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

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**Abstract**

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

**Keywords**

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

## 1. Introduction

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

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

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

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

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

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

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

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

## 2. Materials and methods

### 2.1. Study design and sampling

This was a prospective study performed at the Veterinary University Hospital (Department of Veterinary Medical Science) of the *Alma Mater Studiorum* - University of Bologna, Northern Italy. Cats died or euthanized for ethical reasons from April 2016 to May 2017 were included in the study if the owners had given consent to perform the necropsy. A complete post-mortem examination was carried out to each cat included and samples of spleen, bone marrow, blood clot and hair were taken and stored at -20 °C for further investigation. Signalment and anamnestic data were retrieved from medical records. During their lifetime, some cats were tested for the presence of feline immunodeficiency virus (FIV) antibodies (anti-p24 and anti-gp40) and feline leukemia virus (FeLV) antigen (p27) in plasma, serum, or whole blood samples using a commercial point-of-care enzyme linked immunosorbent assay (ELISA) based test (SNAP FIV/FeLV Combo Plus test, IDEXX, Westbrook, Maine, USA).**2.2. Detection of *Anaplasma* spp. and *Ehrlichia* spp. DNA**

DNA from biological samples was extracted using the NucleoSpin Tissue Mini Kit (Macherey-Nagel) according to the manufacturer's protocol. In particular, the hair samples were cut into three segments (proximal, central and distal) and lysed at 56°C overnight with 250 µl of lysis buffer (Buffer T1) and 50 µl of proteinase K supplied in the extraction kit as reported by Urbani et al. (2020). Extracted DNA was stored at -20 °C. The presence of all known *Anaplasma* spp. and *Ehrlichia* spp. DNA was evaluated using a previously described end-point PCR assay amplifying an approximately 600 bp fragment of the heat shock (groEL) gene (Barber et al., 2010). The PCR assay was performed as previously described (Dondi et al., 2014). Briefly, amplification was carried out using a commercial Taq-polymerase (Taq DNA Polymerase Kit, Qiagen, Hilden, Germany), following the manufacturer's instructions. The thermal cycling consisted of an initial denaturation

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

### 2.3. Sequence analysis

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

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

### 2.4. Statistical analysis

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

## 3. Results

### 3.1. Study population and sampling

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

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

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

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

None of the cats included in the study were positive in bone marrow and blood samples, including the three cats tested positive in spleen or hair samples. Comparable results were obtained in Greek cats by Mylonakis et al. (2018). To date, the peripheral whole blood is considered the sample of choice for *Anaplasma* and *Ehrlichia* species DNA detection in cats (Lappin et al., 2020; Pennisi et al., 2017; Schäfer and Kohn, 2020). Molecular detection of *A. phagocytophilum* in blood had high sensitivity and specificity but can be falsely negative due to the low numbers of morulae in circulating neutrophilic granulocytes or to the absence of the pathogen in blood in chronic infections (Schäfer and Kohn, 2020). The absence of positive results in blood samples in this study, could be due to the limits of sensitivity which do not make it ideal for *A. phagocytophilum* DNA detection or to the origin of the samples tested that were not taken from live cats but from died cats as intracardiac blood clots, often from thawed carcasses, affecting the result of the molecular test. Differently, some studies suggested that *E. canis* may persist longer in spleen than in peripheral blood and splenic aspirate PCR resulted superior to blood PCR for the *E. canis* DNA detection in dogs (Harrus et al., 2004). *A. phagocytophilum* may have similar persistence in cats, making this matrix suitable for diagnostic purposes. Foreign DNA can be sequestered in the hair of infected animals (Belinchón-Lorenzo et al., 2013). Iniesta et al. (2013) proposed two potential mechanisms for the origin of foreign DNA in the hair of animals infected by *Leishmania major*: a "transepidermal elimination" prevalentely in the site of inoculation with direct incorporation of the parasite DNA among keratinocytes of skin and its passage into the formed hair during the keratinization process, and/or DNA incorporatations into the hair by migration from the bloodstream. Similar mechanisms can also be hypothesized for other vector-borne pathogens circulating in the host's bloodstream. Furthermore, in the Iniesta et al. study (2013), the number of animals infected by *L. major* tested positive for the pathogen DNA in the hair and the DNA load detected in this sample, progressively increased with the time elapsed from infection. Therefore, the

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

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

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

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

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Declarations of interest: none.

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## Figure legends

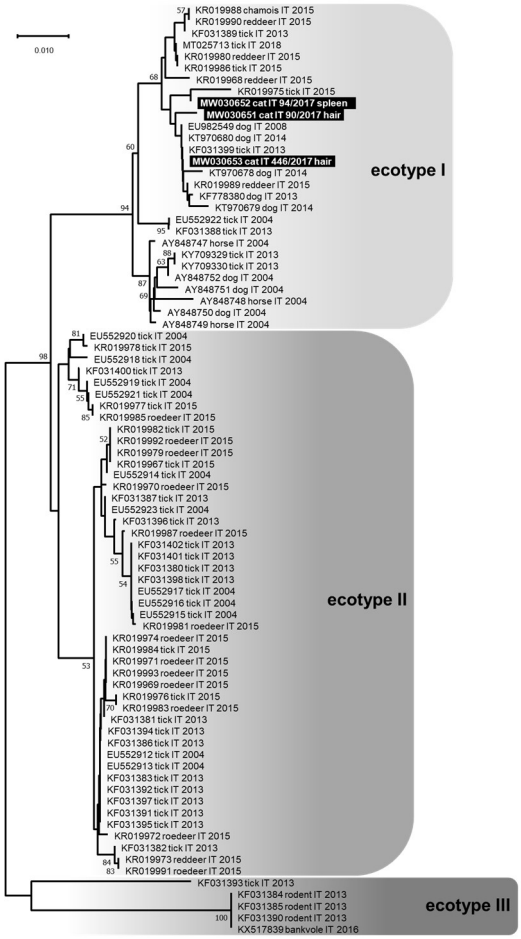


**Figure 1.** Unrooted phylogenetic tree based on partial heat shock gene (groEL) nucleotide sequences (546 bp) of *Anaplasma phagocytophilum*.

Phylogeny was evaluated using the Minimum Evolution method implemented on the software MEGA X version 10.1.7 (Kumar et al., 2018) on multiple alignment constructed with nucleotide sequences obtained in this study and 77 *A. phagocytophilum* reference sequences identified in ticks and mammals hosts in Italy retrieved from GenBank. The best-fit model of nucleotide substitution was determined using the Find Best DNA/Protein Model function implemented in MEGA X. Tamura 3-parameter model with gamma distribution resulted optimal for the sequence data. Statistical support was provided by bootstrapping with 1000 replicates. Bootstrap values ≥ 50% are indicated on the respective branches. Highlighted in black: Sequences generated in this study. Main clusters are labelled following the ecotype classification proposed by Jahfari and colleagues (Jahfari et al., 2014).

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

| Variables | Total | Positive to *A. phagocytophilum* DNA | Negative to *A. phagocytophilum* 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) | |

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

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

NA = not available.