NOTE

'*Candidatus* Mycoplasma haemodidelphidis' sp. nov., '*Candidatus* Mycoplasma haemolamae' sp. nov. and *Mycoplasma haemocanis* comb. nov., haemotrophic parasites from a naturally infected opossum (*Didelphis virginiana*), alpaca (*Lama pacos*) and dog (*Canis familiaris*): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas

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The 16S rRNA sequence of newly characterized haemotrophic bacteria in an opossum (*Didelphis virginiana*) and alpaca (*Lama pacos*) was determined. In addition, the 16S rRNA sequence of a haemotrophic parasite in the dog (*Canis familiaris*) was determined. Sequence alignment and evolutionary analysis as well as secondary structural similarity and signature nucleotide sequence motifs of their 16S rRNA genes, positioned these organisms in the genus *Mycoplasma*. The highest scoring sequence similarities were 16S rRNA genes from haemotrophic mycoplasma species (*Haemobartonella* and *Eperythrozoon* spp.). However, the lack of several higher-order structural idiosyncrasies used to define the pneumoniae group, suggests that these organisms and related haemotrophic mycoplasmas represent a new group of mycoplasmas. It is recommended that the organisms be named '*Candidatus* Mycoplasma haemodidelphidis', '*Candidatus* Mycoplasma haemolamae' and *Mycoplasma haemocanis* comb. nov., to provide some indication of the target cell and host species of these parasites, and to reflect their phylogenetic affiliation.

Keywords: 16S rRNA, *Rickettsiales*, *Anaplasmataceae*, *Mollicutes*, phylogeny

Haemobartonella and *Eperythrozoon* spp. are wall-less bacteria that attach to and grow on the surface of red blood cells. They infect a wide range of vertebrate animals (Ristic & Kreier, 1984). These haemotrophic bacteria were previously classified in the order *Rickettsiales* as a member of the family *Anaplasmataceae*.

Abbreviation: M & WR, mollicutes and walled relatives group.

A secondary structural representation of a generic haemotrophic mycoplasma 16S rRNA is available in IJSEM Online (<http://ijs.sgmjournals.org>).

The GenBank accession numbers for the 16S rRNA sequences of '*Candidatus* Mycoplasma haemodidelphidis' and '*Candidatus* Mycoplasma haemolamae' are AF178676 and AF306346.

However, sequence analysis of the 16S rRNA gene showed that these bacteria were members of the genus *Mycoplasma* (Rikihisa *et al.*, 1997; Neimark & Kocan, 1997). It was recently proposed that the classification of these taxa should be changed to reflect this phylogenetic affiliation. Thus, *Haemobartonella felis* and *H. muris* were transferred to the genus *Mycoplasma* as '*Candidatus* Mycoplasma haemofelis' and '*Candidatus* Mycoplasma haemomuris', respectively, and *Eperythrozoon suis* and *Eperythrozoon wenyonii* were transferred to the genus *Mycoplasma* as '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii', respectively. The trivial name haemo-

Fig. 1. Phylogenetic tree showing position of *'Candidatus Mycoplasma haemodidelphidis'*, *'Candidatus Mycoplasma haemolamae'* and *Mycoplasma haemocanis* (bold) among haemotrophic mycoplasmas, mollicutes and walled relatives (Table 1). Species names and accession numbers in GenBank are given at each axis. *C. ramosum* and *C. innocuum* served as outgroups. Bootstrap percentage values are given at the nodes of the tree and the scale bar shows the distance equivalent to 1 substitution per 10 nucleotides.

plasmas was given to these haemotrophic mycoplasmas (Neimark *et al.*, 2001). However, it was pointed out by the List Editor in the subsequent Notification List that the *Candidatus* designation is unacceptable because the basonyms would lose standing in nomenclature; the *Candidatus* designation is for new, incompletely described taxa, in order to give them a provisional status. Thus, *'Candidatus Mycoplasma*

haemofelis' has been revised to *Mycoplasma haemofelis* comb. nov., nom. nov., *'Candidatus Mycoplasma haemomuris'* has been revised to *Mycoplasma haemomuris* comb. nov., nom. nov., *'Candidatus Mycoplasma haemosuis'* has been revised to *Mycoplasma haemosuis* comb. nov., nom. nov. and *'Candidatus Mycoplasma wenyonii'* has been revised to *Mycoplasma wenyonii* comb. nov. (Neimark *et al.*, 2002).

The objective of the present investigation was to determine the taxonomic position of red blood cell parasites identified in an opossum, alpaca and dog and evaluate their relatedness to mycoplasmas. The light and electron microscopic features of an *Eperythrozoon*-like parasite associated with a severe anaemic episode in an immune-compromised opossum were recently described (Messick *et al.*, 1999). Red blood cell parasites found in llamas (*Lama glama*) in the USA were reported in 1990 (McLaughlin *et al.*, 1990; Reagen *et al.*, 1990). The clinical spectrum of infection in the llama varies from asymptomatic to severe disease, depending on host susceptibility. Recently, an alpaca with severe anaemia and overwhelming numbers of haemotrophic parasites was also identified (Messick & Walker, in preparation). The diagnosis of haemotrophic bacteria in dogs is well documented with cases reported throughout the United States, Australia, Europe, Canada and Great Britain (Seneviratna *et al.*, 1973). In a recent report, the clinical and haematologic abnormalities, as well as light and electron microscopic identification of characteristic eperythrocytic organisms in the peripheral blood of a dog, were described (Brinson & Messick, 2001).

Haemotrophic bacteria in peripheral blood from an infected opossum, alpaca and dog were lysed, DNA captured and then released using the Generation Capture Column Kit of Gentra Systems. Standard amplification reactions were carried out in a thermal cycler (MJ Research). The 16S rRNA gene of each of these red blood cell parasites was amplified using a universal primer set. The 16S rRNA gene of the red blood cell parasite of the alpaca was also amplified using specific primers for *M. haemosuis* (*E. suis*) (Messick *et al.*, 1999), whereas the specific primers for *M. haemofelis* (*H. felis*) amplified DNA of the parasite from the dog. The primer sets designed to be specific for *M. haemofelis* (*H. felis*) (Messick *et al.*, 1998; Berent *et al.*, 1998) and *M. haemosuis* (*E. suis*) (Messick *et al.*, 1999) failed to amplify DNA of the red blood cell parasite of the opossum.

PCR products were subjected to electrophoresis, the bands were extracted from the gel with a spin-column and DNA was sequenced directly in duplicate in both directions using a dye terminator cycle sequencing core kit and an automated sequencer. The nucleotide sequences were deposited in GenBank database. Sequence comparison to the GenBank database using BLASTN, showed all high scoring matches were from *Mycoplasma* species. The highest scoring sequences obtained were 16S rRNA genes from the newly recognized haemotrophic species of mycoplasmas (*Haemobartonella* and *Eperythrozoon* spp.) and species belonging to the pneumoniae group.

Alignments of 16S rRNA sequences were performed using PILEUP from the Sequence Analysis package, version 10.0 (Genetics Computer Group, Madison, WI, USA). The alignments were aided by use of secondary structure models of the 16S rRNA mole-

cules of *M. haemofelis* (*H. felis*) and a generic Gram-positive organism (Gutell, 1994; Woese *et al.*, 1983). Pairwise evolutionary distances were computed from the percentage similarities by the correction of Jukes and Cantor (Devereux *et al.*, 1984), modified to consider gaps (gap weight = 1.0). A phylogenetic tree was created from the distance matrices using the neighbour-joining method; 1365 nucleotides were included in the numerical analysis. The same branching order was found using different methods for distance correction (Kimura, Jin-Nei gamma and Tajima-Nei) and tree construction. The dataset was resampled 1000 times and bootstrap percentage values are given at the nodes of the phylogenetic tree shown in Fig. 1.

The sequence similarity for the 16S rRNA genes of '*Candidatus* Mycoplasma haemodidelphidis' and '*Candidatus* M. haemolamae' to organisms belonging to the haemotrophic group was 89.9–91.7% for the haemosuis cluster, *M. wenyonii* (*E. wenyonii*), *M. haemosuis* (*E. suis*) and '*Candidatus* M. haemominutum' (*H. felis*-California strain; Foley & Pedersen, 2001). The haemotrophic parasite infecting the dog was 99.2% homologous to the sequence previously reported for the Illinois/felis strain of *M. haemofelis* (*H. felis*, GenBank accession no. U95297). This parasite belonged to the haemofelis cluster that included *M. haemofelis* and *M. haemomuris* (*H. muris*). As previously reported, the presence of two separate clusters of haemotrophic mycoplasmas was also supported by a characteristic truncation of about 10 bp in a segment corresponding to positions 453–481 in the 16S rRNA gene sequence of *Escherichia coli*. This truncation was present in the haemofelis cluster and absent in the haemosuis cluster (Johansson *et al.*, 1999). The phylogenetic relations between the three haemotrophic bacteria described herein, the haemoplasmas (*Eperythrozoon* and *Haemobartonella* spp.) and representatives of some of mollicutes and their walled relatives are shown in the evolutionary tree in Fig. 1. The tree shows that '*Candidatus* M. haemodidelphidis', '*Candidatus* M. haemolamae', *M. haemocanis* and related haemotrophic mycoplasmas share a node in common with the pneumoniae group, but form a separate and distinct group.

The 16S rRNA gene of the three haemotrophic bacteria in this study share many of the unusual sequence and secondary structural features that are used to identify the mollicutes and walled relatives group (M & WR) (Woese *et al.*, 1980; Weisburg *et al.*, 1989, 1991). A secondary structural representation of the 16S rRNA gene of a haemotrophic mycoplasma, showing features discussed in the text, is available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>), and is discussed below. Perhaps the most convincing shared derived character is the absence of a helix at positions 1025–1036 [a] (Woese *et al.*, 1983). A uridine residue at position 888 [b] (Woese *et al.*, 1980) also distinguishes the small-subunit rRNA of these haemotrophic mycoplasmas. The specific clustering of the haemotrophic bacteria to the hominis,

Table 1. Species and strains used for phylogenetic analysis

Species	Strain	Accession no.*	Reference
<i>Mycoplasma sualvi</i>	Mayfield B	M23936	Weisburg <i>et al.</i> (1989)
<i>Mycoplasma hominis</i>	PG21	M24473	Weisburg <i>et al.</i> (1989)
<i>Mycoplasma mycoides</i>	UM30847	M23943	Weisburg <i>et al.</i> (1989)
<i>Spiroplasma citri</i>	Maroc	M23942	Weisburg <i>et al.</i> (1989)
<i>Mycoplasma cavipharyngis</i>	117C ^T	AF125879	Johansson <i>et al.</i> (1999)
<i>Mycoplasma fastidiosum</i>	4822 ^T	AF125878	Johansson <i>et al.</i> (1999)
' <i>Candidatus M. haemominutum</i> '	California	U88564	Rikihisa <i>et al.</i> (1997)
<i>M. wenyonii</i>		AF016546	Neimark & Kocan (1997)
<i>M. haemosuis</i>	Zachary	AF029394	Messick <i>et al.</i> (1999)
' <i>Candidatus M. haemodidelphidis</i> '	Illinois	AF178676	Messick <i>et al.</i> (2000); this study
' <i>Candidatus M. haemolamae</i> '	Michigan	AF306346	This study
<i>M. haemofelis</i>	Illinois/felis	U95297	Messick <i>et al.</i> (1998)
<i>M. haemocanis</i>	Illinois/canis	AF197337	Brinson & Messick (2001)
<i>M. haemomuris</i>	Shizuoka	U82963	Rikihisa <i>et al.</i> (1997)
<i>Ureaplasma urealyticum</i>	27	L08642	Robertson <i>et al.</i> (1993)
<i>Mycoplasma pneumoniae</i>	FH	M29061	Weisburg <i>et al.</i> (1989)
<i>Acholeplasma laidlawii</i>	JA1	M23932	Weisburg <i>et al.</i> (1989)
<i>Anaeroplasmabactoclasticum</i>	6-1	M25050	Weisburg <i>et al.</i> (1989)
<i>Asteroleplasma anaerobium</i>	161T	M22351	Weisburg <i>et al.</i> (1989)
<i>Clostridium innocuum</i>	B-3	M23732	Weisburg <i>et al.</i> (1989)
<i>Clostridium ramosum</i>	113-I	M23731	Weisburg <i>et al.</i> (1989)

pneumoniae and spiroplasma groups to the exclusion of others was strongly supported by several stringent features (Woese *et al.*, 1983). The most notable of these was the presence of two adjacent pairs, 127–128 and 233–234, in the form YY·RR [c], the addition of a single nucleotide after position 1361 [d], and low G + C content (43.4 mol %) of its 16S rRNA. The elimination of the helix between positions 1126 and 1144 [e], a higher-order structural feature that defines the pneumoniae group (Woese *et al.*, 1983), was also shared by these three organisms. Interestingly, this truncation is also characteristic for members of the *M. synoviae* cluster in the hominis group (Pettersson *et al.*, 1996).

In contrast, several sequence and secondary structural features used to identify the mycoplasmas were found to be divergent for '*Candidatus Mycoplasma haemodidelphidis*', '*Candidatus M. haemolamae*' and *M. haemocanis*. Position 1383 [f] is typically a uridine residue among M & WR (Woese *et al.*, 1985), however these haemotrophic mycoplasmas have reverted to or never changed from the ancestral cytidine residue composition, and a terminal adenosine rather than a guanosine residue was found in the otherwise highly conserved eubacterial oligonucleotide (UCCCCG) that covers this position. The M & WR sequence AUAUAUG (Woese *et al.*, 1980) covering position 705 [g] had a uridine rather than a guanosine residue in the terminal position. '*Candidatus M. haemodidelphidis*', '*Candidatus M. haemolamae*' and *M. haemocanis* lacked the cytidine residue [h] insert following position 915 (AAACGGAA) and the helix located between position 416 and 427 [i] lacked the loop-

proximal U·A in the 16S rRNA secondary structure, both of which are characteristic features of the pneumoniae group (Woese *et al.*, 1983).

Other haemotrophic mycoplasmas (*M. haemofelis* – all strains, '*Candidatus M. haemominutum*', *M. haemomuris*, *M. haemosuis* and *M. wenyonii*) cluster to the pneumoniae group and have identical 16S rRNA sequence and secondary structural features to those described herein for the haemotrophic mycoplasmas of the opossum, alpaca and dog. A few of these features appear to be characteristic of the haemoplasmas. The presence of an adenosine residue at position 1385 (UCCCCA) and uridine residue at position 710 (AUAURYU) are unique features in the 16S rRNA sequences of all the haemotrophic mycoplasmas and are an extremely uncommon finding among eubacteria in general. This feature is shared by *M. cavipharyngis* and *M. fastidiosum*, a newly recognized cluster within the pneumoniae group that forms a sister lineage to the haemotrophic bacteria (Johansson *et al.*, 1999). As others have suggested, the tendency for mycoplasma rRNAs to be relatively variable at sites that are otherwise highly conserved among eubacteria may be a reflection of their rapid evolutionary pace (Weisburg *et al.*, 1989).

It has been suggested that the haemotrophic mycoplasmas and their sister lineage (*M. cavipharyngis* and *M. fastidiosum*) may have descended from a common ancestor (Johansson *et al.*, 1999). Most of the unique sequence features in the 16S rRNA that characterized the haemotrophic mycoplasmas were also observed within the sister lineage. However, position 1383 in the

16S rRNA of *M. cavipharyngis* and *M. fastidiosum* was a uridine residue, which contrasted sharply with the ancestral cytidine residue found at this position in all the haemotrophic mycoplasmas. Unlike the haemotrophic mycoplasmas, these organisms also have added a fifth loop proximal C·G pair between positions 416 and 427 in their 16S rRNA and are capped by a loop of three nucleotides consisting of uridine and adenosine residues. The loop formed at this site contained five uridine residues for the cluster of *M. haemocanis*, *M. haemofelis* and *M. haemomuris*. However, only four nucleotides consisting of uridine and cytidine residues were found in the cluster of 'Candidatus *M. haemodidelphidis*', 'Candidatus *M. haemolamae*', *M. wenyonii*, *M. haemosuis* and 'Candidatus *M. haemominutum*'.

The haemotrophic mycoplasmas have many of the signatures of the M & WR group, however they lack several of the pneumoniae group signature sequences. It has recently been shown that 18 unique nucleotides out of 30 are in common for the pneumoniae group and the haemotrophic mycoplasmas (Johansson *et al.*, 1999). Thus, the haemotrophic mycoplasmas are most closely related to members of the pneumoniae group, albeit only peripherally. In the present study, the organisms that attached to the surface of the red blood cells of the opossum and the alpaca were found to be new haemotrophic mycoplasma species. Since haemotrophic mycoplasmas remain unculturable, an official species designation is impossible. Based upon genomic data, structural and secondary characteristics of the 16S rRNA gene, as well as light and electron microscopic features, it is recommended that the newly identified parasite of the opossum and alpaca be named 'Candidatus *Mycoplasma haemodidelphidis*' and 'Candidatus *Mycoplasma haemolamae*', respectively. For the haemotrophic parasite of the dog, we propose transferring *H. canis* to the genus *Mycoplasma* as *Mycoplasma haemocanis*.

'Candidatus *Mycoplasma haemodidelphidis*' sp. nov.

'Candidatus *Mycoplasma haemodidelphidis*' (hae.mo.di.del'phid.is. Gr. neut. n. *haema* blood; L. fem. gen. n. *didelphidis* of the opossum; N.L. fem. gen. n. *haemodidelphidis* of opossum blood. [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank no. AF178676)]).

In Wright–Giemsa-stained peripheral blood films, rod-, coccus- or ring-shaped organisms are attached to red blood cells. Produces a parasitaemia and severe anaemia in the opossum. By electron microscopy, the bacteria are in shallow depressions on the red blood cell surface, vary in diameter from 300 to 750 nm, and are enclosed by a single limiting membrane. 'Candidatus *M. haemodidelphidis*' is most closely related to the haemotrophic mycoplasmas (former *Haemobartonella* and *Eperythrozoon* species) based on sequence analysis of its 16S rRNA gene, having a distant, shared common ancestry with members of the pneumoniae group.

'Candidatus *Mycoplasma haemolamae*' sp. nov.

'Candidatus *Mycoplasma haemolamae*' (hae.mo.la'mae. Gr. neut. n. *haema* blood; L. fem. gen. n. *lamae* of the alpaca; N.L. fem. gen. n. *haemolamae* of alpaca blood. [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank no. AF306346)]).

Coccus- and ring-shaped basophilic organisms are present on red blood cells in Wright–Giemsa-stained peripheral blood smears. Cells are 0.4–1.0 µm in diameter, causing only slightly deformation of the red blood cell membrane by scanning and transmission electron microscopy (McLaughlin *et al.*, 1990; Reagan *et al.*, 1990). Multiple clinical problems have been reported in infected llamas including poorly to non-regenerative anaemia, inflammatory disease, hypoproteinaemia, chronic weight loss and failure to thrive. Younger animals are more susceptible to acute infection, leading to anaemia and massive parasitaemia, especially when stressed or when they have concurrent disease. In some cases, hypoglycemia is associated with the parasitaemia and may be profound (McLaughlin *et al.*, 1990). Based on sequence analysis of its 16S rRNA gene, 'Candidatus *M. haemolamae*' is most closely related to *Mycoplasma wenyonii*, a parasite of cattle, and other haemotrophic mycoplasmas.

Mycoplasma haemocanis comb. nov. [basonym *Haemobartonella canis* (ex Kikuth 1928)]

Mycoplasma haemocanis (hae.mo.ca'nis. Gr. neut. n. *haema* blood; L. fem. gen. n. *canis* of the dog, M.L. fem. gen. n. *haemocanis* of dog blood). [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank no. AF197337)]).

Wright–Giemsa-stained peripheral blood smears reveal coccus-shaped organisms singly or in chains. Some chains may split and produce Y-shaped forms or were arched, producing a 'violin-bow' form (Lumb, 1961). By electron microscopy, the organisms are found in deep depressions, attaching to the intact plasma membrane of the red blood cells. A single limiting membrane encloses the organisms, which vary in size from 0.3 to 2.0 µm in diameter (Venable & Ewing, 1968). The acute form of the disease in the dog, characterized by the presence of many red blood cell parasites and a rapidly developing anaemia, is most often found in immunocompromised or splenectomized dogs. The clinical signs of acute infection may include anorexia, lethargy, weight loss and fever. In severe cases, an acute haemolytic anaemia may result in death, but more frequently the dog recovers. A latent or chronic infection may be present in healthy dogs that have not undergone a splenectomy. The sequelae to infection in these dogs are poorly defined. Based on sequence analysis of its 16S rRNA gene, *M. haemocanis* is most closely related to *M. haemofelis*, a haemotrophic parasite of cats. Although, the question arose in late 1950s whether or not the organism causing canine and feline haemobartonellosis were the same,

this has never been resolved (Griesemer, 1958; Lumb, 1961).

Acknowledgements

We are grateful to Dr Carl Woese for his comments and suggestions during the writing of this manuscript. We are also indebted to Dr Robin Gutell for providing us with the secondary structure of the 16S rRNA gene of *Haemobartonella felis* and to Dr Tony L. Goldberg for kindly providing bootstrap values for the phylogenetic tree.

Note added in proof

The name *Mycoplasma haemosuis* comb. nov., nom. nov. (Neimark *et al.*, 2002) has been further corrected to *Mycoplasma suis* comb. nov. in Validation List no. 85 in this issue (pp. 691–692).

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