# 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

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 antimycobacterial 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. IS*6110*, IS*1081* 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 |
|---|---|---|---|---|---|---|---|---|
| *mtp40* | +* | + | +* | +* | − | − | − | − |
| Niacin accumulation | + | + | V | V | + | − | − | − |
| Nitrate reduction | + | + | − | + | − | − | − | − |
| TCH | R | S | S | S | S | S† | S | S |
| Pyrazinamide | S | S | S | S | S | S | R | R |
| *pncA* c 57 | CAC (His) | CAC | CAC | CAC | CAC | CAC | GAC (Asp) | GAC |
| *katG* c 463 | CGG (Arg) CTG (Leu) | CGG/CTG | ND | ND | CTG | CTG | CTG | CTG |
| *oxyR* n 285 | G | G | G | G | G | A | A | A |
| *gyrA* c 95 | AGC (Ser) ACC (Thr) | − | ACC | ACC | ACC | ACC | ACC | ACC |
| *gyrB* 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 the 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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