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FIRST MOLECULAR IDENTIFICATION OF '*CANDIDATUS* MYCOPLASMA HAEMOMINUTUM' FROM A CAT WITH FATAL HAEMOLYTIC ANAEMIA IN HUNGARY

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Although haemobartonellosis was previously reported in Hungary, until now the diagnosis (based on morphological identification in blood smears) has only been suggestive of the occurrence of the large species, recently reclassified as *Mycoplasma haemofelis*. However, in July 2007 a cat was presented at a small animal clinic with severe haemolytic anaemia, icterus and haemoglobinuria. While biochemical parameters were within the reference range, the cat had leukocytosis and rapidly decreasing haematocrit values, and eventually died 7 days after the sudden onset of aggravating clinical signs. From blood samples of the cat '*Candidatus* Mycoplasma haemominutum' was identified by molecular methods, according to its 100% 16S rRNA gene sequence homology with two Swiss isolates and one isolate from the UK. The rapid termination of the disease and the high pathogenicity of the causative agent observed in this case are unusual, taking into account that PCR results were negative for immunosuppressive viruses. This is the first record of this feline haemoplasma species in Hungary.

Key words: *Candidatus* Mycoplasma haemominutum, feline infectious anaemia, real-time PCR

Haemotropic mycoplasmas (haemoplasmas) are Gram-negative, epierythrocytic bacteria causing more or less severe haemolytic anaemia in various hosts (Ristic and Kreier, 1984). Previously belonging to the order Rickettsiales, recently it has been verified that they are more closely related to and should be reclassified within the genus *Mycoplasma* (order Mycoplasmatales: Neimark et al.,

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2001). While formerly only one species, *Haemobartonella felis* was recognised in cats, subsequently it became evident that at least three genetically and to some extent morphologically different feline species exist: *Mycoplasma haemofelis* [formerly *Haemobartonella felis*], '*Candidatus* Mycoplasma haemominutum' (Foley and Pedersen, 2001) and '*Candidatus* Mycoplasma turicensis' (Willi et al., 2005).

The worldwide distribution of haemoplasmas is not completely known. In Hungary the first report of haemobartonellosis (Túry et al., 1977) as well as the clinical findings described later (Csikós and Fehér, 1992) were based on identification of the organisms (measuring 0.6 to 1 μm) in blood smears, suggesting the occurrence of *M. haemofelis* in this country. In contrast, '*Candidatus* M. haemominutum' has cells of approximately 0.3 μm in diameter (Foley and Pedersen, 2001). This latter haemotropic mycoplasma is also regarded as less pathogenic, usually causing no or only mild anaemia and not associated with mortality (Foley et al., 1998). However, here we describe a case of fatal anaemia in a cat from which '*Candidatus* M. haemominutum' was identified by real-time PCR and sequencing, at the same time providing the first molecular evidence for the occurrence of this species in Hungary.

Materials and methods

Case history and sampling

An 8-year-old, unvaccinated, castrated male cat (that had never been taken abroad) was presented at a small animal clinic in Hungary (Szekszárd, Tolna county). The animal had been showing anorexia, lethargy and weight loss for 3 weeks, but received no treatment during that time. Three days prior to its examination the cat ceased eating and drinking, started vomiting, and became apathic. EDTA and heparin-anticoagulated blood samples were collected and abdominal ultrasonography was performed.

Thin blood films were prepared and stained with Giemsa for haematological evaluation. Biochemical parameters were measured from heparinised blood using a VIS-2000 spectrophotometer (Opto-Elektronika, Hungary). Urinalysis was performed with test strips (Deka Phan Leuco, Pliva-Lachema Diagnostica, Brno, Czech Republic).

Nucleic acid extraction and real-time PCR for species identification

DNA was extracted with the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). First, 100 μl of EDTA blood collected on the first day of clinical presentation was diluted with the same amount of phosphate-buffered saline (PBS without $MgCl_2$ and $CaCl_2$, Invitrogen, Basel, Switzerland), then 300 μl of the lysis buffer containing guanidinium thio-

cyanate and Triton X-100 (Roche Diagnostics) were added. The homogenised sample was loaded into wells of the automated MagNA Pure LC Instrument (Roche Diagnostics). An extraction control of PBS was included to monitor for external contamination. The elution volume was 100 µl and the sample was stored at –20 °C until evaluation.

Species-specific quantitative real-time TaqMan PCR assays were performed for *M. haemofelis*, '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' using the ABI Prism 7700 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland) as described previously (Willi et al., 2005; Willi et al., 2006*a*). PCR reactions for the presence of feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV) and RT-PCR for feline coronaviruses (FCoV) were done as referred to elsewhere (Gut et al., 1999; Leutenegger et al., 1999; Tandon et al., 2005).

Amplification and sequencing of 16S rRNA gene

A conventional PCR was performed using species-specific primers for '*Candidatus* M. haemominutum' (forward: 5'-AAG TCG AAC GAA GAG GGT TTA CTC-3', reverse: 5'-TTW AAT ACG GTT TCA ACT AGT ACT TTC TCC-3') which amplify 1345 bp of the gene as described earlier (Willi et al., 2006*a*). The 25 µl reaction mixture contained 5 µl of 5× High Fidelity PCR buffer (Finnzymes: BioConcept, Allschwil, Switzerland), 0.5 µM of each primer, 0.2 mM dNTP mixture (Sigma-Aldrich, Buchs, Switzerland), 1 U Phusion High Fidelity DNA polymerase (Finnzymes), 15.25 µl nuclease-free water and 2.5 µl template. Amplification was performed using a TPersonal 48 Thermocycler (Biometra GmbH, Goettingen, Germany). The thermal profile consisted of an initial denaturation step at 98 °C for 3 min, then 35 cycles of 10 s at 98 °C, 30 s at 63 °C, and 1 min at 72 °C, with a final elongation at 72 °C for 10 min. An aliquot of the PCR product was visualised in a 1% agarose gel stained with ethidium bromide after electrophoresis; the rest was purified with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich). After evaluation of the DNA concentration using the GeneTools Software (SynGene, Cambridge, UK) cycle sequencing was performed with approximately 25 ng of DNA, 3.3 pmol of the two above-mentioned product-specific primers and an additional internal primer (5'-AGC AAT ACC ATG TGA ACG ATG AA-3'), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems). Cycling conditions were as follows: 1 min at 96 °C, then 25 cycles at 96 °C for 30 sec and 50 °C for 15 sec, finally incubation at 60 °C for 4 min. Products were purified using the SigmaSpin™ Post-Reaction Purification Columns (Sigma-Aldrich), and analysed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequence obtained was aligned to one consensus sequence by SeqScape (Version 1.1, Applied Biosystems) and then compared to reference sequences deposited in the GenBank®.

The new sequence was submitted to the GenBank (accession number: EU128752). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al., 2004).

Results

Clinical and laboratory findings

To the best of the owner's knowledge, there was no record of tick bite nor infestation of the cat with fleas, neither was the cat bitten by others. The animal had mild icterus, pale mucosal membranes and severe exsiccosis. The urine was brown; the urinalysis revealed haemoglobin- and bilirubinuria. Ultrasonographic findings included a diffusely enlarged liver, distended hepatic veins, and very little peritoneal fluid which could not be aspirated and thus analysed. The signs of icterus aggravated in the course of a few days, in the same way as anaemia as indicated by progressively decreasing haematocrit values (Table 1). In thin blood films of the cat small microorganisms (< 0.4 μm) were seen in association with red blood cells (Fig. 1). The white blood cell count was elevated, with neutrophilia (21.9 G/l) and lymphopenia (0.73 G/l). Apart from these changes the total protein level and all liver and kidney parameters were within the reference range (Table 1). The cat received daily palliative treatment together with antibiotics (amoxicillin at 10 mg/kg body weight subcutaneously every 24 hours and doxycycline at 5 mg/kg body weight orally every 12 hours), but eventually died within one week after the onset of rapidly aggravating clinical signs (before the causative agent was molecularly identified).

Table 1

Haematological and blood chemistry values of the cat with haemolytic anaemia

Parameters	Values of the cat	Reference range
White blood cells	24.3 G/l	6–19 G/l
Haematocrit	31 > 29 > 25 > 22%*	30–45%
Glucose	5.8 mmol/l	3.8–6 mmol/l
Creatinine	112 μmol/l	40–140 μmol/l
Total protein	68 g/l	60–80 g/l
AP	268 IU/l	20–280 IU/l
ALT	72.1 IU/l	< 80 IU/l
GGT	2.6 IU/l	1–10 IU/l

*At days 1, 2, 3 and 4 of clinical presentation, respectively. Abbreviations: AP – alkaline phosphatase, ALT – alanine aminotransferase, GGT – gamma-glutamyl transpeptidase, IU – international unit

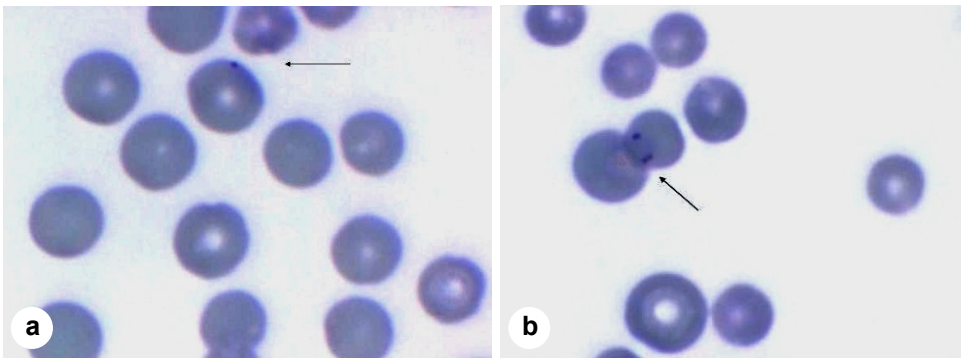

Fig. 1. '*Candidatus* M. haemominutum' in blood smears of the cat showing (a) epierythrocytic position and (b) dividing form (with anisocytosis)

PCR and sequencing

The blood sample of the cat was positive in the quantitative real-time TaqMan PCR assay specific for '*Candidatus* M. haemominutum'. The bacterial load amounted to 4×10^5 copies/ml of blood. The cat was negative for other feline haemotropic mycoplasmas tested. Sequencing of the nearly complete 16S rRNA gene (1354 bp) directly from the PCR product showed 100% homology with two '*Candidatus* M. haemominutum' isolates from Switzerland and one from the UK (Fig. 2; accession numbers in the GenBank DQ157141, DQ157147 and AF 271154, respectively). Real-time PCR results for FeLV, FIV and RT-PCR for FCoV were negative.

Discussion

This is the first report of a molecularly verified '*Candidatus* M. haemominutum' infection in a cat from Hungary. Although feline haemoplasmas have been documented in several western (Tasker et al., 2003*a*; Willi et al., 2005; Willi et al., 2006*b*; Just and Pfister, 2007; Willi et al., 2007) and southern European countries (Criado-Fornelio et al., 2003; Kurtdede and Ural, 2004), there is no molecularly confirmed information on the occurrence of these organisms in Central-Eastern Europe.

Regarding the cat in the present study, it was not revealed how it may have contracted the causative agent, as none of the potential routes of infection (Willi et al., 2007) could be confirmed. Haematological abnormalities caused by '*Candidatus* M. haemominutum' are usually minor (Foley and Pedersen, 2001), unlike those reported here. Among the other pathological changes, liver enlargement may as well have resulted from red blood cell damage, i.e. erythrophagocytosis and consequent reticuloendothelial hyperplasia. Since hepatocytes are sensitive to hypoxia (Lemasters et al., 1983), the rapid development of

haemolytic anaemia may explain the absence of increased plasma level of liver enzymes, although this has been demonstrated as soon as 6 days post-infection during haemobartonellosis (Kurtdede and Ural, 2004).

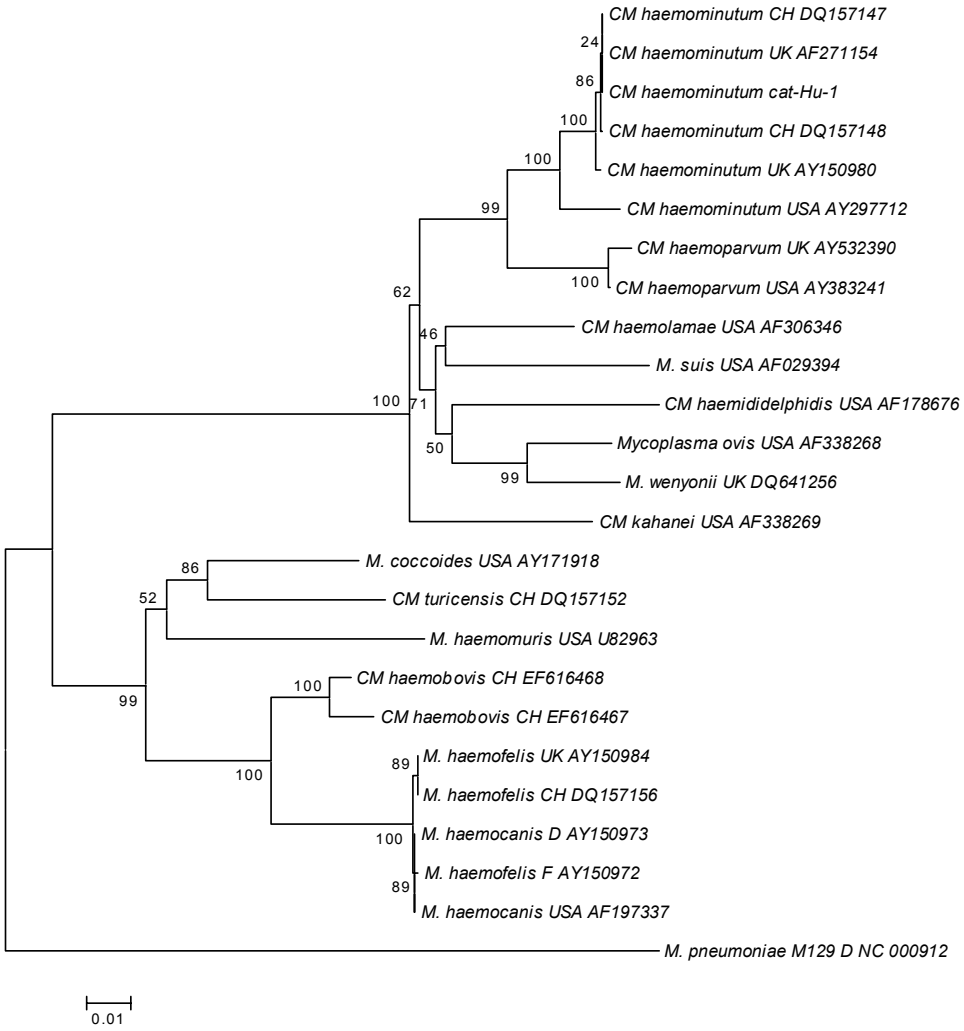

Fig. 2. Bootstrap phylogenetic tree demonstrating the relationship of the Hungarian isolate of '*Candidatus* M. haemominutum' (cat-Hu-1) to other haemoplasma species based on 16S rRNA gene sequences, using a Neighbor-Joining method. The numbers at the nodes were generated from 1000 Bootstrap resamplings. The bar represents the mean number of differences per 100 sites

Despite palliative treatment and application of antibiotics known to reduce bacterial loads during '*Candidatus* M. haemominutum' infection (Willi et al., 2006*a*) the cat died within 4 days after its presentation at the clinic. '*Candidatus*

M. haemominutum' is usually regarded as mildly pathogenic (Foley et al., 1998). Co-infection with the more pathogenic *M. haemofelis* and '*Candidatus* M. turicensis' was not detected by PCR, although the former should not be excluded, since *M. haemofelis* loads were shown to vary extremely over time (Tasker et al., 2006), and were found to be highly positive and negative within only a few days in a cat also co-infected with '*Candidatus* M. haemominutum' (Willi et al., 2006*a*). The development of distinct anaemia in '*Candidatus* M. haemominutum' infected cats has also been described with concomitant feline retroviral (FeLV, FIV) infections (George et al., 2002) or other deprived conditions (De Lorimier and Messick, 2004). Here PCR results were negative for FeLV, FIV and FCoV which may be immunosuppressive (Willis, 2000; De Groot-Mijnes et al., 2005) and thus may aggravate the manifestation of diseases. The assays applied here to detect FeLV or FIV have been shown to be highly sensitive, and they also detect proviraemia, therefore an infection of the cat with these viruses is unlikely.

Although the effects of other unrevealed aggravating factors cannot be completely ruled out, the present findings may attest the existence of more pathogenic strains of '*Candidatus* M. haemominutum'. This is further supported by the results of those studies which, concerning this species, gave an account of its higher incidence in clinically ill animals than in healthy ones (Tasker et al., 2003*a*; Willi et al., 2006*a*), and showed the lack of other recognisable causes of anaemia in several naturally infected cats (Reynolds and Lappin, 2007).

The haemoplasma load in the blood of the anaemic cat was higher when compared to the median copy number (7.8×10^4 copies/ml) of '*Candidatus* M. haemominutum' in another report (Willi et al., 2006*a*), but in that survey no correlation could be established between haematocrit values and bacterial loads. Although '*Candidatus* M. haemominutum' was successfully demonstrated in blood smears of the Hungarian cat, this diagnostic method is not always reliable for the evaluation of the presence of haemotropic mycoplasmas, since (as calculated from the above values) in most cases the proportion of infected red blood cells is low, and these microorganisms are small. Accordingly, in a previous study (Criado-Fornelio et al., 2003) microscopic observation could confirm relevant epierythrocytic bacteria in only two of nine PCR-positive cat samples. Therefore, molecular biological techniques are more suitable for the diagnosis of feline haemoplasma infection.

The 16S rRNA gene of the Hungarian '*Candidatus* M. haemominutum' isolate showed 100% identity to isolates from Switzerland and the UK. This is not surprising in the light of recent reports demonstrating that feline and canine haemotropic mycoplasma isolates from various parts of the world, including Europe, Australia, South Africa and the USA, showed only little variations within their 16S rRNA gene sequence (Tasker et al., 2003*b*; Willi et al., 2006*b*).

Concerning the situation in Europe, there are only few molecular epidemiological studies (and all are from western countries) which compare the prevalence of feline haemoplasmas in similar populations. From these '*Candidatus* M. haemominutum' seems to be the most prevalent species, with incidence rates of 8.3% or 20.3% in the United Kingdom and 7.0% or 8.7% in Switzerland, in healthy or ill cats, respectively (Tasker et al., 2003*a*; Willi et al., 2006*a*). In another report from Southern Europe (Criado-Fornelio et al., 2003) only symptomatic cats – suspected to have infection with haemotropic *Mycoplasma* spp. – were evaluated, and *M. haemofelis* was identified in most (20%) of them, with lower (10%) prevalence of '*Candidatus* M. haemominutum'. However, such information is completely lacking from Central-Eastern Europe. Therefore, further studies are encouraged to evaluate geographical differences in the occurrence of these pathogens in other countries of the continent.

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