'*Candidatus Mycoplasma haematoparvum*', a novel small haemotropic mycoplasma from a dog

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A novel small haemoplasma was detected following cytological examination of blood smears from a splenectomized dog with haemic neoplasia. The 16S rRNA and *rnpB* genes of the organism were partially sequenced and a phylogenetic tree constructed. The organism was most closely related to the small feline haemoplasma, '*Candidatus Mycoplasma haemominutum*' (94 % 16S rRNA gene nucleotide sequence identity; 75 % *rnpB*) and was only distantly related to *Mycoplasma haemocanis* (78 % 16S rRNA gene nucleotide sequence identity; 65 % *rnpB*). As this organism has not been cultured *in vitro*, the candidate species name '*Candidatus Mycoplasma haematoparvum*' is proposed.

Haemotropic mycoplasmas, or haemoplasmas, are uncultivable wall-less bacteria that are associated with the surfaces of erythrocytes from a wide range of vertebrate hosts. The organisms are visible, using light-microscopic examination of blood smears, as rods, cocci or rings. Haemoplasmas within the haemosuis subcluster of mycoplasmas include '*Candidatus Mycoplasma haemominutum*', *Mycoplasma suis*, *Mycoplasma wenyonii*, '*Candidatus Mycoplasma haemolamae*' and '*Candidatus Mycoplasma haemodidelphidis*' (Messick *et al.*, 2002; Neimark *et al.*, 2001, 2002); these are haemoplasmas of cats, pigs, cattle, alpacas and opossums, respectively. In contrast, haemoplasmas belonging to the haemofelis subcluster include *Mycoplasma haemofelis*, *Mycoplasma haemomuris* and *Mycoplasma haemocanis*, which infect cats, mice and dogs, respectively.

Until recently, the only canine haemoplasma identified worldwide was *M. haemocanis* (previously *Haemobartonella canis*), which was first reported in 1928 (Kikuth, 1928). This is a relatively large haemoplasma (0.3–2.0 µm in diameter), which characteristically forms long chains across the erythrocyte surface (Messick *et al.*, 2002; Venable & Ewing, 1968). Chronic, subclinical infection may develop in immunocompetent dogs, which might then develop signs of haemolytic anaemia following splenectomy. Signs

have occasionally been reported in dogs that are immunocompromised for other reasons, and, rarely, disease has been reported in immunocompetent dogs (Austerman, 1979; McNaught *et al.*, 1935; North, 1978).

We have recently identified a novel haemotropic mycoplasma in a splenectomized dog with haemic neoplasia (Sykes *et al.*, 2004). Of the canine and feline haemoplasmas, this organism most closely resembled '*Candidatus M. haemominutum*' in that it was uniformly small in size (0.3 µm in diameter) and chains of organisms were not seen. Splenectomy was performed, together with multiple blood-product transfusions, just prior to diagnosis of acute lymphocytic leukaemia. Chemotherapy with prednisone and chlorambucil was instituted; 2 weeks after chemotherapy was instituted, numerous organisms were seen on the surfaces of approximately 70 % of the erythrocytes. Reticulocytosis was identified 1 week later, and a few haemoplasma-like organisms were detected. Treatment with doxycycline was initiated; 11 days later, no organisms were visible on blood smears. The aim of this work was to characterize this novel haemoplasma in more detail by sequencing the partial 16S rRNA gene and partial *rnpB* (RNase P) gene, which have been used to examine the relationship between other haemoplasmas (Birkenheuer *et al.*, 2002; Messick *et al.*, 2002; Neimark *et al.*, 2001; Tasker *et al.*, 2003). On the basis of these results, we propose a candidate species name for this organism, '*Candidatus Mycoplasma haematoparvum*'.

DNA was extracted from 200 µl anticoagulated whole blood in EDTA collected when organisms were first visualized (3 weeks after splenectomy) using the QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's instructions. DNA was eluted with 200 µl elution buffer. Previously extracted DNA from cat blood containing

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rnpB* gene sequences of '*Candidatus Mycoplasma haematoparvum*' are AY383241 and AY380803, respectively.

A figure showing a larger phylogenetic tree of 16S rRNA gene sequences, using *Asteroleplasma anaerobium* as the outgroup and including non-haemotropic mollicutes, is available as supplementary material in IJSEM Online.

M. haemofelis and cat blood containing 'Candidatus *M. haemominutum*' was used as a positive control. DNA extracted from healthy dog blood was used as a negative control. A 1457 bp fragment of the 16S rRNA gene was amplified using primers 8F (5'-AGAGTTTGATCCTGG-CTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Pitulle *et al.*, 1999). These primers amplify almost the complete length of the 16S rRNA gene. Each 50 µl reaction volume contained 1 × reaction buffer, 1.25 U *Taq* DNA polymerase (Brinkmann Instruments), 1.5 mM MgCl₂, 0.4 µM each primer, 200 µM each dNTP and 10 µl DNA template. Cycling conditions were as follows: 95 °C for 10 min, followed by 35 cycles of amplification (1 min at 95 °C, 1 min at 48 °C and 2 min at 72 °C), and a final extension of 5 min at 72 °C in a Dyad thermocycler (MJ Research).

A 188 bp fragment of the *rnpB* gene was also amplified using primers 80F1 [5'-GAGGAAAGTCCRYGCTWGC-AC-3' (R=A or G, Y=C or T, W=A or T)] and 290R1 (5'-TCCCYTACCRAAATTTTRGGTTTCT-3') (Birkenheuer *et al.*, 2002). Each 50 µl reaction volume contained 1 × reaction buffer, 1.25 U *Taq* DNA polymerase, 1.5 mM MgCl₂, 0.4 µM each primer, 200 µM each dNTP and 5 µl DNA template. Cycling conditions were as follows: 95 °C for 5 min, followed by 45 cycles of amplification (1 min at 95 °C, 1 min at 45 °C and 1 min at 72 °C), and a final extension of 5 min at 72 °C.

PCR amplicons were cloned into the pGEM-T Easy vector system (Promega) and transformed into *Escherichia coli*, according to the manufacturer's instructions. Plasmid DNA was isolated using standard methods. DNA sequencing was performed by using the dideoxynucleotide chain-termination method with an ABI 377 automated sequencer (Perkin-Elmer–Applied Biosystems), using M13 primers. Internal sequencing primers 5'-GCTGCGGTAAACA-TAGGTC-3' and 5'-CCTGTCATTCTGATAGCCTCC-3' were used to obtain the partial sequence of the large 16S rRNA gene fragment.

DNA sequence editing, analysis and construction of phylogenetic trees were performed with MacVector 7 and AssemblyLIGN (Accelrys) software. Phylogenetic trees were created from the distance matrices by using the neighbour-joining method. The dataset was resampled 1000 times and bootstrap percentage values are given at the nodes of the phylogenetic tree shown in Fig. 1. A figure showing a larger tree, using *Asteroleplasma anaerobium* as the outgroup and including other mollicutes, is available as supplementary material in IJSEM Online.

The partial nucleotide sequence of the 16S rRNA gene was compared with those of 'Candidatus *M. haemominutum*' (GenBank accession no. U88563), *M. haemofelis* (U95297) and *M. haemocanis* (AF197337). The 16S rRNA gene sequence of the novel haemoplasma was most closely related to that of 'Candidatus *M. haemominutum*' (94% identity at nucleotide sequence level). Nucleotide sequence

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of 'Candidatus *M. haematoparvum*' among haemotropic mycoplasmas. Bootstrap percentages are given at the nodes of the tree. Bar, 10 substitutions per 100 nucleotides. A figure showing a larger tree, using *Asteroleplasma anaerobium* as the outgroup and including other mollicutes, is available as supplementary material in IJSEM Online.

identity with *M. haemofelis* and *M. haemocanis* was considerably lower (76 and 78 %, respectively). More detailed phylogenetic analysis including comparisons with sequences available for a number of other haemoplasma and mycoplasma species in the GenBank database confirmed that the novel haemoplasma was most closely related to 'Candidatus *M. haemominutum*' (Fig. 1).

Comparison of the nucleotide sequence of the *rnpB* gene fragment with those of 'Candidatus *M. haemominutum*' (GenBank accession no. AY150990), *M. haemofelis* (AF407210) and three *M. haemocanis* isolates (AF407209, AF407211 and AF407213) revealed nucleotide sequence identities of 75, 65 and 65 %, respectively.

On the basis of the results of 16S rRNA gene sequence analysis, organisms belonging to the genera *Haemobartonella* and *Eperythrozoon* have now been reclassified as haemotropic *Mycoplasma* species ('haemoplasmas') (Neimark *et al.*, 2001, 2002). These organisms are most closely related to the fastidious, glucose-fermenting mycoplasmas *Mycoplasma caviopharyngis* and *Mycoplasma fastidiosum* (Johansson *et al.*, 1999). Until recently, only one canine haemoplasma had been described, and was recently renamed *M. haemocanis* (Messick *et al.*, 2002). Acute and severe anaemia has been described in infected dogs, especially those that are immunocompromised or splenectomized; chronic, subclinical infection is typical in immunocompetent dogs. In this report, we have characterized an additional canine haemoplasma. The infected dog was both splenectomized and immunocompromised due to concurrent lymphoid neoplasia and immunosuppressive drug therapy, and haemolysis was suggested by the appearance of marked reticulocytosis following the appearance of organisms on blood smears (Sykes *et al.*, 2004).

Analysis of the 16S rRNA gene sequence of this novel

haemoplasma revealed that it was most closely related to 'Candidatus M. haemominutum', a small, low-virulence feline haemoplasma (Foley & Pedersen, 2001). This organism can induce anaemia in cats that are concurrently infected with feline leukaemia virus, and there is some evidence that it may enhance the oncogenicity of this virus (George *et al.*, 2002). Whether this novel canine haemoplasma is capable of inducing disease in immunocompetent dogs, and the extent to which it may cause disease in immunosuppressed dogs, await further study. Preliminary results of experimental infection of cats suggest that this organism may be capable of infecting cats (J. E. Sykes and others, unpublished).

The 16S rRNA gene of *M. haemofelis* is >99% similar to that of *M. haemocanis* (previously *H. canis*), an organism that causes anaemia in splenectomized dogs. In fact, using this method of classification, some *M. haemofelis* isolates are more similar to *M. haemocanis* than they are to other *M. haemofelis* isolates, so it has been suggested that *M. haemofelis* and *M. haemocanis* may, in reality, be the same organism infecting different species of animals (Birkenheuer *et al.*, 2002; Brinson & Messick, 2001). However, cats inoculated with blood from a dog infected with *M. haemocanis* have remained asymptomatic, but blood from these cats, when inoculated into dogs, reproduced the disease (Lumb, 2001). Blood from *M. haemofelis*-infected cats, when inoculated into splenectomized and non-splenectomized dogs, did not result in anaemia or cytologically detectable parasitaemia, and inoculation of susceptible cats with this blood also did not result in anaemia or parasitaemia (Lumb, 2001; Splitter *et al.*, 1956). Because of these results, and morphological differences between these organisms, discrimination of these organisms on the basis of their *rnpB* gene sequences has been suggested (Birkenheuer *et al.*, 2002; Tasker *et al.*, 2003). Using this method, sequence identity between *M. haemofelis* and *M. haemocanis* is lower (94.3–95.5%) than that noted within strains of *M. haemofelis* (99.4%) and *M. haemocanis* (97.7–100%). The *rnpB* gene sequence identity between the novel haemoplasma and 'Candidatus M. haemominutum', *M. haemocanis* and *M. haemofelis* was considerably lower (75, 65 and 65%, respectively).

According to the guidelines for the naming of uncultivated prokaryotes (Murray & Stackebrandt, 1995), we propose a *Candidatus* designation for this newly recognized canine haemoplasma, and recommend that it be named 'Candidatus Mycoplasma haematoparvum'.

Description of 'Candidatus Mycoplasma haematoparvum'

'Candidatus Mycoplasma haematoparvum' [hae.ma.to.par'vum. Gr. neut. n. *haema* -atos blood; L. neut. adj. *parvum* small; N.L. neut. adj. *haematoparvum* small (mycoplasma) from blood] [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AY383241)].

In Wright–Giemsa-stained peripheral blood smears, cells appear as small (approx. 0.3 µm) coccus-shaped organisms present singly or in doublets and attached to erythrocytes. The light-microscopic appearance resembles that of 'Candidatus M. haemominutum' (Foley & Pedersen, 2001). Although further study is required, the organism appears to induce a parasitaemia and haemolysis in dogs; immunocompromise and/or splenectomy may be necessary for disease to occur. On the basis of sequence analysis of the 16S rRNA gene, 'Candidatus M. haematoparvum' is most closely related to 'Candidatus M. haemominutum', a low-virulence haemoplasma of cats.

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