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Haemotropic *Mycoplasma* species in pet cats in Latvia: a study, phylogenetic analysis and clinical case report

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Abstract

Objectives The aim of this study was to evaluate whether haemotropic *Mycoplasma* species are detected in pet cats in Latvia, to perform a phylogenetic analysis of the detected pathogens and to report a clinical case of feline infectious anaemia.

Methods Peripheral blood samples (n = 125) from pet cats were submitted; 99 samples were adequate to test for the presence of *Mycoplasma* species DNA by nested PCR. A clinical case was added in the later stages of the study. Positive isolates were subjected to phylogenetic analysis.

Results The prevalence of ‘*Candidatus Mycoplasma haemominutum*’ was 15% (n = 15/99), that of *Mycoplasma haemofelis* was 5% (5/99) and that of ‘*Candidatus Mycoplasma turicensis*’ was 2% (n = 2/99). Cases of coinfection included ‘*Candidatus M haemominutum*’ + *M haemofelis* (4%; n = 4/99) and ‘*Candidatus M haemominutum*’ + ‘*Candidatus M turicensis*’ (1%; n = 1/99). This is the first published report of *M haemofelis* infection in the Baltic states. Two different ‘*Candidatus M turicensis*’ isolates were discovered after phylogenetic analysis.

Conclusions and relevance This report is the first of an autochthonous feline infectious anaemia case in the Baltic region. The prevalence of *Mycoplasma* species was similar to that in other northern European countries. Phylogenetic analysis revealed variability of the isolates; one of the ‘*Candidatus M turicensis*’ genotypes was detected for the first time in Europe.

Keywords: Nested PCR; phylogenetic analysis; infectious anaemia; geographical distribution

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Introduction

Worldwide, the prevalence of vector-borne diseases depends on the prevalence of vectors and pathogens that themselves are affected by anthropogenic, socioeconomic and climate factors known to change over time.^{1–5}

Canine babesiosis and granulocytic anaplasmosis are considered endemic in the Baltic countries.^{6–9} To our knowledge, no studies have been performed to evaluate the presence of vector-borne pathogens in cats in the Baltics.

Mycoplasma haemofelis (MH) is clinically the most important, while ‘*Candidatus Mycoplasma haemominutum*’ (CMH) and ‘*Candidatus Mycoplasma turicensis*’ (CMT) are more frequently isolated from asymptomatic

cats but can cause clinical disease in immunocompromised cats.^{10–13} The routes of infection are not clear; vectors (ticks and fleas), as well as transmission via blood, have been suspected.¹²

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In this study, we evaluated the prevalence of haemotropic *Mycoplasma* species in pet cats in Latvia and performed a phylogenetic analysis of the sequenced pathogens, as it is known that a greater variety in endemic pathogen species can lead to increased variability and severity in clinical disease, both in animals and people.²

Materials and methods

Blood sample collection

From May to September 2016, with the owners' written consent, EDTA-stabilised venous blood samples (1.0 ml) were collected from the jugular or medial saphenous veins of pet cats admitted to veterinary clinics in Latvia. Convenience sampling resulted in the collection of 125 blood samples; 99 were included in the study. For this study cats were not separated into healthy or sick cats; shelter or free-roaming cats were excluded. For each sampled cat, veterinarians were asked to note the age, sex, breed of the cat, haematology parameters (erythrocyte and leukocyte count, haemoglobin concentration and haematocrit), access to the outdoors (yes/no) and ectoparasite control (yes/no). Blood samples were shipped biweekly to the research facility on cold packs and stored at +8°C until testing. DNA extraction was carried out 2–10 days after the samples were obtained.

In September 2017, a clinic contacted us regarding a cat that had been diagnosed with haemotropic *Mycoplasma* species infection. We opted to collect medical history, treatment plan and disease history, as well as an EDTA-stabilised venous blood sample from this cat to report it as a clinical case.

DNA isolation and PCR

DNA isolation from blood was performed according to the method of Tiškina et al.⁹ Primary and nested PCR were performed in a final volume of 25 µl reaction

mixtures containing 1 × Taq Buffer with (NH₄)₂SO₄, 2.5 mM MgCl₂, 200 µM dNTP mixture, 0.2 µM of each primer and 0.8 U Taq DNA polymerase (recombinant) (Thermo Fisher Scientific) and 2 µl DNA template (2 µl of products from the primary PCR were used as a template for the nested PCRs). The negative control was a PCR mix with DNA substituted by water. The positive controls for PCR were genomic DNA samples of MH and CMH. The PCR conditions were as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of amplification (denaturation at 95°C for 20 s, primer annealing at 50°C and at 55°C for 30 s for primary and nested PCR, respectively, and elongation at 72°C for 1 min and 40 s for primary and nested PCR, respectively) and a final elongation step at 72°C for 5 mins. The primers used in this study are listed in Table 1.

PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's standard protocol and analysed on an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer). The same primers listed in Table 1 were used for sequencing. Sequences were aligned with those deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>).¹⁹ The sequences generated in this study were submitted to GenBank under the following accession numbers: MG456679–MG456700.

Mycoplasma species sequences that were deposited in GenBank until February 2019 were included in the neighbour joining phylogenetic analysis (MEGA7 software version 7.0.20).^{20–22} Evolutionary distances were calculated according to the Kimura two-parameter model.²³ Included codon positions were first + second + third + non-coding; gaps and missing data were eliminated from the data set. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The robustness of the generated phylogenetic

Table 1 Primers used in this study^{14–18}

Pathogen species and reference	Target gene	PCR	Primer name	Primer sequence (5'–3')	Product size (bp)	Primer annealing temperature (°C)
Universal for <i>Mycoplasma</i> species ^{14,15}	16S rRNA	Primary	8F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	1457	50
<i>Mycoplasma haemofelis</i> ^{15,16}	16S rRNA	Nested	<i>H felis</i> F1 <i>H felis</i> R3	GACTTTGGTTTCGGCCAAGG CGAAGTACTATCATAATTATCCCTC	393	55
' <i>Candidatus</i> <i>Mycoplasma haemominutum</i> ' ^{15,17}	16S rRNA	Nested	1183F 1290R	GCATAATGTGTCGCAATC GTTTCAACTAGTACTTTCTCCC	130	55
' <i>Candidatus</i> <i>Mycoplasma turicensis</i> ' ^{15,18}	16S rRNA	Nested	Mt1Fw Mt2Rv	GTATCCTCCATCAGACAGAA CGCTCCATATTTAATTCCAA	488	55

Spectrophotometry was used to measure the concentration and purity of the DNA (ND-1000 UV–VIS Spectrophotometer; NanoDrop Technologies)⁹

bp = base pairs

tree was evaluated by bootstrap tests.²⁴ Association of the taxa clustering in the bootstrap test (500 replicates) is represented by the percentage next to the branches (values >50 are displayed). The scale bar represents the units of the number of base substitutions per site (Figures 1–3). The 16S rRNA gene sequence variant classification for MH (MH-1 to MH-10), CMT (CMT-1 to CMT-16) and CMH (CMH-1 to CMH-8) was generated after multiple sequence alignment preparation and the exclusion of sites with missing data and gaps.

Statistical analysis

Fisher's exact test (GraphPad Prism software, version 5) was used to calculate associations among age, sex, breed, red blood cell count, haematocrit, haemoglobin content, leukocyte count, platelet count and positivity against *Mycoplasma* species. A *P* value ≤0.05 was considered to be statistically significant.

Results

Overall sample analysis

Twenty-six samples had to be excluded, thus 99 feline blood samples were included in the study, from the capital city of Riga (*n* = 62) and from its vicinity (*n* = 11); the remaining samples were from cities of Jelgava (*n* = 1), Jurmala (*n* = 1), Liepaja (*n* = 1), Valmiera (*n* = 1) and Koknese (*n* = 1). Nineteen samples were missing addresses on the information sheet. The mean age of the cats was 8.05 years (range 3 months to 21 years); there were 39 females and 42 males; the sex was unknown for 18 cats. There were 65 domestic shorthairs and 25 were of different breeds; breed information was missing for nine cats. Detailed information on the haematology parameters is presented in Table 2.

Prevalence of infectious agents and phylogenetic analysis of the isolates

Pathogen DNA was isolated from 17 cats (Table 3). The overall prevalence of CMH was 15% (*n* = 15/99), the prevalence of MH was 5% (*n* = 5/99) and the prevalence of CMT was 2% (*n* = 2/99). Among these cats were several cases of coinfection: CMH + MH (4%; *n* = 4/99) and CMH + CMT (1%; *n* = 1/99). Being a male cat was statistically associated with positivity for CMH (*P* = 0.0004); there were no statistically significant associations between age and breed.

The mean age of the *Mycoplasma*-positive cats was 8.8 years, 12 of the cats were male and sex was not mentioned for five cats. Seven cats were domestic shorthairs, four were purebred (British Shorthair, Scottish Fold, Oriental and Burmilla) and breed was not noted for seven cats. Ectoparasite control (collar) use was reported in one cat.

***Mycoplasma haemofelis* infection case** Upon admission to the clinic in Liepaja city, a 2-year-old cat was anaemic and febrile, and microscopic evaluation of the peripheral blood revealed multiple small epicellular organisms. Infection with MH was confirmed by PCR (LABOKLIN). The status of the retroviral infections was not tested. Treatment with doxycycline (Doxylan 100mg PO [10mg/kg]; Lannacher Heilmittel) and a mix of vitamins and macroelements (B12, B6 and C, haemic iron, copper [Hemovet, VetPlanet] 1 tablet q24h PO) was continued for 3 weeks, and each week a complete blood count was performed. The anaemia fully resolved after 6 weeks (haematocrit 33.7%, haemoglobin 9.7g/dl and a red blood cell count $7.03 \times 10^{12}/l$) (Table 4).

Three months after the initial presentation, the cat had no clinical signs of disease, and the blood results were within the reference intervals. Four months after the clinical disease episode, we performed nested PCR for *Mycoplasma* species using our methodology, and the cat was positive for CMH but negative for MH and CMT.

***Mycoplasma haemofelis* phylogenetic analysis** Phylogenetic analysis of 16S rRNA gene fragment sequences from GenBank showed that MH sequences belonged to 10 different genotypes (marked MH-1 to MH-10) based on the variation of nucleotides in 11 positions (Table 5). All five MH sequences from this study belonged to the MH-1 genotype (Figure 1), which has a widespread occurrence in pet cats and wild animals in Europe, Asia, South and North America and Australia.

'Candidatus *Mycoplasma turicensis*' phylogenetic analysis After analysis of the Latvian CMT 16S rRNA gene sequences and those available in GenBank, we identified 16 variants (Table 6). Our sequences belonged to the variants CMT-2 and CMT-13. CMT-2 has been isolated from pet cats and wild animals in Europe (Switzerland, France, Italy and the UK), Asia (Taiwan), South America (Brazil) and Australia. CMT-13 is a novel genetic variant in Europe, and, until now, it has been isolated only in South Africa, South America (Brazil) and Australia (Figure 2).

'Candidatus *Mycoplasma haemominutum*' phylogenetic analysis Analysis of CMH 16S rRNA gene sequences of from our study and those available in GenBank revealed eight variants of the gene (Table 7). Of the 15 sequences obtained in this study, nine (including that sequenced from the clinical case described earlier) belonged to CMH-1, one was CMH-4 and five contained a mix of CMH-1 and CMH-4 variants (Figure 3). CMH-1 has been previously detected in several European countries (UK, France, Switzerland, Hungary, Italy and Spain), as well as Africa, Asia, South and North America and Australia (Figure 3). CMH-4 has been less widely detected in

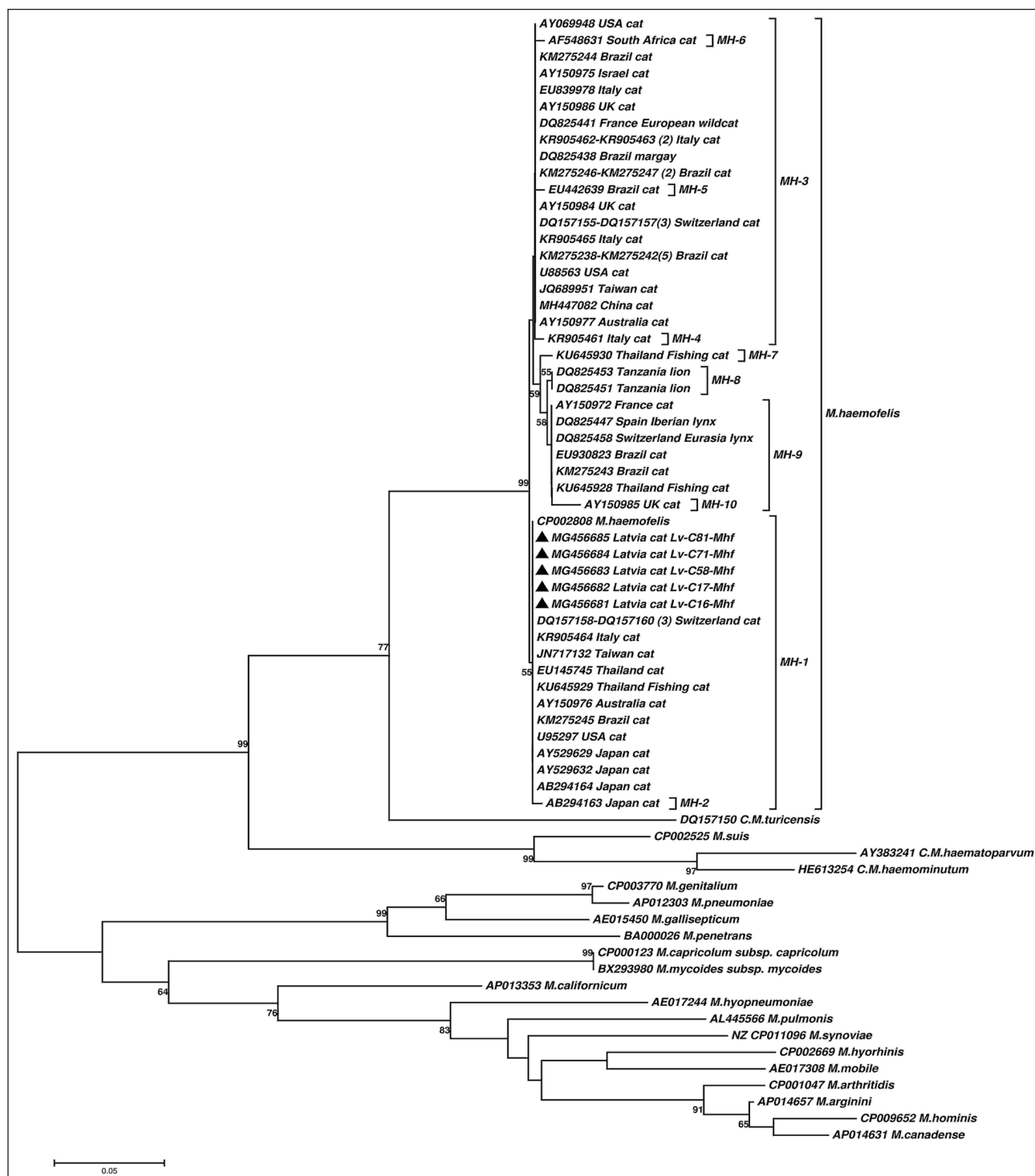


Figure 1 Phylogenetic analysis of *Mycoplasma haemofelis* 16S rRNA gene sequences

Europe (Italy and Switzerland), Asia (Thailand) and North America (USA).

Discussion

In Latvian cats, we identified three of the four known haemotropic *Mycoplasma* species. Compared with a

Danish study, our results did not show statistical significance between positivity for *Mycoplasma* species and the breed or age of cats; however, being a male cat was significantly associated with positivity for CMH.¹¹ As reported by others, CMH was isolated with a greater frequency than MH and CMT, and coinfections were

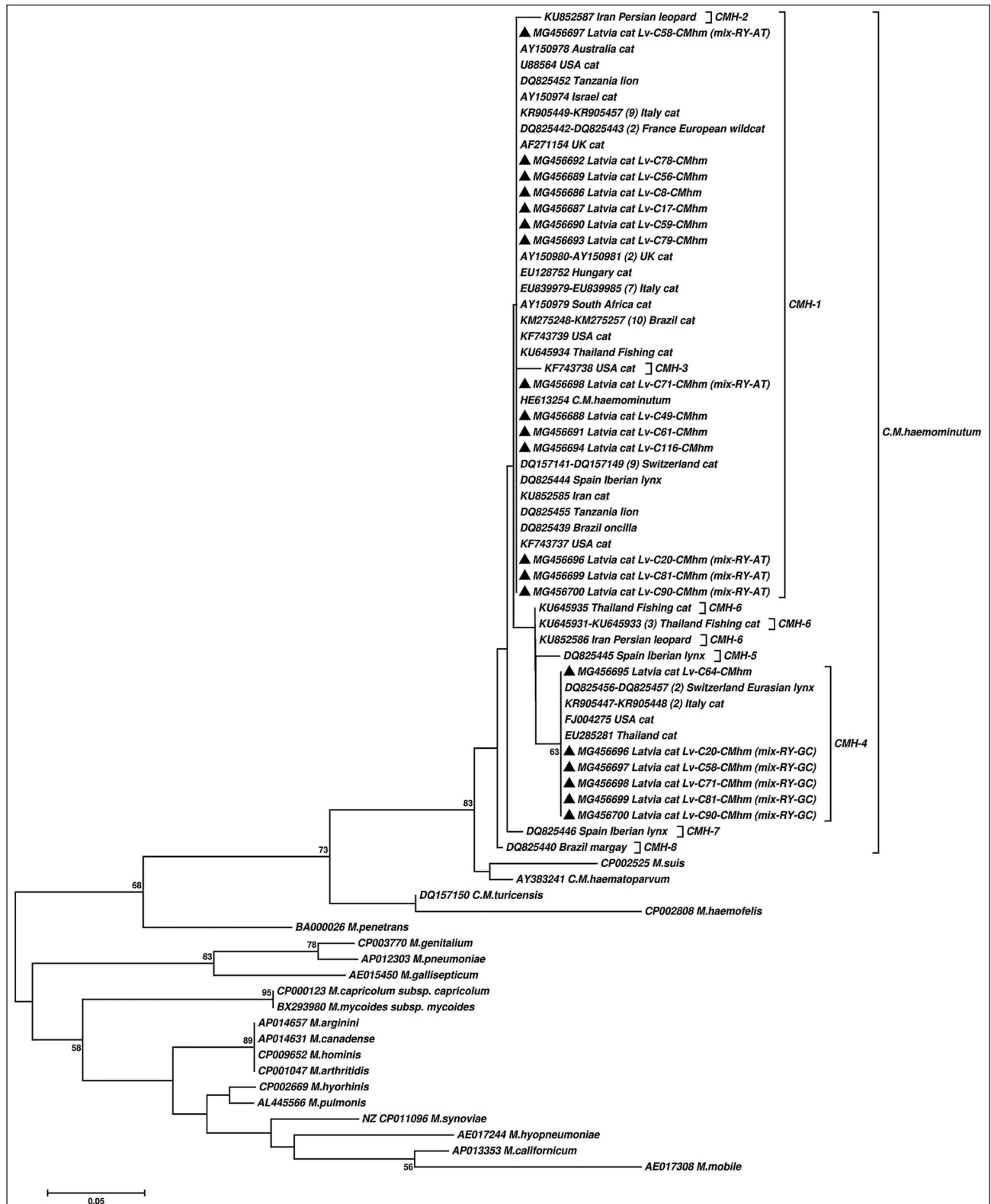


Figure 2 Phylogenetic analysis of '*Candidatus Mycoplasma turicensis*' 16S rRNA gene sequences

common.^{10,12,25} In laboratory settings, coinfections have been shown to result in longer clinical disease with more severe anaemia, but no such differences were noted in

cats from Brazil.^{1,26} Our results concur with suggestions stated in other studies that PCR positivity and/or chronic carrier status are not necessarily linked to anaemia.¹³

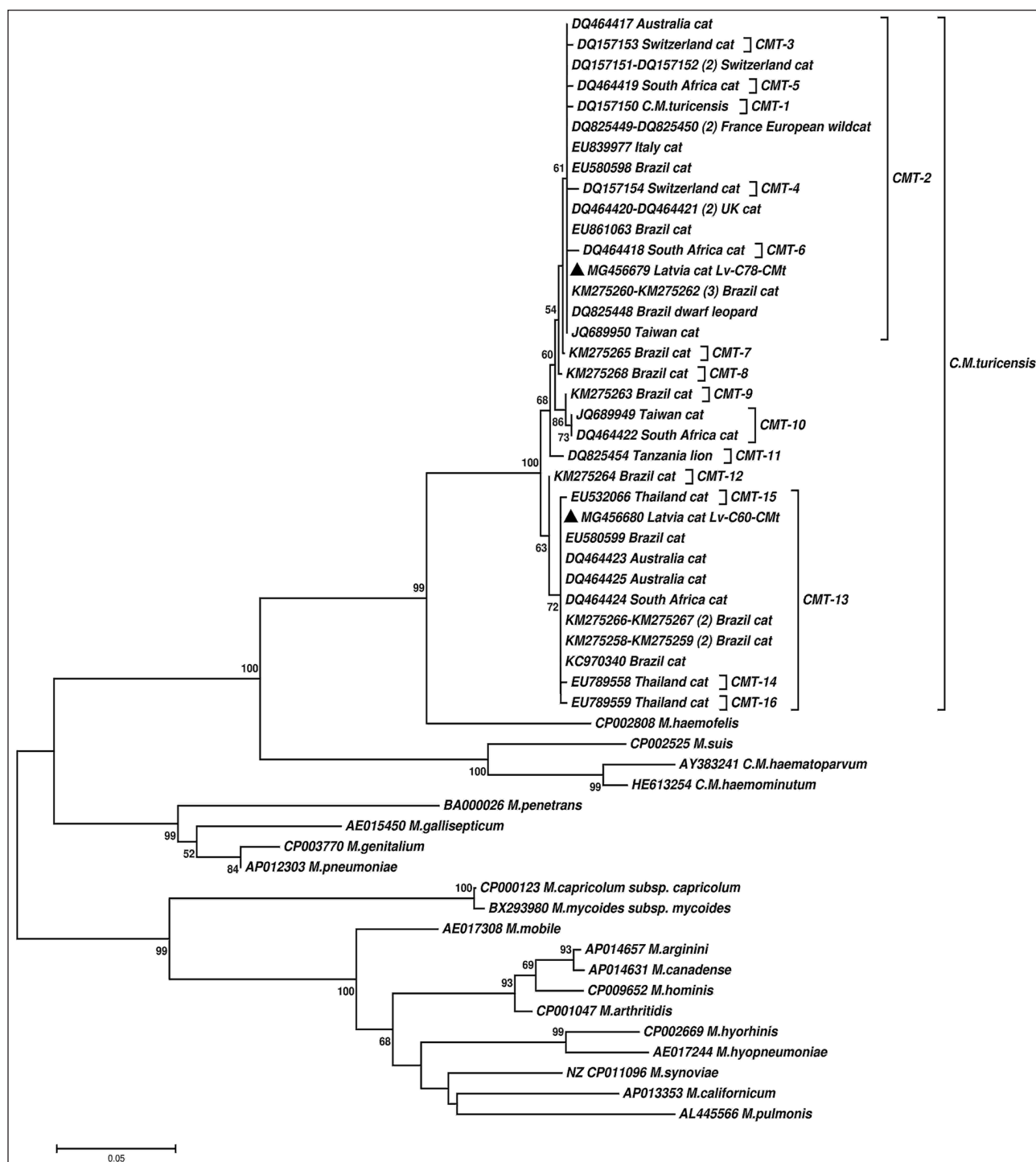


Figure 3 Phylogenetic analysis of '*Candidatus Mycoplasma haemominutum*' 16S rRNA gene sequences

Infectious causes should be excluded in anaemic cats in Latvia. A clinically sick cat presented with typical clinical and laboratory signs. Although it was positive in our case, microscopic observation of the pathogen is not always possible and should not be used as a tool for the exclusion of disease.^{11,12} Treatment with doxycycline was effective, although prolonged treatment of up to

8 weeks might be needed to eliminate infection.¹² Results of the repeated PCR were unexpected, suggesting a newly acquired infection with CMH and possible resolution of the MH infection, but this information must be viewed in the light of the discordant results obtained in different laboratories, likely with different methodologies and with a time lapse of 4 months. It is

Table 2 Haematology parameters of cats tested for the presence of haemotropic *Mycoplasma* species

Parameter	PCR-negative cats (n = 82)	PCR-positive cats (n = 17)	P value
Mean RBC count ($\times 10^{12}/l$)	8.2	7.55	1.2335
Mean haematocrit (%)	35.59	33.37	0.9914
Mean haemoglobin (g/dl)	13.39	12.46	1.2286
Mean leukocyte count ($\times 10^9/l$)	11.27	15.19	1.6174

RBC = red blood cell

Table 3 List of pathogens isolated from feline blood in this study

DNA number of sample	Pathogen species (sequence variant classification number)	GenBank accession number (isolate name)
Lv-C16	<i>M haemofelis</i> (MH-1)	MG456681 (Lv-C16-Mhf)
Lv-C8	<i>C M haemominutum</i> (CMH-1)	MG456686 (Lv-C8-CMhm)
Lv-C20	<i>C M haemominutum</i> (mix) (CMH-1 + CMH-4)	MG456696 (Lv-C20-CMhm)
Lv-C49	<i>C M haemominutum</i> (CMH-1)	MG456688 (Lv-C49-CMhm)
Lv-C56	<i>C M haemominutum</i> (CMH-1)	MG456689 (Lv-C56-CMhm)
Lv-C59	<i>C M haemominutum</i> (CMH-1)	MG456690 (Lv-C59-CMhm)
Lv-C61	<i>C M haemominutum</i> (CMH-1)	MG456691 (LV-C61-CMhm)
Lv-C64	<i>C M haemominutum</i> (CMH-4)	MG456695 (Lv-C64-CMhm)
Lv-C79	<i>C M haemominutum</i> (CMH-1)	MG456693 (LV-C79-CMhm)
Lv-C90	<i>C M haemominutum</i> (mix) (CMH-1 + CMH-4)	MG456700 (Lv-C90-CMhm)
Lv-C116	<i>C M haemominutum</i> (CMH-1)*	MG456694 (Lv-C116-CMhm)
Lv-C17	<i>M haemofelis</i> (MH-1) + <i>C M haemominutum</i> (CMH-1)	MG456682 (Lv-C17-Mhf); MG456687 (Lv-C17-CMhm)
Lv-C58	<i>M haemofelis</i> (MH-1) + <i>C M haemominutum</i> (CMH-1 + CMH-4)	MG456683 (Lv-C58-Mhf); MG456697 (Lv-C58-CMhm)
Lv-C71	<i>M haemofelis</i> (MH-1) + <i>C M haemominutum</i> (CMH-1 + CMH-4)	MG456684 (Lv-C71-Mhf); MG456698 (Lv-C71-CMhm)
Lv-C81	<i>M haemofelis</i> (MH-1) + <i>C M haemominutum</i> (CMH-1 + CMH-4)	MG456685 (Lv-C81-Mhf); MG456699 (Lv-C81-CMhm)
Lv-C78	<i>C M turicensis</i> (CMT-2) + <i>C M haemominutum</i> (CMH-1)	MG456679 (Lv-C78-CMt); MG456692 (Lv-C78-CMhm)
Lv-C60	<i>C M turicensis</i> (CMT-13)	MG456680 (Lv-C60-CMt)

*Isolate from the clinically sick cat

M haemofelis = *Mycoplasma haemofelis*; *C M haemominutum* = '*Candidatus Mycoplasma haemominutum*'; *C M turicensis* = '*Candidatus Mycoplasma turicensis*'; *C M haemominutum* (mix) = sample contain two sequence variants of *C M haemominutum*

Table 4 Risk of exposure and haematology parameters in the clinically sick cat

Haematology parameters	Date of haematology testing			Reference interval
	29 July 2017	4 August 2017	4 September 2017	
RBC count ($\times 10^{12}/l$)	2.35	4.08	7.03	5.5–8.00
HCT (%)	14.2	29.8	33.7	0.33–0.55
Haemoglobin (g/dl)	4	6.8	9.7	10.00–18.00
WBC count ($\times 10^9/l$)	13.4	8.86	11.12	6.00–18.00
TBC count ($\times 10^9/l$)	191	159	163	200–500
Other parameters				
Ectoparasite control	No			
Outdoor access	Yes			
Recent cat fights	Yes			
Recently removed ticks	Yes			

RBC = red blood cell; HCT = haematocrit; WBC = white blood cell; TBC = platelet

Table 5 Polymorphisms in the 16S rRNA gene fragment of *Mycoplasma haemofelis*

Sequence variant	<i>M haemofelis</i> polymorphic nucleotide positions 5'-3'*										
	138	149	177	196	204	213	214	216	301	331	334
MH-1	G	C	T	C	C	A	T	G	A	G	A
MH-2	–	–	–	–	–	–	–	–	G	–	–
MH-3	–	–	C	–	–	–	–	–	–	–	–
MH-4	–	–	C	A	–	–	–	–	–	–	–
MH-5	–	–	C	–	T	–	–	–	–	–	–
MH-6	–	–	C	–	–	–	–	–	–	A	–
MH-7	–	T	C	–	–	–	–	–	–	–	G
MH-8	A	T	–	–	–	–	–	–	–	–	–
MH-9	A	T	C	–	–	–	–	–	–	–	–
MH-10	A	T	C	–	–	G	A	T	–	–	–

*Polymorphic nucleotide positions are assigned according nucleotide position in complete sequence of the 16S rRNA gene of *M haemofelis* (GenBank: CP002808)

Table 6 Polymorphism analysis of the 16S rRNA gene fragment of '*Candidatus Mycoplasma turicensis*'

Sequence variant	'C M turicensis' polymorphic nucleotide positions 5'-3'*																					
	152	153	188	206	246	275	299	310	317	319	341	344	348	349	351	353	387	418	419	494	501	572
CMT-1	G	A	A	G	T	A	T	G	C	T	C	T	G	G	C	G	T	A	T	C	A	G
CMT-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–
CMT-3	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–
CMT-4	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	–	–	G	C	–	–	–
CMT-5	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	C	–	–	–
CMT-6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	C	T	–	–
CMT-7	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	C	–	–	–
CMT-8	A	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	C	–	–	–
CMT-9	A	–	–	A	–	–	–	–	–	–	T	–	–	–	–	–	C	–	C	–	–	–
CMT-10	A	–	–	A	C	–	–	–	–	–	T	–	–	–	–	–	C	–	C	–	–	–
CMT-11	A	–	G	–	–	–	–	–	–	–	–	–	–	–	–	A	C	–	C	–	–	A
CMT-12	A	G	–	A	–	–	–	–	T	–	–	–	–	–	–	–	C	–	C	–	–	A
CMT-13	A	G	–	A	–	–	–	A	T	–	–	C	–	–	–	–	C	–	C	–	–	A
CMT-14	A	G	–	A	–	–	–	A	T	–	–	C	–	C	–	–	C	–	C	–	–	A
CMT-15	A	G	–	A	–	–	–	A	T	–	–	C	–	–	–	–	C	–	C	–	G	A
CMT-16	A	G	–	A	–	–	–	A	T	–	–	C	A	–	–	–	C	–	C	–	–	A

*Polymorphic nucleotide positions are assigned according nucleotide position in the partial sequence of the 16S rRNA gene of '*C M turicensis*' (GenBank: DQ157150)

Table 7 Polymorphism in the 16S rRNA gene fragment of '*Candidatus Mycoplasma haemominutum*'

Sequence variant	'C M haemominutum' polymorphic nucleotide positions 5'-3'*					
	1244	1245	1247	1257	1258	1271
CMH-1	T	A	G	A	C	T
CMH-2	–	–	–	C	–	–
CMH-3	–	–	–	–	T	–
CMH-4	–	G	–	–	–	C
CMH-5	A	G	–	–	–	–
CMH-6	–	G	–	–	–	–
CMH-7	–	–	A	–	–	–
CMH-8	–	–	T	–	–	–

*Polymorphic nucleotide positions are assigned according nucleotide position in the complete sequence of the 16S rRNA gene of '*C M haemominutum*' (GenBank: HE613254).

possible that CMH infection was present but undetected at the time of the clinical disease episode. To prove complete elimination of an infection, several repeated PCRs should be performed.^{12,13}

The CMH primers used in this study amplified a relatively short fragment of the 16S rRNA gene (130 base pairs [bp]) located at the 3' end, which restricted our phylogenetic analysis. Our CMH sequences belonged to the widely distributed CMH-1 and CMH-4 gene variants. The length of the 16S rRNA gene fragment for CMT was significantly longer (488bp), allowing us to perform more detailed phylogenetic analysis. The CMT-13 genotype that was detected in our study has only previously been reported in Australia, Brazil and South Africa, and this study is the first to report it in European countries. The MH 16S rRNA genotype variant MH-1 observed in this study is widely present in Europe.

The limitations of the study included the fact that the convenience sampling resulted in a sample pool mostly from the central part of the country. Convenience sampling has been used in several other studies.^{12,13} MH was detected at the commercial laboratory, and we were not able to perform phylogenetic analysis of the sequence at the time of the clinical disease.

Conclusions

Mycoplasma species were detected in 17% of cats tested in this study. This study is the first publication on clinical feline infectious anaemia caused by MH in the Baltic states. Phylogenetic analysis adds significantly to the information about the distribution of the pathogens in cats.

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Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval This work involved the use of non-experimental animals only (including owned or unowned animals and data from prospective or retrospective studies). Established internationally recognised high standards ('best practice') of individual veterinary clinical patient care were followed. Ethical approval from a committee was therefore not specifically required for publication in *JFMS Open Reports*. Although not

required, where ethical approval was still obtained, it is stated in the manuscript.

Informed consent Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work (either experimental or non-experimental animals) for the procedure(s) undertaken (either prospective or retrospective studies). For any animals or humans individually identifiable within this publication, informed consent (either verbal or written) for their use in the publication was obtained from the people involved.

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