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Molecular detection of *Anaplasma phagocytophilum* in hair and spleen of cats revealed a possible underestimation of feline vector-borne pathogens

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Balboni A, U.L. (2021). Molecular detection of *Anaplasma phagocytophilum* in hair and spleen of cats revealed a possible underestimation of feline vector-borne pathogens. RESEARCH IN VETERINARY SCIENCE, 137, 144-149 [10.1016/j.rvsc.2021.05.003].

Availability:

This version is available at: <https://hdl.handle.net/11585/857022> since: 2022-02-12

Published:

DOI: <http://doi.org/10.1016/j.rvsc.2021.05.003>

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(Article begins on next page)

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6 This is the final peer-reviewed accepted manuscript of:

7 **Balboni A, Urbani L, Morini M, Dondi F, Battilani M. Molecular detection of**
8 **Anaplasma phagocytophilum in hair and spleen of cats revealed a possible**
9 **underestimation of feline vector-borne pathogens. Research in Veterinary**
10 **Science. 2021, 137: 144-149**

11 The final published version is available online at:
12 <https://doi.org/10.1016/j.rvsc.2021.05.003>

13

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19 **Molecular detection of *Anaplasma phagocytophilum* in hair and spleen of cats revealed a**
20 **possible underestimation of feline vector-borne pathogens**

21

22 Andrea Balboni*, Lorenza Urbani, Maria Morini, Francesco Dondi, Mara Battilani

23

24 Department of Veterinary Medical Sciences, Alma Mater Studiorum-University of Bologna, Via
25 Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy.

26

27 e-mail addresses:

28 Andrea Balboni: a.balboni@unibo.it; Lorenza Urbani: lorenza.urbani2@unibo.it; Maria Morini:

29 maria.morini@unibo.it; Francesco Dondi: f.dondi@unibo.it; Mara Battilani:

30 mara.battilani@unibo.it

31

32 * Corresponding author:

33 Andrea Balboni

34 Department of Veterinary Medical Sciences, Alma Mater Studiorum-University of Bologna, Via
35 Tolara di Sopra 50, 40064, Ozzano dell'Emilia (BO), Italy.

36 e-mail: a.balboni@unibo.it

37 ORCID: orcid.org/0000-0002-8049-6645

38

39 **Declaration of Competing Interest**

40 Declarations of interest: none.

41 **Abstract**

42 Feline Vector-Borne Diseases show increased global prevalence and some *Anaplasma* and
43 *Ehrlichia* species may pose a risk to human health. The diagnosis of *Anaplasma* and *Ehrlichia*
44 species infection in cats is achieved by the combined use of different methods as cytologic
45 examination evidencing intracytoplasmic morulae, serologic tests and molecular assays. The
46 peripheral whole blood is considered the sample of choice for *Anaplasma* and *Ehrlichia* species
47 DNA detection in cats, but false negative results are reported leading to underestimation of
48 infection prevalence. In order to have a more accurate assessment of the spread of feline vector-
49 borne pathogens, the presence of *Anaplasma* spp. and *Ehrlichia* spp. DNA in 37 owner and shelter
50 cats subjected to necropsy were prospectively investigated by testing in end-point PCR spleen, bone
51 marrow, blood clot and hair samples. The bacteria identified were genetically characterized. Three
52 shelter cats tested positive for *A. phagocytophilum* DNA in spleen (one cat) or in hair samples (two
53 cats). None of the cats tested positive in bone marrow and blood samples. From the results obtained,
54 it can be assumed that the use of spleen or hair samples could allow a more reliable detection of *A.*
55 *phagocytophilum* DNA in cats with blood tested negative. In the phylogeny constructed with a
56 fragment of the heat shock (groEL) gene nucleotide sequences, all the identified *A.*
57 *phagocytophilum* clustered with bacteria infecting a wide range of hosts, including humans,
58 showing a potential zoonotic role.

59

60 **Keywords**

61 *Anaplasma phagocytophilum*, cat, hair, spleen, vector-borne pathogens

62

63 **1. Introduction**

64 Feline Vector-Borne Diseases (FVBD) emerged in recent years showing increased global
65 prevalence (Lappin, 2018; Pennisi et al., 2017; Quorollo et al., 2019). In particular, *Anaplasma* and
66 *Ehrlichia* species (Anaplasmataceae family, Rickettsiales order) infections have been reported in
67 cats and their presence in domestic felines may pose a risk to human health by acting as reservoirs
68 or amplifying hosts. Indeed, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* may infect cats
69 and are the main responsible for human granulocytic anaplasmosis (HGA) and human
70 monocytotropic ehrlichiosis (HME), respectively (Ismail and McBride, 2017). Nevertheless,
71 *Anaplasma* and *Ehrlichia* species infections have been generally less studied in cats than in dogs
72 (Otranto et al., 2017; Pennisi et al., 2017; Quorollo, 2019), probably due to the sub-clinical and
73 aspecific nature of clinical signs, the frequent occurrence of comorbidities, and to the scarce
74 perception of practitioners (Morelli et al., 2019).

75 In Europe, *E. chaffeensis* is not present, but several studies increasingly reported *Anaplasma* species
76 (in particular *A. phagocytophilum*, *A. platys* and *A. platys*-like) and *Ehrlichia canis* or *E. canis*-like
77 circulation in cats, mainly using serological and/or molecular assays (Adaszek et al., 2013; Attipa et
78 al., 2017; Ayllón et al., 2009; Bergmann et al., 2015; Bjöersdorff et al., 1999; Ebani and Bertelloni,
79 2014; Heikkilä et al., 2010; Maia et al., 2014; Morelli et al., 2019; Persichetti et al., 2018;
80 Schaarschmidt-Kiener et al., 2009; Shaw et al., 2005; Solano-Gallego et al., 2006; Spada et al.,
81 2014; Tabar et al., 2008; Vilhena et al., 2013; Zobba et al., 2015).

82 Currently, the diagnosis of *Anaplasma* and *Ehrlichia* species infection in cats is achieved by the
83 combined use of different methods: i) Cytologic examination of Romanowsky-stained peripheral
84 blood and buffy-coat smear, as well as lymph nodes and spleen aspirates, evidencing
85 intracytoplasmic inclusion bodies (morulae) in granulocytes, mononuclear cells or platelets (Allison
86 and Little, 2013; Pennisi et al., 2017); ii) serologic tests as indirect fluorescent antibody (IFA) assay
87 and enzyme-linked immunosorbent assay (ELISA) techniques (Allison and Little, 2013; Pennisi et
88 al., 2017); iii) rapid in-clinic ELISA tests validated to detect antibodies to member of the genera

89 *Anaplasma* and *Ehrlichia* in dog serum (SNAP Multi-Analyte Test and the SNAP 4Dx Plus Assay;
90 IDEXX Laboratories) that have also shown to detect antibodies against *A. phagocytophilum* in
91 feline serum (Allison and Little, 2013; Hegarty et al., 2015; Lappin et al., 2015; Lappin et al., 2020;
92 Liu et al., 2018; Schäfer and Kohn, 2020); and iv) molecular assays used to detect the presence of
93 pathogen DNA mainly in blood samples (Lappin, 2018).

94 All the diagnostic tests are affected by not optimal sensibility and specificity in the different stages
95 of infection (Allison and Little, 2013). Direct tests are more performing in the acute phase of
96 infection because morulae and pathogen DNA are readily detected in whole blood samples, whereas
97 indirect tests are more performing during a later stage of infection because antibodies made in
98 response to pathogens can take several weeks postexposure to become detectable (Lappin, 2018;
99 Quorollo, 2019). In particular, molecular assays applied to blood samples are highly sensitive and
100 specific but false-negative results may occur (Allison and Little, 2013; Quorollo, 2019; Sainz et al.,
101 2015). Negative results in molecular tests only indicate that the respective nucleic acid sequence is
102 not detected in a particular sample at a particular point of time, and should not be interpreted as
103 conclusive evidence of absence of infection (Allison and Little, 2013; Sainz et al., 2015). Therefore,
104 underdiagnosed infections using molecular assays are not uncommon and serological positivity
105 associated by rare or absent molecular positivity were reported (Persichetti et al., 2018; Ayllón et
106 al., 2009; Solano-Gallego et al., 2006).

107 Similar problems affect the molecular diagnosis of all the vector-borne infections. Recently, efforts
108 have been made to reduce false-negative results in the molecular diagnosis of leishmaniasis testing
109 biological matrices different to blood samples, comprising non-invasive samples such as hair, both
110 in dogs (Belinchón-Lorenzo et al., 2013; Manna et al., 2004) and in cats (Chatzis et al., 2014;
111 Urbani et al., 2020).

112 In order to have a more accurate assessment of the spread of feline vector-borne infections, in this
113 study the presence on *Anaplasma* spp. and *Ehrlichia* spp. DNA in owner and shelter cats subjected

114 to necropsy (April 2016 - May 2017) were investigated by testing spleen, bone marrow, blood clot
115 and hair samples. The bacteria identified were also genetically characterised.

116

117 **2. Materials and methods**

118 **2.1. Study design and sampling**

119 This was a prospective study performed at the Veterinary University Hospital (Department of
120 Veterinary Medical Science) of the *Alma Mater Studiorum* - University of Bologna, Northern Italy.
121 Cats died or euthanized for ethical reasons from April 2016 to May 2017 were included in the study
122 if the owners had given consent to perform the necropsy. A complete post-mortem examination was
123 carried out to each cat included and samples of spleen, bone marrow, blood clot and hair were taken
124 and stored at -20 °C for further investigation. Signalment and anamnestic data were retrieved from
125 medical records. During their lifetime, some cats were tested for the presence of feline
126 immunodeficiency virus (FIV) antibodies (anti-p24 and anti-gp40) and feline leukemia virus
127 (FeLV) antigen (p27) in plasma, serum, or whole blood samples using a commercial point-of-care
128 enzyme linked immunosorbent assay (ELISA) based test (SNAP FIV/FeLV Combo Plus test,
129 IDEXX, Westbrook, Maine, USA).**2.2. Detection of *Anaplasma* spp. and *Ehrlichia* spp. DNA**
130 DNA from biological samples was extracted using the NucleoSpin Tissue Mini Kit (Macherey-
131 Nagel) according to the manufacturer's protocol. In particular, the hair samples were cut into three
132 segments (proximal, central and distal) and lysed at 56°C overnight with 250 µl of lysis buffer
133 (Buffer T1) and 50 µl of proteinase K supplied in the extraction kit as reported by Urbani et al.
134 (2020). Extracted DNA was stored at -20 °C. The presence of all known *Anaplasma* spp. and
135 *Ehrlichia* spp. DNA was evaluated using a previously described end-point PCR assay amplifying an
136 approximately 600 bp fragment of the heat shock (groEL) gene (Barber et al., 2010). The PCR
137 assay was performed as previously described (Dondi et al., 2014). Briefly, amplification was carried
138 out using a commercial Taq-polymerase (Taq DNA Polymerase Kit, Qiagen, Hilden, Germany),
139 following the manufacturer's instructions. The thermal cycling consisted of an initial denaturation

140 at 94°C for 5 min followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s
141 and elongation at 72°C for 45 s, followed by a final elongation step at 72°C for 10 min. The *A.*
142 *phagocytophilum* 862/2014 strain (KT970678, De Arcangeli et al., 2018) and a no DNA template
143 were run in parallel with the PCR reaction as positive and negative controls, respectively. PCR
144 products were analyzed by 1.5% agarose gel electrophoresis under ultraviolet exposure.

145 **2.3. Sequence analysis**

146 The obtained amplicons were purified and sequenced directly using both forward and reverse
147 primers. The assembled nucleotide sequences were analysed using the BLAST web interface
148 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignments between obtained and reference
149 sequences available from GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) were
150 generated using the ClustalW method implemented in BioEdit sequence alignment editor version
151 7.2.5. Phylogenetic relationships of obtained sequences with reference sequences were evaluated
152 using the Minimum Evolution method implemented on the software MEGA X version 10.1.7
153 (Kumar et al., 2018). Tamura 3-parameter nucleotide substitution model with gamma distribution
154 was used. Bootstrap values were determined by 1000 replicates to assess the confidence level of
155 each branch pattern.

156 The sequence data obtained have been submitted to the DDBJ/EMBL/GenBank databases under
157 accession numbers MW030651-MW030653.

158 **2.4. Statistical analysis**

159 The data were evaluated using standard descriptive statistics and data comparison among subgroups
160 (sex, age, breed, lifestyle and results to FIV-FeLV rapid test) was performed by Chi-squared test
161 considering significant a P value <0.05. Statistical analysis was carried out using commercially
162 available software (MedCalc Statistical Software version 16.8.4).

163

164 **3. Results**

165 **3.1. Study population and sampling**

166 During the study period, 37 cats were included (**Table 1**). Eighteen of the 37 (48.6%) cats were
167 male and 19/37 (51.4%) were female. Cats had a median age of 49 months (range 2 months - 16
168 years and 7 months): 4/37 (10.8%) cats were kitten with less than 6 months of age, 8/37 (21.6%)
169 were young with age comprised between 6 and 24 months, and 24/37 (64.9%) were adult with more
170 than 24 months. Thirty-five of the 37 (94.6%) were domestic shorthaired cats, 1/37 (2.7%) was a
171 Siamese cat and for one cat the breed was not known; 9/37 (24.3%) were owned cats while 28/37
172 (75.7%) lived in a shelter. The rescued cats are mainly subjects who entered the shelter after birth,
173 belonging to unwanted litters, or subjects previously owned and then abandoned. The shelter allows
174 the adoption, even if many cats remain in the centre for life, and it is a well-isolated structure with
175 access to outdoor green spaces that prevents cats can enter into contact with other animals. Nineteen
176 of the 37 (51.4%) cats were tested for the presence of FIV antibodies and FeLV antigen: 6/19
177 (31.6%) were tested positive, three for FIV antibodies, one for FeLV antigen and two were positive
178 for both. Thirty-four of the 37 (91.9%) cats exhibited gross lesions in one or more apparatus
179 compatible to diseases of different severity, nine of these showed lesions attributable to feline
180 infectious peritonitis (FIP). Three of the 37 (8.1%) showed no macroscopic lesions on post-mortem
181 examination. Samples of liver, bone marrow and hair were taken from each cat included in the
182 study, while blood clot was sampled from 30/37 cats because in the remaining 7 cats the blood
183 undertaken lysis process due to the freezing and thawing of the carcasses.

184 **3.2. Detection and sequence analysis of *Anaplasma* spp. and *Ehrlichia* spp.**

185 Positive PCR products of the expected size were detected in 3/37 (8.1%) cats: two cats showed a
186 well-defined amplicon in hair sample (lab ID: 90/2017 and 446/2017) and one cat showed a faint
187 specific amplicon in the spleen sample (lab ID: 94/2017). For cats 90/2017 and 94/2017 all the four
188 biological matrices were taken and tested, while for cat 446/2017 the blood coat was not sampled.
189 The assembled nucleotide sequences obtained for cats 90/2017 and 446/2017 were 520 bp in length
190 while for cat 94/2017 was 232 bp in length. The sequences obtained showed a nucleotide identity of

191 99.1-99.5% among them and BLAST analysis allowed to align them with the reference sequences
192 of *A. phagocytophilum*.

193 Statistical association was found among positivity to *A. phagocytophilum* DNA and age, with all cat
194 tested positive ranging between 6 and 24 months (P = 0.003). No other statistical association was
195 found with signalment and anamnestic data. Although not significant, all cat tested positive come
196 from a shelter and lived outdoors (**Table 1**).

197 The unrooted phylogenetic tree reconstructed with partial groEL gene nucleotide sequences of *A.*
198 *phagocytophilum* from Italy was characterised by three major clusters (**Figure 1**), corresponding to
199 the first three of the four ecotypes proposed by Jahfari et al. (2014). All the three nucleotide
200 sequences obtained in this study clustered in ecotype I with *A. phagocytophilum* sequences
201 identified in ticks and a large number of distinct mammalian hosts (dog, horse, red deer and
202 chamois).

203

204 **4. Discussion**

205 In this study, 3/37 (8.1%) cats from Northern Italy tested positive for the presence of *A.*
206 *phagocytophilum* DNA in spleen (one cat) or in hair (two cats) samples. None of the cats included
207 in the study tested positive for other *Anaplasma* or *Ehrlichia* species DNA. The number of cats
208 tested positive for *A. phagocytophilum* DNA, as well as for *Ehrlichia* spp. DNA, is lower than
209 reported for urban stray colony cats in a nearby geographical area testing blood samples (Spada et
210 al., 2014). The high prevalence values (17.7% for *A. phagocytophilum* and 5.4% for *Ehrlichia* spp.)
211 detected by Spada et al. (2014) is probably due to the lifestyle of the sampled cats because a
212 significant increase of *Anaplasma* spp. and *Ehrlichia* spp. infection in stray cats was reported
213 (Maia et al., 2014). Indeed, the three cats tested positive in this study come from a shelter and lived
214 outdoors, showing living conditions and exposure to risk factors similar to stray cats. No owned
215 cats tested positive. From the signalment and anamnestic data analysed, only the age was
216 significantly associated to the *A. phagocytophilum* infection, with pathogen DNA detected only in

217 young cats of 6-24 months. Contrarily, Ebani and Bertelloni (2014) reported higher seroprevalences
218 in adult and mixed breed cats than in younger and purebred subjects.

219 None of the cats included in the study were positive in bone marrow and blood samples, including
220 the three cats tested positive in spleen or hair samples. Comparable results were obtained in Greek
221 cats by Mylonakis et al. (2018). To date, the peripheral whole blood is considered the sample of
222 choice for *Anaplasma* and *Ehrlichia* species DNA detection in cats (Lappin et al., 2020; Pennisi et
223 al., 2017; Schäfer and Kohn, 2020). Molecular detection of *A. phagocytophilum* in blood had high
224 sensitivity and specificity but can be falsely negative due to the low numbers of morulae in
225 circulating neutrophilic granulocytes or to the absence of the pathogen in blood in chronic
226 infections (Schäfer and Kohn, 2020). The absence of positive results in blood samples in this study,
227 could be due to the limits of sensitivity which do not make it ideal for *A. phagocytophilum* DNA
228 detection or to the origin of the samples tested that were not taken from live cats but from died cats
229 as intracardiac blood clots, often from thawed carcasses, affecting the result of the molecular test.

230 Differently, some studies suggested that *E. canis* may persist longer in spleen than in peripheral
231 blood and splenic aspirate PCR resulted superior to blood PCR for the *E. canis* DNA detection in
232 dogs (Harrus et al., 2004). *A. phagocytophilum* may have similar persistence in cats, making this
233 matrix suitable for diagnostic purposes. Foreign DNA can be sequestered in the hair of infected
234 animals (Belinchón-Lorenzo et al., 2013). Iniesta et al. (2013) proposed two potential mechanisms
235 for the origin of foreign DNA in the hair of animals infected by *Leishmania major*: a
236 “transepidermal elimination” prevalently in the site of inoculation with direct incorporation of the
237 parasite DNA among keratinocytes of skin and its passage into the formed hair during the
238 keratinization process, and/or DNA incorporations into the hair by migration from the
239 bloodstream. Similar mechanisms can also be hypothesized for other vector-borne pathogens
240 circulating in the host’s bloodstream. Furthermore, in the Iniesta et al. study (2013), the number of
241 animals infected by *L. major* tested positive for the pathogen DNA in the hair and the DNA load
242 detected in this sample, progressively increased with the time elapsed from infection. Therefore, the

243 use of hair for the detection of pathogen DNA could therefore allow to reduce the false-negatives
244 that usually affect the molecular diagnosis in animals with chronic infections. The limited number
245 of cats tested in this study does not allow to draw definitive conclusions but, from the results
246 obtained, it can be assumed that the use of biological matrices different to blood, such as spleen or
247 hair, could allow a more reliable detection of *A. phagocytophilum* DNA in cats with blood tested
248 negative. Future investigations are needed to confirm this hypothesis.

249 The nucleotide sequence obtained from one cat (94/2017, spleen sample) was shorter than the
250 others because the amplicon was faint and part of the electropherogram was of low quality.

251 Phylogeny evidenced three clusters including all the *A. phagocytophilum* groEL gene nucleotide
252 sequences identified in Italy, corresponding to the first three of the four ecotypes proposed by
253 Jahfari et al. (2014) for the Europe continent. The fourth ecotype, most likely associated with bird
254 species, not emerged in the phylogenetic tree constructed in this study because groEL nucleotide
255 sequences identified in birds from Italy were not available in GenBank. Ecotype I, including the
256 sequences obtained in this study, had the broadest host range and was reported to include all the *A.*
257 *phagocytophilum* groEL-gene from Genbank identified in humans, demonstrating that members of
258 this ecotype are zoonotic (Jahfari et al., 2014).

259 In conclusion, as reported for *Leishmania* spp. (Belinchón-Lorenzo et al., 2013), we think that hair,
260 easy to collect and store, is a sample to take in account in the molecular diagnosis of *Anaplasma*
261 spp., and that its application should be evaluated for the diagnosis of all vector-borne infections in
262 which the pathogen circulates in the host's bloodstream. Cats with undiagnosed anaplasmosis can
263 play an important epidemiological role acting as source of infection for invertebrate hosts
264 increasing the risk of transmission to humans, posing a threat to public health. Therefore, further
265 studies are needed to compare the use of different biological matrices for the molecular detection of
266 *A. phagocytophilum* and other FVBD pathogens to identify those allowing to minimize false
267 negative results.

268

269 **Funding**

270 This research did not receive any specific grant from funding agencies in the public, commercial, or
271 not-for-profit sectors.

272

273 **Declaration of Competing Interest**

274 Declarations of interest: none.

275

276 **Acknowledgment**

277 We thank Federica Zipari for help with DNA extraction and amplification.

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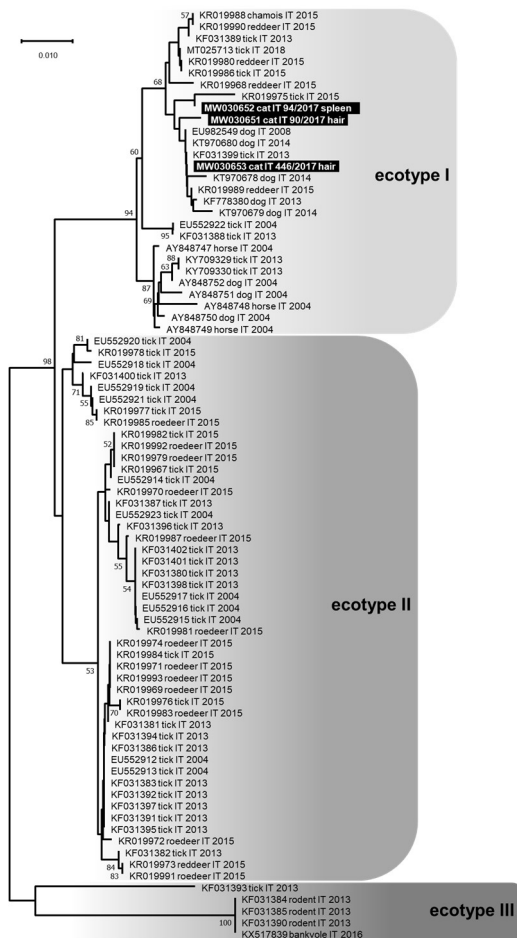
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405 **Figure legends**



406

407 **Figure 1.** Unrooted phylogenetic tree based on partial heat shock gene (groEL) nucleotide
 408 sequences (546 bp) of *Anaplasma phagocytophilum*.
 409 Phylogeny was evaluated using the Minimum Evolution method implemented on the software
 410 MEGA X version 10.1.7 (Kumar et al., 2018) on multiple alignment constructed with nucleotide
 411 sequences obtained in this study and 77 *A. phagocytophilum* reference sequences identified in ticks
 412 and mammals hosts in Italy retrieved from GenBank. The best-fit model of nucleotide substitution
 413 was determined using the Find Best DNA/Protein Model function implemented in MEGA X.
 414 Tamura 3-parameter model with gamma distribution resulted optimal for the sequence data.
 415 Statistical support was provided by bootstrapping with 1000 replicates. Bootstrap values $\geq 50\%$ are
 416 indicated on the respective branches. Highlighted in black: Sequences generated in this study. Main
 417 clusters are labelled following the ecotype classification proposed by Jahfari and colleagues (Jahfari
 418 et al., 2014).

419 **Table 1.** Descriptive statistics and prevalence (%) of infection to *Anaplasma* spp. among the cats
 420 included in the study groups.

421

Variables	Total	Positive to <i>A. phagocytophilum</i> DNA	Negative to <i>A. phagocytophilum</i> DNA	P value
Cats included in the study	37	3 (8.1)	34 (91.9)	
Sex				0.961
Male	18 (48.6)	1 (5.6)	17 (94.4)	
Female	19 (51.4)	2 (10.5)	17 (89.5)	
Age (months)				0.003
Kittens <6	4 (10.8)	0 (0)	4 (100)	
Young 6–24	8 (21.6)	3 (37.5)	5 (62.5)	
Adult >24	24 (64.9)	0 (0)	24 (100)	
NA	1 (2.7)	0 (0)	1 (100)	
Breed				0.126
Domestic shorthaired cat	35 (94.6)	3 (8.6)	32 (91.4)	
Purebred	1 (2.7)	0 (0)	1 (100)	
NA	1 (2.7)	0 (0)	1 (100)	
Lifestyle				0.747
Owned cat	9 (24.3)	0 (0)	9 (100)	
Feline community (shelter)	28 (75.7)	3 (10.7)	25 (89.3)	
IgG anti-FIV and/or Ag FeLV ^a				0.545
Positive	6 (16.2)	0 (0)	6 (100)	
Negative	13 (35.1)	3 (23.1)	10 (76.9)	
NA	18 (48.7)	0 (0)	18 (100)	

422

423 P value <0.05 was considered significant. Not available data were excluded from the statistical
 424 analysis.

425 ^a The presence of feline immunodeficiency virus (FIV) antibodies (anti-p24 and anti-gp40) and
 426 feline leukemia virus (FeLV) antigen (p27) was detected on plasma, serum, or whole blood samples
 427 using a commercial point-of-care enzyme linked immunosorbent assay (ELISA) based test (SNAP
 428 FIV/FeLV Combo Plus test, IDEXX, Westbrook, Maine, USA), following the manufacturer
 429 instruction.

430 NA = not available.