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RESEARCH ARTICLE

Evaluation of *Anaplasmataceae* Family Agents Infection in Domestic Cats

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ABSTRACT

The present study aimed to determine the occurrence of *Anaplasmataceae* family agents and to assess comparative hematology in cats from District Federal, Brazil using molecular methods. Blood samples from two hundred cats from different regions of District Federal were submitted to two different PCR protocols to amplify the 16S rRNA gene of *Anaplasmataceae* agents and *Anaplasma platys*. Besides, all samples were analyzed by complete blood cell count (CBC). The occurrence of *Anaplasmataceae* agents was 13% (26/200), whereas that of *Anaplasma platys* was 3.5% (7/200). There was no significant difference ($P<0.05$) of hematological parameters between positive and negative animals. The most frequent hematological alterations seen in *Anaplasmataceae* positive cats were lymphopenia (40%) and hyperproteinemia (28%). The infection of *Anaplasmataceae* family agents and *Anaplasma platys* occur in cats from District Federal, Brazil, but is uncommon. Cats seem to be more resistant or adapted to these infectious agents than dogs, as no significant hematological alteration was observed.

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INTRODUCTION

Research regarding cat diseases caused by *Anaplasmataceae* family bacteria has increased through years. Bacteria from this family are rarely diagnosed in cats during routine veterinary practice, although they are commonly found in dogs (Almazán *et al.*, 2016), horses (Magnarelli *et al.*, 2000), ruminants (Alim *et al.*, 2011), deers (Dawson *et al.*, 1994) and rodents (Nicholson *et al.*, 1998) worldwide. The scarce diagnosis of *Anaplasma* (A.) *sp.* in cats may be related to misrecognition of diseases transmitted by ticks, such as *Rhipicephalus sanguineous*, in cats, low pathogenicity of *Anaplasma sp.* in cats or the rapid removal of ticks by cats due their hygienic habits, which decreases the possibility of disease transmission (Eberhardt *et al.*, 2006).

So far, the species belonging to *Anaplasmataceae* family found to infect cats were *A. platys* (Lima, 2010; Correa *et al.*, 2011), *A. phagocytophilum* (Magnarelli *et al.*, 2005; Wardrop *et al.*, 2005; Solano-Gallego *et al.*, 2006; Heikkilä *et al.*, 2010; Lima, 2010), *Ehrlichia (E.) chaffeensis* (Braga *et al.*, 2012) and *E. canis* (Breitschwerdt *et al.*, 2002; Wardrop *et al.*, 2005; Solano-Gallego *et al.*, 2006; Little, 2010). Infected animals usually present unspecific signs such as fever, anorexia, weight loss, apathy and pale mucosa. The severity of the disease depends on the infecting strain, concomitant diseases and/or host immune response (Heikkilä *et al.*, 2010; Martins, 2012).

It is not known if infected cats are reservoirs or accidental hosts (Martins, 2012), but it is important to determine their epidemiological relevance (Oliveira, 2008) as the prevalence of anaplasmosis has been increased in animals and people (Heikkilä, 2010). Therefore, the present study aimed to determine the occurrence of *Anaplasmataceae* agents and *A. platys* in domestic cats from different areas of District Federal, Brazil through PCR and to assess comparative hematological parameters from the sampled animals.

MATERIALS AND METHODS

EDTA-anticoagulated blood samples were obtained from 200 cats of different areas of District Federal: 36 during the 2012 anti-rabies vaccination campaign, 123 from cats that attended the local Veterinary Teaching Hospital (VTH) and 41 from private clinics.

All samples were analyzed by complete blood cell count (CBC) in the same day they were collected using ABC Vet-Horiba© (ABX diagnostics, Brazil). Mean cell

volume (MVC) and mean cell hemoglobin concentration (MCHC) were calculated from hemoglobin, PCV and erythrocytes number. Microhematocrit centrifugation method was used to determine the PCV. Total plasma protein (TPP) was measured using a refractometer. Leukocyte differential cell count and erythrocytes morphologic analysis were realized on blood smears stained with Diff-quick (Instant Prov, NewProv, Brazil).

DNA extraction from whole blood was performed using a commercial kit Illustra blood genomic Prep Mini Spin Kit (GE Healthcare©, Piscataway, NJ) following the manufacturer's recommendations. DNA was stored at -20ºC until molecular analysis.

All samples were tested with a PCR that detects the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme, to verify DNA quality, integrity, and the presence of PCR inhibitors using GAPDH-F (CCTTCATTGACCTCAACTACAT) and GAPDH-R (CCAAAGTTGTCATGGATGACC) oligonucleotides, as previously described by Birkenheuer *et al*. (2003). Next, the samples were screened for *Anaplasmataceae* agents by PCR using EHR16sd (GGTACCYACAGAAGAAGTCC) and EHR16sr (TAGCACTCATCGTTTACAGC) oligonucleotides designed to amplify the 16S rRNA gene (Inokuma *et al.*, 2000). In the reaction, 10 ng of DNA was amplified using 1X PCR buffer, 0.2 mM of each deoxinucleotide, 1.6 mM $MgCl_2$, 1µM concentration of each oligonucleotide and 0.5U Taq DNA polimerase (Invitrogen©, Vila Guarani, São Paulo) with water to 25µl. An initial denaturation step at 95°C for 5min followed by 34 amplification of 30s at 95°C, 30s at 53°C and 90s at 72°C, with a final extension step of 5min at 72°C, producing a 345 base pairs (bp) fragment in positive samples.

Positive animals were submitted to a second PCR to amplify the 16S rRNA gene of *A. platys* using EHR16sr and Platys (GATTTTTGTCGTAGCTTGCTATG) oligonucleotides (Inokuma *et al.*, 2000). The reaction included 10 ng of DNA, 1X PCR buffer, 0.2 mM of each deoxinucleotide (Invitrogen©, Vila Guarani, São Paulo), 2.5 mM $MgCl_2$, 1µM of each oligonucleotide and 0.5U Taq DNA polymerase (Invitrogen©, Vila Guarani, São Paulo) with water to 25µl. The initial denaturation step was 5min at 95°C followed by 40 amplification cycles of 30s at 95°C, 30s at 51°C and 90s at 72°C, with a final extension step of 5min at 72°C, producing a 680 bp fragment in positive samples.

All amplifications were performed on the same thermal cycler (Biorad© C1000™ Thermal Cycler, Hercules, CA). PCR products were visualized by fluorescence transilluminator after electrophoresis in a 1.5% agarose gel stained with ethidium bromide. The reactions used water as negative control and DNA from an *E. canis* or *A. platys* naturally infected dogs diagnosed by morula observation in blood smear as positive control.

Statistical evaluation was carried out using SPSS for Windows (SPSS Inc., Chicago IL, USA). T-test was used to compare mean hematologic parameters of positive and negative cats. Hematological parameters were categorized as above, within and below reference interval and as positive and negative through PCRs and compared using the χ^2 test. Significance of all the tests was assigned as a p value <0.05.

RESULTS

Considering all cats tested, 47.5% (95/200) were female and 47% (94/200) were male. There was no information about the gender of 11 cats. Twenty-five cats (12.5%) were 1-year-old or less, 76 (38%) were more than 1 and less than 10 years old and 23 cats (11.5%) were 10 years old or more. The information about the age of 76 cats was not available.

Samples from 26 cats (13%) were PCR-positive to *Anaplasmataceae*, 10 of them female and 15 males. The gender of one positive cat was not informed. The number of *Anaplasmataceae* family and *A. platys* PCR positive cats in each area of Federal District, Brazil is shown in Table 1. Fig. 1 shows electrophoresis results of *Anaplasmataceae* agents PCR and *A. platys* PCR in agarose gel stained by ethidium bromide (0.01%) where the 345bp and 678bp products can be seen, respectively. There was no significant difference (P>0.05) when comparing mean hematologic parameters of *Anaplasmataceae* positive and negative animals, *A. platys* positive and negative animals and *A. platys* positive and *Anaplasmataceae* positive animals. The mean values and standard deviation of hematologic parameters from these comparisons can be observed in Table 2. Hematologic values for one *Anaplasmataceae* PCR positive cat and 37 negative cats were not available.

Table 1: Number of positive cats in *Anaplasmataceae* family and *A. platys* PCR per origin of blood sample.

Area	*Anaplasmataceae* family n (%)	*Anaplasma platys* n (%)
Vaccination campaign	1/36 (2.7)	1/1 (100)
Veterinary hospital	25/123 (20)	6/25 (24)
Private clinics	0/41 (0)	0 (0)
Total	26/200 (13)	7/26 (27)

Although there was no difference (P>0.05) when comparing mean hematologic parameters, 40% (11/27) of *Anaplasmataceae* positive cats presented lymphopenia, 28% (7/27) presented hyperproteinemia and 12% (3/25) presented thrombocytosis. The most common hematologic alteration found in *A. platys* positive cats was lymphopenia, which was observed in a half of animals (3/6).

DISCUSSION

Considering that only part of the positive cats to *Anaplasmataceae* species were also positive to *A. platys*, it indicates that apart from *A. platys* other *Anaplasmataceae* species may be present infecting cats in DF, as the oligonucleotides tested can amplify *E. canis, E. chaffeensis, E. muris, E. equi, E. phagocytophila, E. platys (A. platys), A. marginale, A. centrale, Wolbachia pipientis, E. sennetsu, E. risticii* and *Neorickettsia helminthoeca* (Inokuma *et al.*, 2000). Cats infected with several species of *Anaplasmataceae* family were already found in other states of Brazil. Guimarães *et al.* (2013) found *Ehrlichia* species DNA in 23% of cats tested with PCR in metropolitan region of Rio de Janeiro, with 11 cats presenting cell inclusions in blood smear, both in platelets and/or leukocytes. On the other hand, Correa *et al*. (2011) did not found positive animals in Rio de Janeiro when testing blood of 91 cats with a PCR designed to

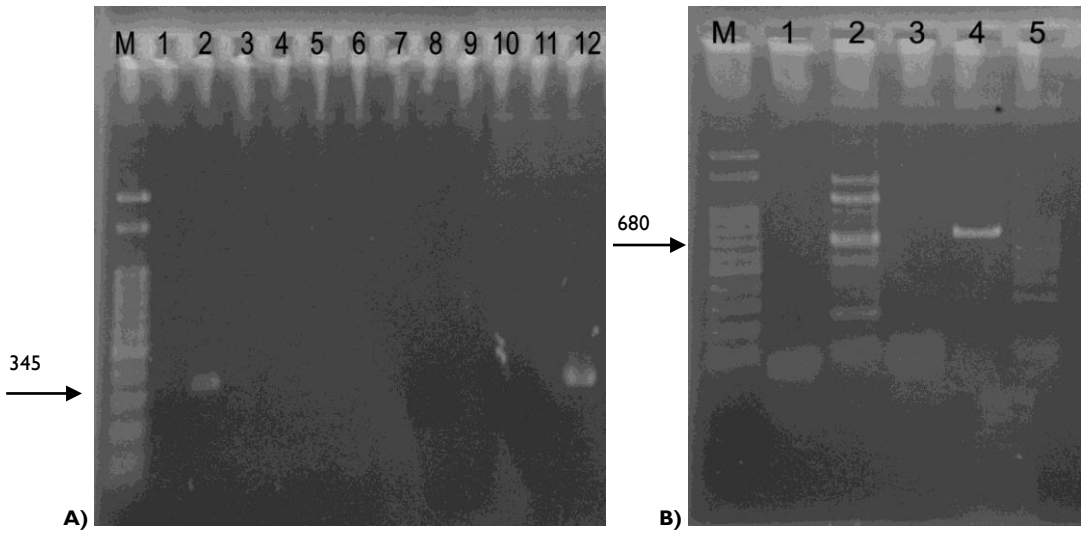

Fig. 1: A) *Anaplasmataceae* family PCR results using the oligonucleotides EHR16sd and EHR16sr. Lane (m): molecular weight marker (100 pb, Invitrogen®), Lane (1): negative control (water); Lane (2): positive control; Lanes (3) to (11): negative animals; Lane (12): positive animal. B) *Anaplasma platys* PCR results using the oligonucleotides Platys e EHR16sr. Lane (m): molecular weight marker (100 pb, Invitrogen®), Lane (1): negative control (water); Lane (2): positive control; Lane (3): negative animal; Lanes (4) and (5): positive animals.

Table 2: Mean values and standard deviation of hematologic parameters in *Anaplasmataceae* family positive and negative cats, *A. platys* positive and negative cats and *A. platys* positive cats and in *Anaplasmataceae* family agents positive cats, except *A. platys* (*A.* positives – *A. platys*), from all areas

Parameter	*Anaplasmataceae* family		*Anaplasma platys*		*A. platys* positives	*A* positives – *A. platys*	Reference interval
	Positives	Negatives	Positives	Negatives			
PCV (%)	36.0±4.2	32±2.8	31.5±2.1	34.5±6.3	31.5±2.1	35±5.6	24 - 45
Erythrocytes (10^6/µL)	8.2±2.2	9.1±2.3	8.2±2.2	8.6±1.6	8.2±2.2	9.51±0.3	5 - 10
Hemoglobin (g/dL)	13.3±1.2	11.25±0.7	13.3±1.2	11.55±1.2	13.3±1.2	13±0.8	8 - 15
MVC (fl)	42.2±10.38	35.9±6.3	40.09±13.37	37.6±3.9	40.09±13.37	34.1±1	39 - 55
MCHC (%)	39.7±4.6	35.2±0.7	42.3±0.96	36.0±0.6	42.3±0.96	35.3±1.6	30 - 36
Platelets (10^3/µL)	227±83.4	362.5±74.2	477.5±437.7	350.5±91.2	477.5±437.7	250±50.9	195 - 624
TPP (g/dL)	7.4±0.3	6.7±0.7	7.1±0.1	7.4±0.28	7.1±0.1	7.7±0.1	6 - 8
Leukocytes (10^3/µL)	10.2±1.1	19.2±1.9	19.1±13.7	14.4±4.8	19.1±13.7	10.5±0.7	5.5 - 19.5
Neutrophils (10^3/µL)	7.5±0.7	16.2±0.9	14.1±8.8	11.1±6.1	14.1±8.8	7.5±0.9	2.5 - 12.5
Lymphocytes (10^3/µL)	1.8±0.8	1.6±1.1	3.6±3.4	1.6±1.1	3.6±3.4	1.8±0.7	1.5 - 7
Eosinophil (10^3/µL)	0.9±1.2	0.8±0.3	1.0±1.4	1.4±0.4	1.0±1.4	0.9±1.1	0-1.5
Monocytes (10^3/µL)	0.14±0.2	0.5±0.1	0.28±0	0.2±0.2	0.28±0	0.2±0.2	0 - 0.85
Basophils (10^3/µL)	0	0	0	0	0	0	Rare

There was no statistically significant difference (p>0,05) by T test when comparing values from *Anaplasmataceae* family positive cats to negative cats, *A. platys* positive cats to negative cats and *A. platys* positive cats to *Anaplasmataceae* family positive cats, except *A. platys* positives. Packed Cell Volume (PCV), Mean Cell Volume (MVC), Mean Cell Hemoglobin Concentration (MCHC), Total Plasma Protein (TPP).

detect *E. chaffeensis, E. ewingii* and *E. canis*. Oliveira *et al*. (2009) found 20% (3/15) of *E. canis* positive cats through *nested*-PCR and no positive cats to *E. ewingii* in Viçosa, Minas Gerais. In São Luís, Maranhão, Braga *et al.* (2012) PCR tested 200 cats to *Ehrlichia* species and found two (1%) positives, one with DNA sequence 98% like *E. canis* and the other had DNA sequences 97% like *E. chaffeensis*. In Curitiba, Paraná 1.4% (2/142) of cats were *Ehrlichia*-positive and none were *A. platys* positive (Montaño *et al.*, 2013). It is important to consider that 25 positive animals in the present study were patients attended in the local VTH, therefore it is expected to find more positive animals in this group as owners usually bring animals to attendance when there are alterations in their pets related to clinical disease.

There are few studies about *A. platys* molecular detection in Brazil. The first description was an accidental finding in Pernambuco and the DNA sequence was like that found in dogs from different countries (Lima, 2010). The first epidemiologic study was made by Correa *et al.* (2011) who found *A. platys* DNA in 13.18% of 91 cats in Rio de Janeiro, an occurrence higher than the 3.5% found herein. Santarém *et al.* (2005) found inclusions like *A. platys* in thrombocytes from a cat, however there was no molecular confirmation. *A. platys* was already detected infecting cats in Thailand (Salakij *et al*., 2012) and in United States (Qurollo *et al*., 2014).

There was no significant difference (P>0.05) when comparing mean hematologic parameters of *Anaplasmataceae* positive and negative animals. This suggests that cats may be more resistant or adapted than dogs to these infectious agents (Breitschwerdt *et al*., 2002; Oliveira, 2008). However, some infected cats have already been described as presenting clinical signs and hematological findings, like fever, lethargy, anemia and thrombocytopenia, like the ones observed in dogs infected with granulocytic *Ehrlichia* (Björsdorff *et al*., 1999; Little, 2010; Braga *et al.*, 2013). Also, the absence of difference between positive and negative animals on hematological parameters may be due to the convenience sample, as most of the animals included in the present study were patients attending a VTH, which could have any other disease leading to alterations in CBC, therefore compromising further analysis.

Other *Anaplasmataceae* family species, such as *E. canis* and *A. phagocytophilum,* should be considered infecting cats, as these bacteria have been found infecting dogs and cats in the region of Distric Federal (Vieira *et al.*, 2011; André *et al.*, 2014). Also, to date no study has been done on *E. chaffeensis* (Braga *et al.*, 2012) and *E. ewingii* (Oliveira *et al.*, 2009) species infecting animals in District Federal, although these species had already been found infecting animals in other regions of Brazil.

Conclusions: The occurrence of *Anaplasmataceae* infection in cats is possible, although uncommon. In District Federal, it was observed 13% of cats positive to *Anaplasmataceae* family, of which 27% were *A. platys.* Different *Anaplasmataceae* species other than *A. platys* may be present infecting cats. Cats may be resistant or adapted to *Anaplasmataceae* infection, as hematologic alterations from positive and negative animals presented no significant difference.

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Authors contribution: GP conceived and designed the study. ML executed the experiment and analyzed the samples. ML, LA and GP analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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