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Detection of *Anaplasma* sp. phylogenetically related to *A. phagocytophilum* in a free-living bird in Brazil

Detecção de *Anaplasma* sp. filogeneticamente associado a *A. phagocytophilum* em uma ave de vida livre no Brasil

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Abstract

Wild animals play an important role in carrying vectors that may potentially transmit pathogens. Several reports highlighted the participation of wild animals on the *Anaplasma phagocytophilum* cycle, including as hosts of the agent. The aim of this study was to report the molecular detection of an agent phylogenetically related to *A. phagocytophilum* isolated from a wild bird in the Midwest of the state of Paraná, Brazil. Fifteen blood samples were collected from eleven different bird species in the Guarapuava region. One sample collected from a *Penelope obscura* bird was positive in nested PCR targeting the 16S rRNA gene of *Anaplasma* spp. The phylogenetic tree based on the Maximum Likelihood analysis showed that the sequence obtained was placed in the same clade with *A. phagocytophilum* isolated from domestic cats in Brazil. The present study reports the first molecular detection of a phylogenetically related *A. phagocytophilum* bacterium in a bird from Paraná State.

Keywords: Anaplasmosis, wild animals, birds, hemoparasites, PCR.

Resumo

Animais selvagens possuem participação importante como carreadores dos vetores responsáveis por transmitir doenças e vários relatos destacam a participação de animais silvestres no ciclo do *Anaplasma phagocytophilum*, inclusive como hospedeiros do agente. O presente trabalho tem por objetivo relatar pela primeira vez a detecção molecular da infecção por um agente filogeneticamente associado a *A. phagocytophilum* em uma ave silvestre no interior do Paraná, Brasil. Foram colhidas 15 amostras de sangue originadas de onze espécies diferentes de aves, todas provenientes da região de Guarapuava. Apenas uma amostra pertencente a uma ave da espécie *Penelope obscura* foi positiva para o ensaio de nested PCR baseado no gene 16S rRNA. A árvore filogenética baseada na análise por máxima verossimilhança demonstrou que a sequência obtida no presente estudo se posicionou no mesmo clado com cepas de *A. phagocytophilum* isoladas de gatos domésticos no Brasil. O presente trabalho relata pela primeira vez a detecção molecular de *Anaplasma* sp. filogeneticamente relacionado à *A. phagocytophilum*, em um animal da espécie *P. obscura*, assim como a presença do parasita em uma ave silvestre do Estado do Paraná, Brasil.

Palavras-chave: Anaplasmoze, animais selvagens, aves, hemoparasitas, PCR.

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Several studies about the incidence of hemoparasites among birds have been conducted in Brazil, especially focusing in parasites such as *Plasmodium* spp., *Haemoproteus* spp., and *Trypanosoma* spp. (BENNETT & LOPES, 1980; ADRIANO & CORDEIRO, 2001; LOBATO et al., 2011; SEBAIO et al., 2010, 2012 ; TOSTES et al., 2015).

The genus *Anaplasma* comprises Gram-negative and obligatory intracellular bacteria that infect cytoplasmic vacuoles of eukaryote cells (DUMLER et al., 2001). The main clinical symptom of anaplasmosis caused by *Anaplasma phagocytophilum* is unspecific fever in humans (DUMLER & WALKER, 2001). However, the majority of infected patients may present not only fever, but also headache, myalgia, thrombocytopenia, and elevation in the levels of hepatic enzymes (DUMLER et al., 2005). Nevertheless, anaplasmosis is still considered a disease of difficult diagnosis when no laboratory tests are carried out, as clinical symptoms are unspecific (MASSUNG et al., 2003). The course of infection in humans can be mild or severe, depending on the health condition of the patient, and it has been reported that about 40% of the cases may require hospitalization (RYMASZEWSKA & GREINDA, 2008). Human granulocytic anaplasmosis caused by *A. phagocytophilum* was reported for the first time in 1994 (BAKKEN et al., 1994; CHEN et al., 1994), and this condition has motivated studies on the characteristics of the causative agent.

A. phagocytophilum can naturally infect sheep, cattle, horses, dogs, cats, and humans (MASSUNG et al., 2002) by invading granulocytes, especially neutrophils (EBANI et al., 2008). Although *A. phagocytophilum* may have worldwide distribution, reports on its occurrence are concentrated in the United States, especially in the Midwestern, northeastern, and western regions of the country (CFSPH, 2013). Wild animals play an important role in carrying vectors that may potentially transmit pathogens (EBANI et al., 2008). Several reports highlighted the participation of wild mammals, such as deer, foxes, rodents, and carnivores in *A. phagocytophilum* cycles, including their role as hosts of these bacteria (LIZ et al., 2000; FUENTE et al., 2008; VERONESI et al., 2011; ANDRÉ et al., 2012, JAHFARI et al., 2014). Wild and domestic animals may share pathogens, as described by Zhan et al. (2010), who found goats, sheep, and wild rodents in the same area infected by the same strain of *A. phagocytophilum*. Such occurrences can bring the agent in closer contact with humans.

Some studies indicated that wild birds may be responsible for the distribution of vectors infect by *A. phagocytophilum* (DANIELS et al., 2002; OGDEN et al., 2008; HILDEBRANDT et al., 2010; PALOMAR et al., 2012). However, only a few of them considered that birds might have direct participation in the epidemiology of the disease (SKORACKI et al., 2006; IOANNOU et al., 2009; JAHFARI et al., 2014). In 2012, Machado et al. (2012) showed, for the first time, that Brazilian wild birds can act as hosts for *Anaplasma* sp. The authors reported detection of *Anaplasma* sp. phylogenetically associated with *A. phagocytophilum* in blood samples from Southern carcaras (*Caracara plancus*) and American black vultures (*Coragyps atratus*) using molecular methods.

The transmission of the bacteria from birds to ticks was observed in previous studies (KEESING et al., 2012), and although bird infection is not believed to lead to the development of significant clinical signs, specific antibodies, or bacteremia (JOHNSTON et al.,

2013), a correlation between the participation of birds in the dispersion of the pathogen, and increases in the number of cases of human and animal granulocytic anaplasmosis over the past decade have been described in the literature (IOANNOU et al., 2009).

Analysis conducted in Europe showed that *A. phagocytophilum* genotypes could be clustered in at least four major groups called ecotypes. Each ecotype has unique geographical and host characteristics. One of these ecotypes, interestingly, was associated only with birds (JAHFARI et al., 2014).

Currently, there are no reports that elucidate the epidemiological situation of *Anaplasma* spp. in wild animals in the state of Paraná, southern Brazil. The aim of the present study was to report the first molecular isolation of *Anaplasma* sp. from a blood sample of a *Penelope obscura* (native bird species) found in the Guarapuava region, in Midwestern state of Paraná.

Between August and November 2014, 15 blood samples were collected from eleven different bird species. The animals were sent to the Wildlife Care Center of the State University of Midwest (UNICENTRO), situated in Guarapuava, state of Paraná, Brazil. Blood samples were collected during physical examination for clinical care. The number of samples collected from each bird species is shown in Table 1.

None of the birds presented tick infestations at physical inspection. Blood samples were collected by venipuncture, were placed in tubes containing 5% EDTA, and were then stored at -20 °C until they were used. Molecular assays were performed at the Immunoparasitology Laboratory of Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP, Jaboticabal). DNA extraction was performed using the QIAamp DNA Blood and Tissue Mini Kit (QIAGEN®), according to the manufacturer’s recommendations. A nested cPCR assay based on the 16S rRNA gene (MASSUNG et al., 1998) was performed to determine the presence of *Anaplasma* sp. DNA in the samples.

The amplified products were purified and sequenced in an automatic sequencer (ABI Prism 310 genetic analyzer® - Applied Biosystems/Perkin-Elmer) for confirmation. Sequences obtained were used for subsequent phylogenetic analysis. Trimming was performed using the Phred software (EWING & GREEN, 1998), which evaluates electropherograms produced during

Table 1. Number of birds sampled, according to the species and popular name.

Species	Popular name	Number of samples
<i>Ramphastos dicolorus</i>	Red-breasted toucan	3
<i>Pyrhura frontalis</i>	Maroon-bellied parakeet	1
<i>Caracara plancus</i>	Southern caracara	1
<i>Tigrisoma lineatum</i>	Rufescent tiger-heron	1
<i>Psittacara leucophthalmus</i>	White-eyed parakeet	1
<i>Amazona amazonica</i>	Orange-winged amazon	1
<i>Falco sparverius</i>	American kestrel	1
<i>Tyto furcata</i>	Barn owl	1
<i>Penelope obscura</i>	Dusky-legged guan	2
<i>Pionus maximiliani</i>	Scaly-headed parrot	2
<i>Rupornis magnirostris</i>	Roadside hawk	1

the sequencing. The quality of the peaks corresponding to each sequenced base was observed, and a probability of error was attributed to each of the samples. Bases with quality scores above 20 were considered to be used in the present study. The CAP3 software (<http://pbil.univ-lyon1.fr/cap3.php>) was used for alignment of the consensus sequence. BLAST software was used to analyze nucleotide sequences (BLASTn) in order to compare the sequences obtained with similar ones found in international databases (GenBank) (BENSON et al., 2002). Sequences saved in "FASTA" mode were aligned with other homologous sequences from the same sequenced gene retrieved from the GenBank databank using Multiple Alignment with Fast Fourier Transform (MAFFT) software v.7 (KATO & STANDLEY, 2013). Maximum likelihood analysis was performed using the RaxML cluster black box (STAMATAKIS et al., 2008), via the CIPRES portal (MILLER et al., 2010) with 1,000 bootstrapping replicates. The proportion of invariable sites was estimated using the GTRGAMMA+I evolution model. Phylogenetic tree editing and rooting (via an external group) was performed using the Treegraph 2.0.56-381 beta software (STÖVER & MÜLLER, 2010).

Amplification of a fragment of the 16S rRNA gene of *Anaplasma* spp. was obtained by nested cPCR in a single sample (6.66%) that was collected from a *P. obscura*. Phylogenetic inference based on Maximum Likelihood showed that the genotype detected in the *P. obscura* specimen from the Gurarapuava region, Brazil, was close to that of *Anaplasma* spp. that was detected in domestic cats in Brazil (KF964049) (Figure 1).

Ehrlichia spp. detected in wild birds by Machado et al. in 2012 was phylogenetically related to *Ehrlichia* sp. detected in wild Felidae by André et al. (2010), suggesting that wild birds may participate in the transmission cycle of hemoparasites between species. Machado et al. (2012) detected *Anaplasma* sp. phylogenetically associated with *A. phagocytophilum*, also by amplification of the 16S rRNA gene, in 14.28% of 21 bird samples, a higher percentage than the one found in the present study (6.66%). The possible reasons for this variation may be the use of fewer samples, the difference among species sampled,

or lower incidence of the pathogen in the area where the present study was conducted.

Ixodes sp. ticks, which are more commonly found in the northern hemisphere, are considered the main vectors of *A. phagocytophilum* (RYMASZEWSKA & GREINDA, 2008; WOLDEHIWET, 2010). However, in Brazil, this pattern has not been established. Although no ticks at any phase of life were found in the *P. obscura* specimen described in the present study, this bird species that belongs to the order galliformes remains on the ground most of the time, and this habit may facilitate tick infestation. A relationship between the occurrence of ticks in certain bird species and ground-feeding habits has already been reported (HASLE, 2013; DIAKOU et al., 2016).

Amblyomma longirostre ticks parasitizing *P. obscura* in the state of Paraná were described by Arzua et al. (2005), and this tick species was also reported parasitizing others wild bird species in southern and southeastern Brazil (SOARES et al., 2009; SANCHES et al., 2013). Moreover, *Rickettsia* was detected in *A. longirostre* found in wild birds from Paraná (PACHECO et al., 2012), suggesting a potential participation of this tick species in the transmission of hemoparasites among wild birds in this state.

According to Doan et al. (2013), evaluation of 16S rRNA gene is a sensitive molecular tool for the detection of circulating *Anaplasma* spp. genotypes. However, Paulauskas et al. (2012) stated that the use of *msp4* gene shows greater reliability in terms of phylogenetic positioning, as gene 16S rRNA is highly conserved, with few polymorphic positions. These findings reinforce the results obtained in the present study, once we reported the infection by an *Anaplasma* sp. genotype in a free-living bird specimen using the 16S rRNA gene. The sample was negative to additional target genes.

We believe that new genotypes of *Ehrlichia* and *Anaplasma* circulate in wild and domestic animals in Brazil. The pre-established PCR protocols targeting genes other than 16S rRNA have shown low accuracy in the identification of new Anaplasmataceae genotypes in Brazil (MURPHY et al., 1998; MASSUNG et al., 1998; KOCAN et al., 2000).

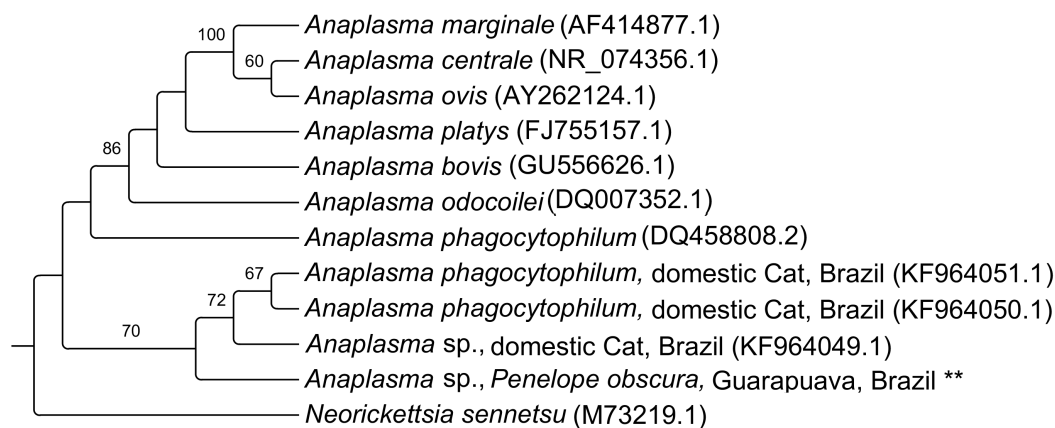


Figure 1. Phylogenetic position of the sequence obtained for *Anaplasma* sp. The tree was constructed using the maximum likelihood method and the GTRGAMMA+I model. Numbers indicate bootstrap values above 50 for branching support. Access numbers are indicated next to the sequences. **Sequence of the present study.

This pattern of positive results to Anaplasmataceae 16S rRNA gene in PCR assays and negative results to other target genes was already described in previous studies that aimed at detecting Anaplasmataceae in domestic and wild animals in Brazil (ANDRÉ et al., 2012, 2014, 2015; SACCHI et al., 2012). Blood samples showing low bacteremia, and an inadequate PCR protocol for a variant of *Anaplasma* sp. species may explain why variable results were obtained with different target genes (ANDRÉ et al., 2012).

The samples analyzed in this study were collected during the clinical inspection of birds sent to the Wildlife Care Center of UNICENTRO for medical care. Because of this, few birds were sampled, and only one sample from each animal could be collected. The detection threshold for genetic material of the pathogen in nested PCR, associated with possible low levels of bacteremia may also explain why we obtained negative results. These limitations reinforce the need for testing more samples, besides using more sensible techniques to elucidate the hypothesis raised in our study.

Further information regarding the vector is necessary. Moreover, other genes should be used for more concise results about the possible *Anaplasma* variant that may be found in wild animals in the state of Paraná. As information on infection by *Anaplasma* spp. in animals from Paraná State is lacking, data of the present study may contribute to the knowledge about the geographic distribution of Anaplasmataceae agents.

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