jiménez, M. S., van Embden, J. D. A., Marin, J. F. & Martín, C. (1997). Identification by spoligotyping of a caprine genotype in *Mycobacterium bovis* strains causing human tuberculosis. *J Clin Microbiol* **35**, 3328–3330.

Haddad, N., Ostyn, A., Karoui, C., Masselot, M., Thorel, M. F., Hughes, S. L., Inwald, J., Hewinson, R. G. & Durand, B. (2001). Spoligotype diversity of *Mycobacterium bovis* strains isolated in France from 1979 to 2000. *J Clin Microbiol* **39**, 3623–3632.

Hannan, M. M., Desmond, E. P., Morlock, G. P., Mazurek, G. H. & Crawford, J. T. (2001). Pyrazinamide-monoresistant *Mycobacterium tuberculosis* in the United States. *J Clin Microbiol* **39**, 647–650.

Hewlett, D., Jr, Horn, D. L. & Alfalla, C. (1995). Drug-resistant tuberculosis: inconsistent results of pyrazinamide susceptibility testing. *JAMA* **273**, 916–917.

Kamerbeek, J., Schouls, L., Kolk, A. & 8 other authors (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* **35**, 907–914.

Karlson, A. G. & Lessel, E. F. (1970). *Mycobacterium bovis* nom. nov. *Int J Syst Bacteriol* **20**, 273–282.

Kasai, H., Ezaki, T. & Harayama, S. (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *J Clin Microbiol* **38**, 301–308.

Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria (1990 Revision)*. Bacteriological Code. Washington, DC: American Society for Microbiology.

- Liébana, E., Aranaz, A., Francis, B. & Cousins, D. (1996). Assessment of genetic markers for species differentiation within the *Mycobacterium tuberculosis* complex. *J Clin Microbiol* **34**, 933–938.
- Liébana, E., Aranaz, A., Domínguez, L., Mateos, A., Gonzalez-Llamazares, O., Rodríguez-Ferri, E. F., Domingo, M., Vidal, D. & Cousins, D. (1997). The insertion element IS6110 is a useful tool for DNA fingerprinting of *Mycobacterium bovis* isolates from cattle and goats in Spain. *Vet Microbiol* **54**, 223–233.
- Mostowy, S., Cousins, D., Brinkman, J., Aranaz, A. & Behr, M. (2002). Genomic deletions suggest a phylogeny for the *Mycobacterium tuberculosis* complex. *J Infect Dis* **186**, 74–80.
- Niemann, S., Richter, E. & Rüsch-Gerdes, S. (2000a). Differentiation among members of the *Mycobacterium tuberculosis* complex by molecular and biochemical features: evidence for two pyrazinamide-susceptible subtypes of *M. bovis*. *J Clin Microbiol* **38**, 152–157.
- Niemann, S., Harmsen, D., Rüsch-Gerdes, S. & Richter, E. (2000b). Differentiation of clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis. *J Clin Microbiol* **38**, 3231–3234.
- Niemann, S., Richter, E. & Rüsch-Gerdes, S. (2002). Biochemical and genetic evidence for the transfer of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz *et al.* 1999 to the species *Mycobacterium bovis* Karlson and Lessel 1970 (Approved Lists 1980) as *Mycobacterium bovis* subsp. *caprae* comb. nov. *Int J Syst Evol Microbiol* **52**, 433–436.
- Scorpio, A. & Zhang, Y. (1996). Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to anti-tuberculous drug pyrazinamide in tubercle bacillus. *Nat Med* **2**, 662–667.
- Scorpio, A., Collins, D., Whipple, D., Cave, D., Bates, J. & Zhang, Y. (1997). Rapid differentiation of bovine and human tubercle bacilli based on a characteristic mutation in the bovine pyrazinamidase gene. *J Clin Microbiol* **35**, 106–110.
- van Soolingen, D., Hoogenboezem, T., de Haas, P. E. W. & 9 other authors (1997). A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. *Int J Syst Bacteriol* **47**, 1236–1245.
- Wayne, L. G. (1984). Mycobacterial speciation. In *The Mycobacteria: a Sourcebook*, pp. 25–65. Edited by G. P. Kubica & L. G. Wayne. New York: Marcel Dekker.
- Wayne, L. G. & Kubica, G. P. (1986). Family *Mycobacteriaceae* Chester 1897, 63^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1436–1457. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Williams.