Taxonomy of the canine *Mollicutes* by 16S rRNA gene and 16S/23S rRNA intergenic spacer region sequence comparison

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The taxonomy of canine *Mollicutes* is described, based on phylogenetic analysis of 16S rRNA gene and 16S/23S rRNA intergenic spacer (IGS) region sequences. The nucleotide sequences of the 16S rRNA gene of two untyped mycoplasmas and the IGS region of 11 *Mycoplasma* species were determined and used for phylogenetic analysis. The two untyped *Mycoplasma* strains, HRC 689 and VJC 358, were found to be distinct from all known canine mycoplasmas and all published mycoplasma 16S rRNA gene sequences.

Mollicutes (or mycoplasmas) are wall-less bacteria that are found in a variety of avian, insect, mammalian, plant and reptilian hosts. Several species are pathogenic and a variety of infections, such as anaemia, arthritis, infertility and respiratory disease, have been attributed to infection by mycoplasmas. To date, 15 known species of mycoplasma have been isolated from or detected in dogs: *Acholeplasma laidlawii*, *Mycoplasma arginini*, *Mycoplasma bovigenitalium*, *Mycoplasma canis*, *Mycoplasma cynos*, *Mycoplasma edwardii*, *Mycoplasma feliminutum*, *Mycoplasma felis*, *Mycoplasma gateae*, *Mycoplasma haemocanis*, *Mycoplasma maculosum*, *Mycoplasma molare*, *Mycoplasma opalescens*, *Mycoplasma spumans* and *Ureaplasma canigenitalium* [for review, see Rosendal (1982)]. Some authors also describe *Mycoplasma collis* as a canine mycoplasma (Tully & Razin, 1996; Johansson & Pettersson, 2002). However, we cannot find any report of *M. collis* infection in dogs and reports indicate

that this species was originally isolated from rodents (Hill, 1983); therefore, this species may have been mistakenly identified as being of canine origin. In addition, other publications describe the isolation of untyped *Mycoplasma* spp. from dogs (Kirchner *et al.*, 1990; Chandler & Lappin, 2002) and some document analyses of *Mycoplasma* strains that do not fit the criteria of known designated species, such as *Mycoplasma* sp. strain HRC 689 (Bowe *et al.*, 1982). In the past 20 years, work on canine mycoplasmas has been extremely limited, with only a dozen publications on mycoplasmas in dogs. Current knowledge of the molecular nature of canine mycoplasmas is non-existent and current diagnostic methods still rely on culture characteristics and use of specific antisera for identification of species. As such antisera are not readily available and several canine species share similar colonial morphology and growth characteristics, diagnosis of canine mycoplasmas is difficult and is therefore limited to specialized laboratories. In addition, despite the publication of 16S rRNA gene sequences of some mycoplasma species that are isolated from dogs, an overall representation of the taxonomy of canine mycoplasmas does not exist. In this publication, we have attempted to present such a review, allowing further understanding of the taxonomic positions of the canine mycoplasmas.

Taxonomically, mycoplasmas are divided into five major groups: the anaeroplasmata, asteroplasmata, hominis, pneumoniarum and spiroplasmata groups. The hominis group is then divided further into eight separate clusters: the *Mycoplasma bovis*, *Mycoplasma equigenitalium*, *Mycoplasma hominis*, *Mycoplasma lipophilum*, *Mycoplasma neurolyticum*, *Mycoplasma pulmonis*, *Mycoplasma suis* and *Mycoplasma synoviae* clusters [for review, see Johansson & Pettersson (2002)]. Of the 15 known species of *Mollicutes* that have been isolated from or detected in dogs, several are also found in other host animals; these species have previously been

Published online ahead of print on 3 October 2003 as DOI 10.1099/ijs.0.02869-0.

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Abbreviation: IGS, intergenic spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and 16S/23S rRNA intergenic spacer region sequences included in this study are: *Mycoplasma* sp. strain HRC 689, AF527624; *Mycoplasma* sp. strain VJC 358, AY246564; *Mycoplasma cynos*, AF538682; *Mycoplasma molare*, AF538683; *Mycoplasma opalescens*, AF538961; *Mycoplasma spumans*, AF538684. In addition, the following 16S/23S rRNA intergenic spacer region sequences were determined: *Mycoplasma arginini*, AF443604; *Mycoplasma canis*, AF443605; *M. cynos*, AF443606; *Mycoplasma edwardii*, AF443607; *Mycoplasma felis*, AF443608; *Mycoplasma gateae*, AF443609; *Mycoplasma maculosum*, AF443610; *M. molare*, AF443611; *M. opalescens*, AF443612.

A figure showing growth of *Mycoplasma* sp. strain VJC 358 on solid medium is available as supplementary material in IJSEM Online.

Table 1. Mycoplasmas isolated from dogs, anatomical sites and taxonomic affiliations

Adapted from Rosendal (1976) and Johansson & Pettersson (2002). Abbreviations: CON, conjunctiva; CSF, cerebrospinal fluid; GT, genital tract; SM, synovial membrane; URT, upper respiratory tract.

Species	Hosts	Canine isolation (associated infections)	IGS region size (bp)	Taxonomic group	Taxonomic cluster
<i>Acholeplasma laidlawii</i>	Canine, various*	GT		Anaeroplasmata	
<i>Mycoplasma arginini</i>	Canine, caprine*, feline, ovine*	Kidneys, lung, URT	180	Hominis	Hominis
<i>Mycoplasma bovigenitalium</i>	Bovine*, canine	CON, GT, lung	312	Hominis	Bovis
<i>Mycoplasma canis</i>	Bovine, canine*, humans	CON, GT, lung, URT	288	Hominis	Synoviae
<i>Mycoplasma cynos</i>	Canine	CON, GT, lung (pneumonia), URT	268	Hominis	Synoviae
<i>Mycoplasma edwardii</i>	Canine	GT, lung, URT	293	Hominis	Synoviae
<i>Mycoplasma feliminutum</i>	Feline*, canine	GT, lung, URT		Anaeroplasmata	Acholeplasmata
<i>Mycoplasma felis</i>	Canine, feline*, equine, humans	URT	290	Hominis	Synoviae
<i>Mycoplasma gateae</i>	Canine, feline*	GT, lung, URT	176	Hominis	Hominis
<i>Mycoplasma haemocanis</i>	Canine	Not isolated (anaemia)		Pneumoniae	Haemotrophic Mollicutes
<i>Mycoplasma maculosum</i>	Canine	GT, URT	310	Hominis	Bovis
<i>Mycoplasma molare</i>	Canine	GT, URT	273	Hominis	Neurolyticum
<i>Mycoplasma opalescens</i>	Canine	GT, URT	306	Hominis	Bovis
<i>Mycoplasma spumans</i>	Canine	CON, CSF and SM (arthritis), GT, lung, URT	189	Hominis	Hominis
<i>Ureaplasma canigenitalium</i>	Canine	GT	305	Pneumoniae	<i>Ureaplasma</i> <i>urealyticum</i>

*Principal host(s).

assigned to phylogenetic groups and clusters (Table 1). In addition to the 15 defined species, two untyped mycoplasma strains were included in the analyses in this study: *Mycoplasma* sp. strain HRC 689 was originally isolated from the pharynx of a dog in 1969 and has been associated with colitis in dogs (Bowe *et al.*, 1982) and *Mycoplasma* sp. strain VJC 358 was isolated recently from the trachea of a dog with mild respiratory disease (this study). Both untyped isolates do not fit the growth, biochemical or serological criteria of any defined canine species. These strains have been deposited in the National Collection of Type Cultures as NCTC 11744 and NCTC 11743, respectively.

In this study, we determine the 16S rRNA gene sequences of two untyped mycoplasma isolates from dogs, strains HRC 689 and VJC 358. To confirm phylogenetic analyses and due to the high 16S rRNA gene similarity between *M. canis*, *M. cynos*, *M. felis* and *M. edwardii*, we also sequenced the 16S/23S rRNA intergenic spacer (IGS) regions of *M. arginini*, *M. canis*, *M. cynos*, *M. edwardii*, *M. felis*, *M. gateae*, *M. maculosum*, *M. molare*, *M. opalescens*, *M. spumans* and *Mycoplasma* spp. strains HRC 689 and VJC 358. Phylogenetic analyses of these and previously published sequences enabled the determination of detailed taxonomic positions for all known canine *Mycoplasma* species and indicated that the two untyped mycoplasma

isolates, HRC 689 and VJC 358, are distinct from all described canine mycoplasmas.

Determination of 16S rRNA gene and IGS region sequences

All *Mycoplasma* spp. were cultured on or in *Mycoplasma* solid or liquid medium and *Ureaplasma* spp. on or in *Ureaplasma* solid or liquid medium (*Mycoplasma* Experience) at 37 °C in 95 % N₂/5 % CO₂. DNA was extracted from 5 ml liquid culture by using a DNeasy Tissue kit for isolation of bacterial DNA, according to the manufacturer's instructions (Qiagen). PCR amplifications were performed with reagents from Promega and oligonucleotide primers from Sigma. The 16S rRNA gene was amplified in two sections by using the following PCRs: the first 750 bp of the 16S rRNA gene was amplified with primers RNA5 (5'-AGAGTTTGATCCTGGCTCAGGA-3', positions 1531–1546 in the *Escherichia coli* 16S rRNA gene sequence, GenBank accession no. V00348) and UNI (5'-TAATCCT-GTTTGCTCCCCAC-3', positions 2286–2305) by a method adapted from Dussurget & Roulland-Dussoix (1994). Briefly, a 50 µl reaction that contained 5 µl 10× magnesium-free buffer, 1.5 mM MgCl₂, 0.25 µl (0.25 U) *Taq* DNA polymerase, 0.2 mM PCR nucleotide mix, 0.025 µg each primer and 1 µg mycoplasma DNA was amplified with PCR conditions of 95 °C for 5 min, 30 cycles of 95 °C for

1 min, 53 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 5 min. The last 750 bp of the 16S rRNA gene was amplified in a 50 µl reaction by using the following PCR conditions: 5 µl 10 × magnesium-free buffer, 1.5 mM MgCl₂, 0.25 µl (0.25 U) *Taq* DNA polymerase, 0.2 mM PCR nucleotide mix, 0.025 µg forward primer (MycH: 5'-GTGGGGAGCAAACAGGATTA-3', positions 2288–2308), 0.025 µg reverse primer (MycJ: 5'-TGGTGTGACGGGC-GG-3', positions 2914–2928) and 1 µg mycoplasma DNA. The gene fragment was amplified with PCR conditions of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 5 min. The 16S/23S rRNA IGS regions were amplified in a 50 µl reaction by using the following PCR: 5 µl 10 × magnesium-free buffer, 1.5 mM MgCl₂, 0.5 µl (0.5 U) *Taq* DNA polymerase, 0.2 mM PCR nucleotide mix, 0.025 µg forward primer (MycI: 5'-CACCGCCCGTCACACCA-3', positions 2914–2931), 0.025 µg reverse primer (Myc2: 5'-CAAGGCATC-CACCAAAACTCT-3' positions 3513–3535) and 1 µg mycoplasma DNA, with PCR conditions of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 54 °C for 40 s and 72 °C for 1 min, followed by 72 °C for 5 min, yielding a product of approximately 450 bp (depending on the species) that encompassed the entire IGS region. The IGS region of *M. feliminutum* could not be amplified with this primer set, even at a reduced annealing temperature. All amplicons were ligated into a pGEM-T Easy vector (Promega) and the resulting plasmids were used to transform *E. coli* JM109 cells. Transformants that contained cloned mycoplasma DNA were selected on Luria agar supplemented with 100 µg ampicillin ml⁻¹ (Sigma), 50 µg X-Gal ml⁻¹ (Merck) and 0.01 M IPTG (Sigma). Plasmid DNA was extracted and purified by using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions; 1 µg plasmid DNA was then sequenced by using fluorescently labelled primers (forward primer, pUCM13Fcyn-5: 5'-TGTAACGACGGCCAGT-3'; reverse primer, pgn-cyn5: 5'-CAGCTATGACCATGATT-ACG-3') and a Thermo Sequenase fluorescently labelled primer cycle kit with 7-deaza-dGTP (Amersham Biosciences), according to the manufacturer's instructions. To obtain consensus sequences, three independent clones from each species were sequenced in both directions. The three PCRs overlapped to give the entire 16S rRNA gene and IGS region; entire sequences were compiled, as well as some independent IGS region sequences. All sequences were submitted to GenBank (for accession numbers, see Table 1); sizes determined for the IGS region are also shown in Table 1.

Phylogenetic analysis

Sequences were aligned with CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic calculations were performed on the resulting alignment files of 1451 bp (16S rRNA gene) by using TREE-PUZZLE, version 5.0 (Schmidt *et al.*, 2000); maximum-likelihood and neighbour-joining analyses, with quartet-puzzling as the tree-search algorithm, were used to

compute phylogenetic trees (Strimmer & von Haeseler, 1997; Strimmer *et al.*, 1997) and a bootstrap analysis with 1000 replications was performed. All sequence accession numbers used in phylogenetic analyses are available from GenBank and are shown in Figs 1 and 2. A neighbour-joining phylogenetic tree for canine mycoplasma 16S rRNA genes is shown in Fig. 1; the same tree was obtained with parsimony analyses that were generated by the phylogenetic analysis package PHYLIP (Felsenstein, 1993). Phylogenetic positions within the tree were confirmed by repetition of the procedure with IGS region sequence analysis, in which a 315 bp alignment and a neighbour-joining tree were constructed (Fig. 2).

Except for *M. feliminutum* and *M. haemocanis*, all canine mycoplasmas are positioned within the hominis group of mycoplasmas (Fig. 1). Both untyped *Mycoplasma* spp. included in the analysis are also positioned within the hominis group, with *Mycoplasma* sp. strain HRC 689 being positioned within the *M. bovis* cluster and *Mycoplasma* sp. strain VJC 358 within the *M. hominis* cluster. The positions of all species and strains were confirmed by IGS region sequence analysis, except for strain VJC 358 (Fig. 2), indicating that analysis of the IGS region gives a good indication of taxonomic positioning in most cases. However, bootstrap values for the IGS tree are generally lower than those for the 16S rRNA tree, perhaps reflecting the high number of polymorphisms in this region or the shortness and differences in length of the sequences used in the alignment. Similarly, the inability to confirm the position of strain VJC 358 by IGS analysis could be a reflection of either the short length of nucleotide sequence used in this analysis or lack of discriminatory power of the IGS region for within-group analyses. Positioning of VJC 358 within the within the *M. hominis* cluster was, however, confirmed by parsimony analysis of the 16S rRNA gene; this is, therefore, taken as the correct position for this strain. Comparison of the 16S rRNA gene of strains to all available prokaryotic 16S rRNA gene sequences by FASTA analysis (Pearson, 1990) indicated that all high-scoring matches of both untyped strains are with *Mycoplasma* species and, in addition, these strains are distinct from all known available mycoplasma sequences. Highest sequence similarity to *Mycoplasma* sp. strain HRC 689 was found with *M. bovis genitalium* (96.0 % identity) and highest similarity to *Mycoplasma* sp. strain VJC 358 was with *M. canis* (91.3 %). These two strains may, therefore, represent novel species of mycoplasma from dogs; future studies should examine the properties of these strains in relation to those of other mycoplasmas, to enable the full description of these strains as novel species of mycoplasma. Although *Mycoplasma* sp. strain HRC 689 is a phosphatase-positive, glucose-fermenting mycoplasma with colonial and biochemical characteristics that are identical to those of *M. cynos*, it can be distinguished from other canine mycoplasmas by using specific antisera (University of Florida, USA) or via analysis of the 16S rRNA gene sequence. In contrast, *Mycoplasma* sp. strain VJC 358 ferments glucose

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, including four of the five groups of *Mollicutes* (except the asteroplasmata group) and all hominis group clusters. *Mycoplasma mycoides* subsp. *mycoides* served as the outgroup; bootstrap percentage values from 1000 resamplings are located at nodes of the tree. All species that can be isolated from dogs are shown in bold. GenBank accession numbers of sequences are given in parentheses; those described in this study are marked with an asterisk (*).

and forms slow-growing (5 days), star-like colonies that are morphologically distinct from those of other species on solid media (see Supplementary Figure, available in IJSEM Online). Specific antisera are not yet available for this strain, but sequence analysis of the 16S rRNA gene enables its conclusive distinction from other species.

From the 16S rRNA gene sequence alignment (Fig. 1), it is apparent that the sequences of *M. canis*, *M. cynos* and *M. edwardii* are very similar; these species may be difficult to distinguish, based on analysis of this gene alone. Indeed, the 16S rRNA genes of *M. canis* and *M. edwardii* both have 97% sequence similarity to that of *M. cynos*. The IGS tree (Fig. 2) gives a similar phylogenetic distribution of these

species with lower sequence similarity, indicating that this region may be more useful for molecular-based discrimination of these species. Interestingly, all species that fall within the neurolyticum and synoviae clusters in IGS region-based analysis are able to ferment glucose, whereas those in the hominis cluster hydrolyse arginine. However, such conservation is not seen within the bovis cluster, indicating that phylogenetic analysis of the IGS region does not reflect phenotypic properties. Furthermore, *M. canis*, *M. cynos* and *M. felis* are related closely within the synoviae cluster and are all associated with respiratory disease in dogs or other animals (Rosendal, 1972; Bemis, 1992; ter Laak *et al.*, 1993), yet this cluster also includes the non-pathogenic species *M. edwardii*.

Fig. 2. Phylogenetic tree based on 16S/23S rRNA IGS region sequences. *Mycoplasma mycoides* subsp. *mycoides* served as the outgroup; bootstrap percentage values from 1000 resamplings are located at nodes of the tree. All species that can be isolated from dogs are shown in bold. GenBank accession numbers of sequences are given in parentheses; those described in this study are marked with an asterisk (*).

Of the several species of mycoplasma that are isolated from dogs, *M. canis*, *M. edwardii*, *M. maculosum* and *M. spumans* are isolated most frequently, whereas others are less common (Rosendal, 1973). Mycoplasmas can be isolated routinely from the oral/pharyngeal cavity or urogenital tract of dogs and few species are particularly restricted to either site (Rosendal, 1982); therefore, no attempt was made to collate the taxonomic position of these mycoplasmas to particular isolation sites. Certain species of mycoplasma have been isolated from a range of mammalian hosts, including *M. feliminutum*, *M. felis* and *M. gateae* from cats (Cole *et al.*, 1967; Heyward *et al.*, 1969; Moise *et al.*, 1983), *M. felis* from horses (Wood *et al.*, 1997) and humans (Bonilla *et al.*, 1997) and *M. bovigenitalium* and *M. canis* from cattle (Freundt, 1955; ter Laak *et al.*, 1993). This is not reflected in the taxonomic positions of these species, as those isolated from multiple hosts cluster together with species of mycoplasma that have only been isolated from dogs (*M. cynos*, *M. edwardii*, *M. maculosum*, *M. molare* and *M. opalescens*). The concept of 'canine mycoplasmas' is somewhat misleading, as it infers that such mycoplasmas can only be isolated from dogs. Although this may be the case for some species, certain mycoplasmas have been isolated from more than one host. Other species of mycoplasma may also be present in a range of mammals but may not have been detected, due to the difficulty in identifying these mycoplasmas, lack of research in this area and the frequency of mixed infections.

The data presented here illustrate that species of mycoplasma that can be isolated from dogs are of diverse phylogenetic origin, with the majority lying in a variety of clusters within the hominis group of mycoplasmas. This study represents the first comprehensive review of canine mycoplasma taxonomy. At the current time, molecular-based tests (i.e. PCR) are not available for the majority of species of mycoplasma that are found in dogs. Availability of the 16S rRNA gene and IGS region sequences for all these species should enable the construction of molecular-based tests for the identification of canine mycoplasmas and will hopefully revolutionize diagnosis of these agents. Furthermore, determination that the two untyped *Mycoplasma* spp. strains HRC 689 and VJC 358 are distinct from all known canine mycoplasmas emphasizes that there is still much to discover and learn in this neglected field.

Acknowledgements

We would like to thank Miss G. Kelly, Miss W. Owen, Miss C. Paterson and Miss C. Toomey for technical assistance and The Dogs' Home, Battersea, for funding to Joe Brownlie.

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