

Absence of Anaplasmatataceae DNA in Wild Birds and Bats from a Flooded Area in the Brazilian Northern Pantanal

Luana Gabriela Ferreira dos Santos¹, Tatiana Ometto², Jansen de Araújo², Luciano Matsumya Thomazelli², Leticia Pinto Borges¹, Dirceu Guilherme Ramos³, Edison Luis Durigon², Joao Batista Pinho⁴ and Daniel Moura de Aguiar^{1*}

¹Laboratório de Virologia e Rickettsioses, Hospital Veterinário, Universidade Federal de Mato Grosso, Brazil

²Laboratório de Virologia Clínica e Molecular NB3+, Departamento de Microbiologia, Instituto de Ciências Biomédicas II, Universidade de São Paulo, Brazil

³Laboratório de Parasitologia e Doenças Parasitárias, Hospital Veterinário, Universidade Federal de Mato Grosso, Brazil

⁴Laboratório de Ecologia de Aves, Instituto de Biociências, Universidade Federal de Mato Grosso, Brazil

Abstract

Birds and bats can be considered potential transmitters of some tick-borne diseases, since eventually they carry infected ticks in areas where transit. Pantanal ecosystem is the largest tropical wetland area of the world with more than 582 recorded avian species, contributing to the maintenance of different tick species. The aim of this study was to examine altogether 152 blood samples of several bird and bat species collected in a large flooded area of Pantanal for the presence of members from genera *Ehrlichia*, *Anaplasma* and *Neorickettsia*. None PCR product was obtained, what suggest that wild, domestic birds and bats from Pantanal region are unlikely to play a significant role in the maintenance of tick-borne agents and DNA survey from this species in birds may not be a reliable indicator of exposure.

Keywords: Tick-borne disease; Anaplasmatataceae; Fowl; Poconé; Brazil

Introduction

Birds and bats can act as reservoirs of several pathogens including those transmitted by ticks and flies. Some infections can be transmitted by trematode-vectors, which use freshwater snails and aquatic insects as intermediate hosts and insectivorous birds and bats as definitive hosts. They also can be considered as potential transmitters of some tick-borne diseases, mainly due to its migratory character, since eventually they carry the infected ticks in areas where transit. Agents that stand out are the bacteria from Anaplasmatataceae family that already have been widely associated with disease in humans and animals, with cycle involving invertebrate vectors. Pathogens such as *Ehrlichia*, *Anaplasma* and *Neorickettsia* are being increasingly recognized around the world in order to elucidate the importance of its infections and ability to cause illness in animals and/or human beings [1-4].

Anaplasmatataceae agents comprise obligate intracellular bacteria that can cause disease in humans and animals, whose cycle in the environment involves complex interactions between invertebrate vectors and vertebrate hosts [5]. There is ecological evidence that passerine birds, at least in principle, could be competent reservoirs for *A. phagocytophilum* as many avian species host larval and nymphal *I. scapularis* ticks [6,7]. Exposure of passerines to *A. phagocytophilum*-infected nymphs has been demonstrated [8], including infected larvae found attached to an American robin (*Turdus migratorius*) and a veery (*Catharus fuscescens*) [9]. However, direct evidence demonstrating birds as competent reservoirs of members from the family Anaplasmatataceae are still scarce.

For instance in Brazil, Machado et al. [1] was the only one that reported molecular detection of *A. phagocytophilum* in carnivorous and migratory birds. Additionally they also reported for the first time DNA of *E. chaffeensis* and an *Ehrlichia* species, phylogenetically related to *E. canis* in birds sampled in Mato Grosso and São Paulo state.

Neorickettsia risticii (formerly *Ehrlichia risticii*) belongs to the Anaplasmatataceae family and is the causative agent of Potomac Horse Fever (also known as Equine Monocytic Ehrlichiosis- EME), a severe

febrile disease affecting horses, typically found in endemic countries during the warmest months. In the environment, *N. risticii* infects trematodes from the genus *Acanthatrium* and *Lecithodendrium*. The lifecycles of these trematodes are not very clear, both are known to involve several stages that range from free-living cercaria to forms that infect invertebrates such as the miracidia and sporocysts (that infect freshwater snails) and the metacercaria (that infect aquatic insects) [10].

In enzootic areas of USA, a common pleurocerid snail, *Juga yrekaensis* is suspected to be involved in the life cycle of *N. risticii* [11]. Additionally, DNA from *N. risticii* has been detected in virgulate cercariae in lymnaeid snails (*Stagnicola* spp.) in virgulate xiphidiocercariae isolated from pleurocerid snails *Elimia livescens* and *Elimia virginica*, indicating that other species of freshwater snails may also harbor infected trematodes [12,13]. Adult forms of these trematodes develop in the intestine of insectivorous vertebrates such as bats and birds, therefore it has been suggested that certain species of bats and birds (swallows) may act as wild reservoirs of *N. risticii* [14,15]. Recently, molecular detection of *N. risticii* in bats of the species *Tadarida brasiliensis* was reported in Argentina [16].

In Brazil, spontaneous outbreaks of equine monocytic Ehrlichiosis (EME) were described in south region [17-19]. In Rio Grande do Sul state, snails from the genus *Heleobia*, harboring *Parapleurolophocercous*

***Corresponding author:** Daniel Moura de Aguiar, Laboratório de Virologia e Rickettsioses, Hospital Veterinário, Universidade Federal de Mato Grosso. Av. Fernando Correa, 2367, Boa Esperança, Cuiabá, Brazil, Tel: +55 65 36158627; E-mail: danmoura@ufmt.br

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cercarie, have been identified as carriers of *N. risticii* [18]. Three species are extremely abundant both in permanent streams and small rivers and on temporarily flooded areas of the Pantanal, namely *Marisa planogyra*, *Pomacea linearis* and *Pomacea scalaris* [20]. Apple snails *Pomacea lineata* are widespread in the tropical regions of Brazil as well as in the Pantanal wetland of Mato Grosso in the western part of the country. This species serve as food for many animals, mainly birds, fishes and caimans [21].

The Pantanal ecosystem is the largest tropical wetland area of the world, situated among two Brazilian states (Mato Grosso and Mato Grosso do Sul) and in portions of Bolivia and Paraguay. The Pantanal is characterized by an immense diversity, where more than 80 species of mammals [22] and 582 avian species, contributing to the maintenance of different tick species have already been recorded [23-25]. Furthermore the aquatic environment can favor the establishment of different species of trematodes, metacercariae and snails which jointly with birds and bats support the occurrence of *N. risticii* in the area. Nevertheless, the role of birds and bats in the epidemiology of tick- and water-borne diseases has been poorly studied, and the connection between birds, dispersal of infected ticks, rickettsial agents and zoonosis outbreaks need to be clarified. The aim of this study was to evaluate blood samples of several birds and bats species collected in the northern Brazilian Pantanal for the presence of members from genera *Ehrlichia*, *Anaplasma* and *Neorickettsia*.

Materials and Methods

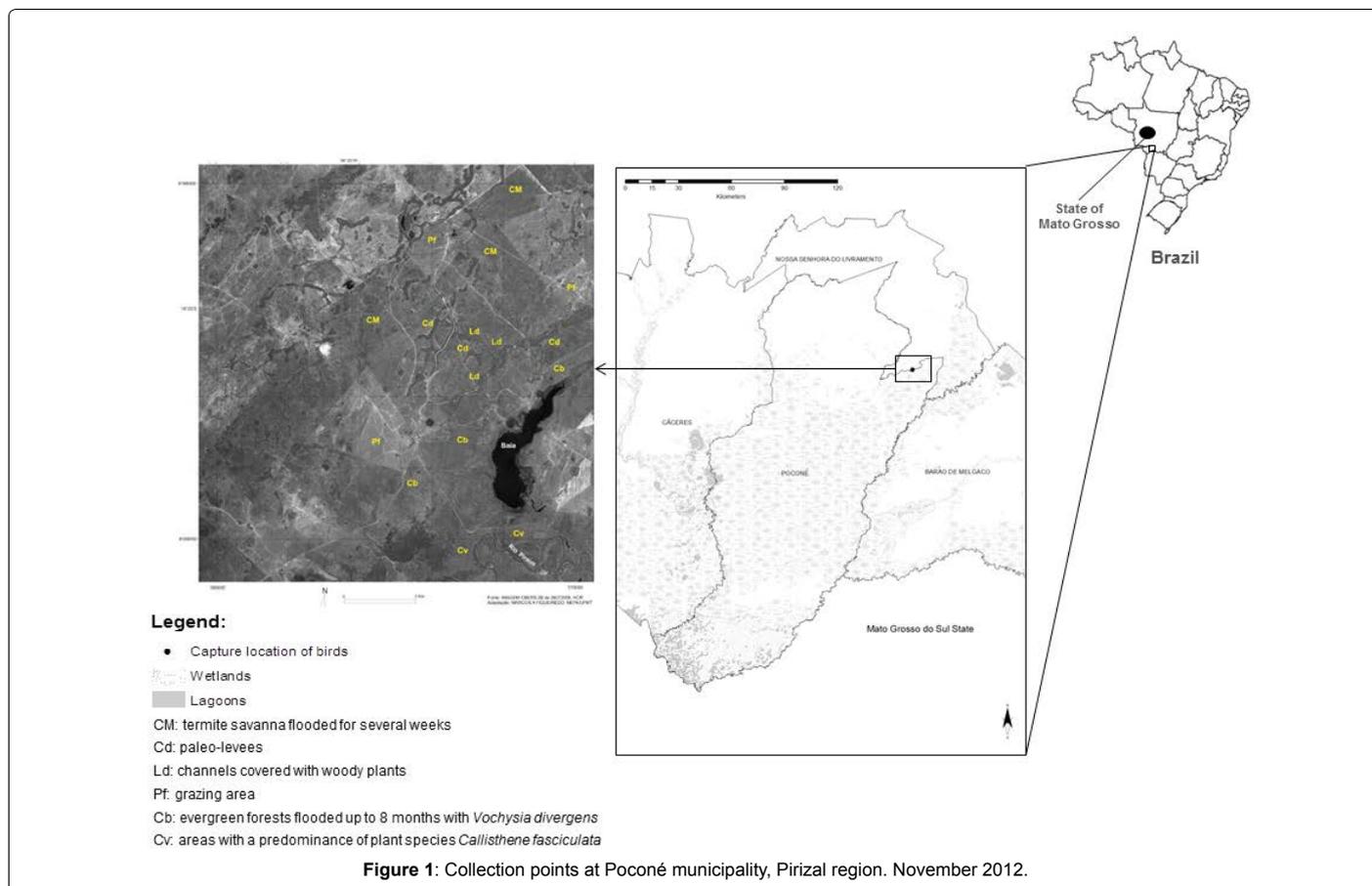
Samples of whole blood stored in cryopreserved bank were

evaluated. Blood samples were collected in the Pocone municipality at the locality called Pirizal (16°15'12"S 56°22'12"W; Figure 1) during November 2012, totaling 152 samples from domestic and wild birds and bats. Regarding tick infestation, no information was obtained from the samples studied. Blood samples were subjected to DNA extraction protocol [26] using guanidine thiocyanate and DNA samples were tested individually for detection of *Ehrlichia* spp., *Anaplasma* spp. and *N. risticii*, according to the protocols described in Table 1.

All products obtained in the reactions were subjected to gel electrophoresis in horizontal 1.5% agarose gel stained with ethidium bromide (0.5 mL/ml - Invitrogen®) and 1X TBE running buffer pH 8.0 (44.58 M Tris-base, 0.44 M boric acid, 12.49 mM EDTA) to 110v/50mA with subsequent visualization under ultraviolet light (UV) in a darkroom. In order to prevent PCR contamination, DNA extraction, reaction set-up, PCR amplification and electrophoresis were performed in separate rooms. DNA of Sao Paulo strain of *E. canis*, *A. platys* from dog blood diagnosed with anaplasmosis and strain Illinois of *N. risticii* gently provide by Dr. S. Dumler was used as positive control in all reaction. Samples were collected under license granted by the Authorization System and Biodiversity Information - SISBIO number 33602-1.

Results and Discussion

One hundred and sixteen species of wild birds were captured and characterized [27-30]. Thirteen domestic fowl (*Gallus gallus*) were also evaluated, although there are no previous reports of Anaplasmatacea pathogens in this avian species. In addition, twenty-three blood



Pathogen	Primer*	Sequence(5' - 3')	References
<i>Ehrlichia</i> spp.	dsb 330 (1 ^s) dsb 720 (1 ^s and 2 nd) dsb 380 (2 nd)	GATGATGTTTGAAGATATSAACAAAT CTATTTACTTCTTAAAGTTGATAWATC ATTTTITAGRGATTTTCCAATACTTGG	[38]
<i>Anaplasma</i> spp	gE3a (1 ^s) gE10R (1 ^s) gE2 (2 nd) gE9F (2 nd)	CACATGCAAGTCGAACGGATTATTC TTCCGTTAAGAAGGATCTAATCTCC GGCAGTATTAAGCAGCTCCAGG AACGGATTATTCCTTATAGCTTGCT	[39]
<i>Neorickettsia risticii</i>	ER3(1 ^s) ER2(1 ^s) ER3a (2 nd) ER2a (2 nd)	ATTTGAGAGTTTGATCCTGG GTTTTAAATGCAGTCTTGG CTAGCGGTAGGCTTAAC CACACCTAACTTACGGG	[40]

* 1^s = first reaction (PCR), 2nd = second reaction (nPCR)

Table 1: Primers and protocols used in the study of *Ehrlichia* spp., *Anaplasma* spp. and *Neorickettsia risticii* in birds and bats from the northern Brazilian pantanal.

Family	Nomenclature	Common name	Feeding	Character*	N*
Columbidae	<i>Columbina picui</i>	Rolinha-picui	Granivorous and Frugivorous	Residents	2
	<i>Patagioenas cayennensis</i>	Pomba-galega	Granivorous and Frugivorous	Residents	
Corvidae	<i>Cyanocorax cyanomelas</i>	Gralha-do-Pantanal	Omnivorous	Residents	1
Cuculidae	<i>Coccyzus euleri</i>	Papa-lagarta-de-euler	Insectivorous	INTRA	1
Dendrocolaptidae	<i>Dendroplex picus</i>	Arapaçu-de-bico-branco	Insectivorous	Residents	4
Emberizidae	<i>Volatinia jacarina</i>	Tiziu (Esporofilo)	Granivorous	INTRA	3
	<i>Sporophila caerulescens</i>	Coleirinho	Granivorous	INTRA	
	<i>Sporophila angolensis</i>	Curio	Granivorous	INTRA	
Furnariidae	<i>Furnarius rufus</i>	João-de-barro	Insectivorous	Residents	14
	<i>Certhiaxis cinnamomeus</i>	Curutié	Insectivorous	Residents	
	<i>Cranioleuca vulpine</i>	Arredio-do-rio	Insectivorous	Residents	
	<i>Synallaxis albilora</i>	João-do-pantanal	Omnivorous	Residents	
Galbulidae	<i>Galbula ruficauda</i>	Ariramba-de-cauda-ruiva	Insectivorous	Residents	1
Icteridae	<i>Cacicus cela</i>	Xexéu (guaxo amarelo)	Omnivorous	Residents	4
	<i>Agelaioides badius</i>	Asa-de-telha	Frugivorous	Residents	
Parulidae	<i>Basileuterus flaveolus</i>	Canário-do-mato	Insectivorous	Residents	2
Phasianidae	<i>Gallus gallus</i>	Galinha	Omnivorous	Residents	13
Picidae	<i>Veniliornis passerinus</i>	Picapauzinho-anão	Insectivorous	Residents	5
	<i>Picumnus albosquamatus</i>	Pica-pau-anão-escamado	Insectivorous	Residents	
Pipridae	<i>Antilophia galeata</i>	Soldadinho	Frugivorous	Residents	8
	<i>Neopelma pallescens</i>	Fruxo-do-cerradão	Insectivorous	Residents	
Poliopitilidae	<i>Poliopitila dumicola</i>	Balança-rabo-de-máscara	Insectivorous	Residents	2
Rhynchocyclidae	<i>Todirostrum cinereum</i>	Ferreirinho-relógio	Insectivorous	Residents	12
	<i>Herpsilochmus longirostris</i>	Chorozinho-de-bico-comprido	Insectivorous	Residents	
	<i>Poecilotriccus latirostris</i>	Ferreirinho-de-cara-parda	Insectivorous	Residents	
	<i>Hemitriccus margaritaceiventer</i>	Olho-de-ouro	Insectivorous	Residents	
Thamnophilidae	<i>Cercomacra melanaria</i>	Chororó-do-pantanal	Insectivorous	Residents	7
	<i>Thamnophilus pelzelni</i>	Choca-do-planalto	Insectivorous	Residents	
Thraupidae	<i>Ramphocelus carbo</i>	Pipira-vermelha	Omnivorous	Residents	21
	<i>Saltator similis</i>	Trinca-ferro	Omnivorous	Residents	
	<i>Paroaria capitata</i>	Galo-da-campina-pantaneiro	Omnivorous	Residents	
	<i>Tachyphonus rufus</i>	Pipira-preta	Omnivorous	Residents	
	<i>Lanio penicillatus</i>	Pipira-da-taoca	Omnivorous	Residents	
Tityridae	<i>Tityra inquisitor</i>	Anhambé-de-boina	Omnivorous	Residents	1
Turdidae	<i>Turdus rufiventris</i>	Sabiá-laranjeira	Omnivorous	Residents	1
Tyrannidae	<i>Myiarchus tyrannulus</i>	Maria-cavaleira-de-rabo-enferrujado	Omnivorous	INTRA	27
	<i>Elaenia chilensis</i>	Guaracava-de-crista-branca	Omnivorous	INTRA	
	<i>Cnemotriccus fuscatus</i>	Guaracavuçu	Insectivorous	INTRA	
	<i>Myiarchus ferox</i>	Maria-cavaleira	Omnivorous	INTRA	
	<i>Myiarchus swainsonii</i>	Irré	Omnivorous	INTRA	
	<i>Myiopagis viridicata</i>	Guaracava-de-crista-alaranjada	Omnivorous	INTRA	
Phyllostomidae	<i>Glossophaga soricina</i>	Morcego-beija-flor	Nectar-feeding	-	23

* INTRA: Intracontinental migrant southern and northern South America.

*N: collected number

Table 2: Birds and bats collected in Pantanal region and main features.

samples from nectar-feeding bat *Glossophaga soricina* (Phyllostomidae) [31] were collected. All wild birds collected and their main features are shown in the Table 2.

Wild birds may play a role as carriers of Anaplasmataceae agents in Brazil as previously described by Machado et al. [1], therefore the choice of collection points were intended to catch birds in different

subtypes of the Pantanal and to cover the major habitat types in each region (data not shown), according to the applied model RAPELD PPBIO [32]. Despite the wide range of collections, in our study no PCR product was obtained from either *Anaplasma* spp., *Ehrlichia* spp. or *N. risticii*.

Machado et al. [1] reported the presence of DNA of *A. phagocytophilum*, *E. chaffeensis* and an *Ehrlichia* species closely related to *E. canis* in carnivorous avian blood samples, but ectoparasites were not found parasitizing birds. Furthermore, the positive birds had carnivorous habits while no other one avian family were detected with Anaplasmataceae DNA. These finding infer that the absence of detection of pathogens in our study may be related to the fact that the birds were not able to develop bacteremia or did not have carnivorous habits, probably because feeding habits of birds may imply in differences in the occurrence of infection, once the eating habits can influence the willingness of birds to certain pathogens and these habits can vary widely between birds depending on the region studied [33]. Johnston et al. [4] in an experimental study showed that the American robin (*Turdus migratorius* (L.)) are able to infect *I. scapularis* larval feeding ticks without developing bacteremia, specific antibodies or significant illness because of exposure.

Due to the fact of the samples were derived from cryopreserved blood banks, information on tick infestation could not be obtained. Studies regarding Anaplasmataceae agents infection in ticks collected from wild birds has been performed for a better understanding of the dynamics between birds and ticks and there is a concordance between the authors that the involvement of birds in the ecology and epidemiology of tick-borne diseases still unclear [34-37].

Bjoersdorff et al. [2] found a large number of migratory birds infested with *Ehrlichia* positive *I. ricinus* ticks and *Ehrlichia* 16S rRNA nucleotide sequences found in these ticks were identical to the HGE *Ehrlichia* found in humans in Sweden and Slovenia, what suggest that birds may play an important role in the dispersal of *I. ricinus* infected and may contribute to the distribution of granulocytic ehrlichiosis. Besides, none *I. ricinus* larvae infected were observed by Bjoersdorff et al. [2] which may suggest that migratory birds are incompetent reservoirs of *Ehrlichia* spp [38] and act just as long-distance carriers of infected ticks. Transovarial transmission of *Ehrlichia* and *Anaplasma* species [39] has not been demonstrated, so reservoir hosts are necessary to maintain the life cycle in the environment [5].

In the environment, *N. risticii* infects trematodes and adult forms may develop in the intestine of insectivorous vertebrates. Cicuttin et al. [16] related the first detection of *N. risticii* in internal organs of insectivorous bats (Brazilian free-tailed bat *Tadarida brasiliensis*) from Argentina and associated this species as reservoirs of these pathogens. *G. soricina* bats captured in the Pantanal region are known for nectar-feeding habits, thus this feature impairs contact with infected trematodes and this species may not act as wild reservoirs of *N. risticii*.

In view of the potential presence of *N. risticii* [40] especially in the Pantanal area, further studies involving bats species in this region should elucidate the potential involvement in the transmission of this agent in Brazil. Nevertheless a more detailed study should be analyzed as the presence of species of trematodes and snail in the Pantanal, since snails of the genera reported in the Pantanal have been found in properties with the occurrence of EME in other regions of Brazil [18,19].

Our results suggest that wild and domestic birds and bats from Pantanal region are unlikely to play a significant role in the maintenance of Anaplasmataceae agents and DNA survey from this species may

not be a reliable indicator of exposure. More studies investigating the ecological factors and ticks burden in avian or bats hosts are needed to improve the understanding on the dynamics of Anaplasmataceae diseases in the relative area. Future efforts should include ticks identification in birds and bats from the Pantanal and experimental evaluations to understand the mechanisms that drives bird exposure risk and susceptibility to ticks and tick-borne diseases.

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