Taxonomic Note

Proposal that the strains of the *Mycoplasma ovine/caprine* serogroup 11 be reclassified as *Mycoplasma bovis*

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This proposal is our response to the recommendation of the International Committee on Systematics of Prokaryotes (Subcommittee on the taxonomy of *Mollicutes*) that we 'write a proposal to classify *Mycoplasma bovis* and ovine/caprine serogroup 11 as a single species'. Physiological and phylogenetic comparisons between 27 strains of *M. bovis* and *Mycoplasma* serogroup 11 showed that (i) growth and patterns of organic acid substrate use completely overlapped among strains; (ii) all had lipase and phosphatase activities; (iii) the strains were indistinguishable in their SDS–PAGE whole-cell protein profiles, which differed from five other species; (iv) strains were indistinguishable in immunoblotting of cell proteins and cross-reactivity in ELISA, but differed from other *Mycoplasma* species; (v) DNA–DNA hybridization did not distinguish between the two groups, and (vi) comparison of 16S and 23S rRNA gene sequences of ten strains of *Mycoplasma* serogroup 11 and six strains of *M. bovis* showed that they shared 98–100% similarity across all strains tested, but only 86–95% to other *Mycoplasma* species. Strains of the *Mycoplasma ovine/caprine* serogroup 11 must therefore be reassigned as *Mycoplasma bovis*.

The taxonomy of the class *Mollicutes* has been the subject of considerable research and the application of molecular phylogenetic methods has clarified the interrelationships of some species of the genus *Mycoplasma* (Brown *et al.*, 1995; Gasparich *et al.*, 2004; Heldtander *et al.*, 1998; Pettersson *et al.*, 1996; Stakenborg *et al.*, 2005; Tasker *et al.*, 2003; Weisburg *et al.*, 1989; Woubit *et al.*, 2004). The nomenclature of the mycoplasmas isolated from ruminants is, however, still under debate (Nicholas *et al.*, 2002). In 1972, a number of vaginal and preputial mycoplasma isolates from an Australian sheep flock presenting symptoms of clinical pneumonia were characterized and

classified as biotype 2-D based on their distinct colony morphology and biochemical characteristics (Carmichael *et al.*, 1972). Two years later, a similar mycoplasma was isolated from an outbreak of vulvovaginitis in Australian ewes and was shown to share biochemical, but not serological properties, with *Mycoplasma agalactiae* (Cottew *et al.*, 1974); the authors also reported some serological reactions with members of the *Mycoplasma mycoides* cluster. In 1978, biotype 2-D was classified as the reference strain of the *Mycoplasma ovine/caprine* serogroup 11 (Erno *et al.*, 1978) based on an earlier classification system devised by Al-Aubaidi *et al.* (1972). No cross-reactions were seen between biotype 2-D and representative strains of the *M. mycoides* cluster and other species from small ruminants using a range of serological tests. Livingston & Gauer (1983) reported the isolation of mycoplasmas identical to the 2-D biotype from apparently healthy sheep flocks in the USA. Since this time, there have

[†]Deceased

Supplementary tables giving comparative 16S and 23S rRNA gene sequence similarities between strains 2-D and other strains of the *Mycoplasma* serogroup 11 and of *Mycoplasma bovis* are available with the online version of this paper.

been further isolations of this mycoplasma from goats with mastitis in India (Prasad *et al.*, 1984), from goats with vulvovaginitis in Nigeria (Chima *et al.*, 1986), from ruminants with unspecified clinical conditions in mainland Europe (Poumarat *et al.*, 1992) and from infertile sheep in the UK (Nicholas *et al.*, 1999). The pathogenic potential of *Mycoplasma* serogroup 11 was shown by Rana *et al.*, (1993) who reproduced vulvovaginitis experimentally in goats.

In an examination of European mycoplasmas, Poumarat *et al.* (1992) identified 13 ruminant isolates that reacted strongly with both *Mycoplasma* serogroup 11 and *M. bovis* antisera, suggesting a close relationship between the two species for the first time. *M. bovis*, first classified by Freundt (1955), is biochemically very similar to *Mycoplasma* serogroup 11 as neither ferments glucose, hydrolyses arginine nor possesses phosphatase activity; however, both digest inspissated serum, reduce tetrazolium and produce film and spots. These biochemical characteristics are also shared by two other ruminant mycoplasmas, *M. agalactiae* and *Mycoplasma bovis*. Interestingly, Poumarat *et al.* (1992) also demonstrated some serological relationships that were shared between these isolates and members of the *M. mycoides* cluster.

Subsequently, further similarities between *Mycoplasma* serogroup 11 and *M. bovis* were described, leading to the suggestion that these might in fact be strains of the same species (Nicholas *et al.*, 2002). It can be argued that the reason for the failure to recognize strains of *Mycoplasma* serogroup 11 as *M. bovis* until quite recently was the erroneous belief in the strict host specificity of mycoplasmas (Edward & Freundt, 1956). As improved media formulations and sensitive molecular biological tools have become available, however, the dogma of host specificity has been successfully challenged with mycoplasmas such as *Mycoplasma canis* (first isolated from dogs) and *Mycoplasma felis* (from cats) being found frequently in calves and horses, respectively (Pitcher & Nicholas, 2005).

An important aspect of being able to clarify the taxonomic relationships of these mycoplasmas relates to their disease-causing effects in ruminants. *Mycoplasma* serogroup 11 induces vulvovaginitis, cervicitis, endometritis, epididymitis and oophoritis (Ruffin, 2001), is pathogenic to the udder of lactating sheep and produces marked biochemical alterations in the milk (Kumar *et al.*, 1993; Rana *et al.*, 1993). *M. bovis* is commonly isolated from the reproductive tracts of cattle and buffaloes and some strains are implicated in mastitis, arthritis, balanoposthitis and genital discharges (Afshar *et al.*, 1966; Jackson & Boughton, 1991; Razin & Freundt, 1984; Thiede *et al.*, 2002). Variations in virulence in bovine udder infections exist for different strains of *M. bovis* (Mackie & Ball, 1984): a 'mastitis strain', isolated from an abnormal dry udder secretion, was highly pathogenic, and a 'vaginal strain', isolated from the vaginal mucus of a cow after abortion, produced a low level infection. The type strain of

M. bovis is, however, non-pathogenic (Jurmanová *et al.*, 1978).

In view of these arguments, the International Committee on Systematics of Prokaryotes (Subcommittee on the taxonomy of *Mollicutes*) recommended that we 'write a proposal to classify *Mycoplasma bovis* and ovine/caprine serogroup 11 as a single species' (Bradbury, 2007). Now that more strains have become available, we have been able to make physiological and phylogenetic comparisons with up to 13 strains of *M. bovis* and 14 strains of the *Mycoplasma* serogroup 11.

We have carried out growth studies and examined the whole-cell protein patterns and immunological profiles of 11 strains of *M. bovis* and seven strains of *Mycoplasma* serogroup 11. We have also reassessed the comparative 16S rRNA gene sequence similarities of six strains of *M. bovis* and 11 strains of *Mycoplasma* serogroup 11, as well as evaluating the available data on DNA-DNA hybridization among representative strains. The *M. bovis* strains used in the new studies were the type strain, NCTC 10122^T (=ATCC 19852^T=PG11^T), and isolates from cattle in the UK with reproductive disease (102B00, 159B01), Germany (398/87, 434/81) and France (2378, 2379, 4031, 4032, 4337, 4339). The strains of *Mycoplasma* serogroup 11 used were the reference strain 2-D (Australia) and six UK isolates (52SR98, 47SR99, 52SR99, 95SR99, 96SR99, 126SR99). For comparison, we have included *M. agalactiae* NCTC 10123^T, *Mycoplasma alkalescens* NCTC 10135^T, *Mycoplasma arginini* NCTC 10129^T, *Mycoplasma ovipneumoniae* NCTC 10151^T and *M. bovis* NCTC 10131^T.

Mycoplasma strains were grown in static culture in a modified Eaton's PPLO broth (Lin *et al.*, 2006) supplemented with glucose (0.5 % w/v), arginine or organic acids (0.2 % w/v) as the energy substrate. Neither arginine nor glucose stimulated growth of the *M. bovis* or *Mycoplasma* serogroup 11 strains, but pyruvate, α -ketobutyrate and lactate supported the growth of several strains (Table 1). Notably, the patterns of organic acid use showed a complete overlap of the *M. bovis* and *Mycoplasma* serogroup 11 strains and did not allow distinction between the species on physiological grounds. Interestingly, while strains 52SR98, 52SR99 (*Mycoplasma* serogroup 11) and *M. bovis* strain 434/81 oxidized both α -ketobutyrate and lactate (Nicholas *et al.*, 2002), they could not grow on either compound. Similarly, lactate was oxidized by *Mycoplasma* serogroup 11 strains 2-D and 47SR99 and by *M. bovis* strains PG11^T and 398/87, but lactate did not support the growth of these strains. All the strains tested also showed lipase and phosphatase activities during growth (data not shown), as previously reported for *M. bovis* and *Mycoplasma* serogroup 11 (Poumarat *et al.*, 1992; Razin & Freundt, 1984).

Sodium dodecyl sulfate PAGE (SDS-PAGE) of cell proteins was carried using the method of Laemmli (1970). Whole-cell suspensions were used as the antigen preparations for

Table 1. Comparative growth stimulation of *M. bovis* and *Mycoplasma* serogroup 11 strains by single organic acids

Organic acids supporting growth	Strains in each group	
	<i>M. bovis</i>	<i>Mycoplasma</i>
	<i>bovis</i>	serogroup 11
Pyruvate and α -ketobutyrate	PG11 ^T (=NCTC 10122 ^T)	Strain 2D
	Strain 398/87	Strain 47SR99
Pyruvate and lactate	Strain 159B01	Strain 96SR99
	Strain 4339	Strain 126SR99
Pyruvate only	Strain 102B00	Strain 52SR98
	Strain 2378	Strain 52SR99
	Strain 2379	Strain 95SR99
	Strain 4032	
None of these acids	Strain 434/81	
	Strain 4031	
	Strain 4337	

immunoblotting and immunoassay. For comparison with *M. bovis* and *Mycoplasma* serogroup 11, strains of *M. bovis* and *M. agalactiae* were grown with 0.2 % (w/v) pyruvate, strains of *M. arginini* and *M. alkalescens* were grown with 0.2 % (w/v) arginine and the *M. ovipneumoniae* strain was grown with 0.5 % (w/v) glucose.

SDS-PAGE protein profiles were essentially identical for the seven *Mycoplasma* serogroup 11 strains and for ten of the *M. bovis* strains. The principal protein bands in all cases were of M_r 30, 40, 45, 50, 75, 86 and 94 kDa. The anomalous *M. bovis* strain was 102B00, which showed a different profile with few of its bands common to the other 17 strains. Comparative protein profiles with five other species showed that the *M. bovis* type strain and the *Mycoplasma* serogroup 11 reference strain were very similar to each other, but differed from the other five species (Fig. 1).

To determine which of the *M. bovis* and *Mycoplasma* serogroup 11 proteins carried epitopes, proteins were transferred electrophoretically on to nitrocellulose membranes for immunoblotting. Membranes were blocked with 1 % (w/v) dried milk powder (Difco) in PBS (37 °C, 1 h), washed briefly with 0.05 % (v/v) Tween 20 in PBS (PBST), reacted with rabbit hyperimmune reference serum (1:100 in PBS; 2 h at 37 °C), washed with PBST, and then incubated with sheep anti-rabbit IgG conjugated to horseradish peroxidase (Sigma; 1:1000 in PBS; 1 h at 37 °C) before visualizing antibody-reactive bands with peroxidase substrate solution 1-Step chloronaphthol (4-CN; Pierce Biotechnology). All seven *Mycoplasma* serogroup 11 strains reacted identically with serum prepared against strain 52SR98, giving predominant immunogenic bands with M_r values of 30, 40, 44, 48, 55, 68 and 110 kDa. Ten of the *M. bovis* strains showed identical immunoblot profiles with serum raised against the type strain (NCTC 10122^T), with strong bands of M_r

Fig. 1. SDS-PAGE patterns of whole-cell proteins from the type strains of seven ruminant mycoplasmas. Lanes: 1, protein markers; 2, *M. agalactiae* NCTC 10123^T; 3, *M. arginini* NCTC 10129^T; 4, *M. ovipneumoniae* NCTC 10151^T; 5, *M. alkalescens* NCTC 10135^T; 6, *M. bovis* NCTC 10131^T; 7, *M. bovis* NCTC 10122^T; 8, *Mycoplasma* serogroup 11 2-D.

30, 40 and 72 kDa. Again, strain 102B00 was anomalous, giving a single antibody-reactive band of 68 kDa.

The cross-reactivity of the whole-cell proteins of 23 strains of *M. bovis*, *Mycoplasma* serogroup 11 and five other mycoplasma species (listed below) against rabbit hyperimmune serum raised against *M. bovis* and *Mycoplasma* serogroup 11 was also assessed using ELISA. ELISA wells (at 37 °C) were coated with 100 µl mycoplasma antigen (7.5 µg protein; 30 min), washed twice with PBS, blocked with 200 µl skimmed milk (1 h), washed three times with PBST and then reacted with 100 µl rabbit serum (1:5000 dilution in PBS) prepared against *M. bovis* NCTC 10122^T or *Mycoplasma* serogroup 11 strain 52SR98. After 30 min, followed by three PBST washings, wells were incubated (1 h) with 100 µl peroxidase-labelled sheep anti-rabbit IgG (1:1000 in PBS), washed four times with PBST and treated with 100 µl ABTS peroxidase substrate solution [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] for 10 min. Absorbance was read at 405 nm (A_{405}) and cross-reactivity (%) expressed relative to the A_{405} of whole-cell proteins from the reference strains, *M. bovis* NCTC 10122^T and *Mycoplasma* serogroup 11 strain 52SR98.

Five of the *Mycoplasma* serogroup 11 strains (including reference strain 2-D) showed 100 % cross-reactivity with serum against *Mycoplasma* serogroup 11 strain 52SR98 and strains 95SR99 and 96SR99 showed 86–87 % cross-reactivity. *Mycoplasma* serogroup 11 strains 2-D and 52SR98 showed 81 % cross-reaction to serum against *M. bovis* strain PG11^T (NCTC 10122^T). *M. bovis* PG11^T also showed 69 % cross-reaction with serum against *Mycoplasma* serogroup 11 strain 52SR98. *M. bovis* strains 159B01, 398/87, 2378 and 2379 gave 93–100 % cross-reactivity against *M. bovis* PG11^T serum, but strains 438/81, 4031, 4032, 4337 and 4339 gave

only 65–82 %, and strain 102B00 showed only 34 % cross-reactivity. *M. alkalescens*, *M. arginini*, *M. bovis* and *M. ovipneumoniae* showed 29–38 % cross-reactivity with the two sera tested and *M. agalactiae* showed 42 % and 51 % cross-reaction, respectively, with serum against *M. bovis* NCTC 10122^T and *Mycoplasma* serogroup 11 strain 52SR98.

It is clear from the protein profiles and immunological reactions that most strains of *M. bovis* and *Mycoplasma* serogroup 11 are similar to each other and differ significantly from the other species tested. By the ELISA cross-reaction tests, it is clearly impossible to distinguish between members of the two groups. These data extend the earlier observation that an indirect immunofluorescence test (Gourlay & Howard, 1983) of five strains of *Mycoplasma* serogroup 11 and two *M. bovis* strains with diagnostic hyperimmune sera to both type strains showed the seven strains to be indistinguishable (Nicholas *et al.*, 2002). Growth of the seven strains on agar plates was inhibited to similar degrees by antisera to both *Mycoplasma* serogroup 11 strain 2-D and *M. bovis* strain PG11^T, with the type strains showing virtually the same sensitivity to each antiserum. In contrast, no immunofluorescence reaction or inhibition was seen with antisera raised against *M. bovis*, *M. agalactiae* or *M. mycoides* subsp. *mycoides* (Nicholas *et al.*, 2002).

It has been shown previously by 16S rRNA gene sequence analysis that the type strain of *M. bovis* and the reference strain for *Mycoplasma* serogroup 11 were essentially indistinguishable, but were quite distinct from eight other *Mycoplasma* species (Nicholas *et al.*, 2002). Furthermore, Sachse *et al.* (2002) identified a 473 nucleotide region of the 23S rRNA gene which contained both highly conserved segments and sequences that were highly variable among *Mycoplasma* species. This meant that nearly all mycoplasmas could be distinguished with higher resolution at the species level than could be achieved using by 16S rRNA gene sequences alone. We have enlarged this inter-group comparison for 16 strains of *M. bovis* and *Mycoplasma* serogroup 11 (see Supplementary Table S1 in IJSEM Online) to show that the 16S and 23S rRNA genes of strain 2-D of *Mycoplasma* serogroup 11 shared 99.3–99.8 % and 98.2–99.5 % gene sequence similarity with 9 other strains of *Mycoplasma* serogroup 11 and six strains of *M. bovis*. Sequences of the 16S rRNA genes of six other mycoplasmas each showed similarity to those of both *Mycoplasma* serogroup 11 and *M. bovis* (see Supplementary Table S2 in IJSEM Online), but at lower similarity levels (86.6–97.4 % gene sequence similarity). The only major anomaly was found with the unusual properties of *M. bovis* strain 102B00 that was isolated from a vaginal swab from a cow which had aborted. This strain had been identified by PCR using specific primers and by growth inhibition tests using rabbit sera to the *M. bovis* type strain; the variability seen in its protein and immunoblotting profile is not uncommon amongst *Mycoplasma* species such as *M.*

ovipneumoniae, which show remarkable heterogeneity despite being easily speciated by molecular and immunological methods (Parham *et al.*, 2006)

We also showed that DNA–DNA hybridization between *Mycoplasma* serogroup 11 and *M. bovis* did not enable distinction between them (Nicholas *et al.*, 2002). Strains 2-D and PG11^T showed 90–94 % cross-hybridization with each other. *M. bovis* strains 57B00 and 438/81 showed 96–98 % hybridization with DNA from *Mycoplasma* serogroup 11 strain 2-D, but only 87–94 % with *M. bovis* PG11^T. Similarly, *Mycoplasma* serogroup 11 strain 3SR99 showed 97–98 % hybridization with DNA from either reference strain, and strain 52SR98 of serogroup 11 showed greater hybridization with *M. bovis* DNA (87 %) than with that from strain 2-D (76 %). DNA–DNA hybridization of three strains of *M. bovis* and five strains of *Mycoplasma* serogroup 11 with DNA from both reference strains (PG11^T and 2-D) showed 93 ± 4 % hybridization, with no significant difference between the strain groups.

Using a range of criteria (growth substrate spectrum, physiological properties, protein profiles, immunological cross-reactions, 16S and 23S rRNA gene sequence similarity and DNA–DNA hybridization), it is impossible to distinguish between *M. bovis* and the strains currently assigned to *Mycoplasma* serogroup 11. We therefore propose that all these strains be reclassified as strains of *M. bovis*. The species description for *M. bovis* may need emendation as the original description was based on a very small number of strains and current proposals suggest descriptions of species should be based on at least five strains (Johansson & Bradbury, 2007). Any such emendation should be considered only after the publication of the volume of Bergey's *Manual* which will include the class *Mollicutes*.

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