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Novel *Anaplasma* and *Ehrlichia* organisms infecting the wildlife of two regions of the Brazilian Amazon

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ABSTRACT

During 2009–2012, wild animals were sampled in the Amazon biome of Brazil. Animal tissues and blood were tested by polymerase chain reaction (PCR) assays targeting DNA of the bacterial family Anaplasmataceae (genera *Anaplasma, Ehrlichia*, *Wolbachia*) and the genus *Borrelia.* Overall, 181 wild animals comprising 36 different species (2 reptiles, 5 birds, and 29 mammals) were sampled. All birds and reptiles were negative by all PCR assays, as well as all mammals for the *Borrelia* PCR assay. Anaplasmataceae agents were searched by PCR assays targeting two different genes, the ribosomal 16S rRNA gene and the protein-coding *dsb* gene. Three *dsb* closely related haplotypes were generated from 3 white-lipped peccaries (*Tayassu pecari*). In a phylogenetic analysis inferred from *dsb* partial sequences, these haplotypes grouped with previously reported *Ehrlichia* haplotypes from jaguar (*Panthera onca*) and horse from Brazil, suggesting that they could all represent a single species, yet to be properly characterized. A unique *dsb* haplotype was generated from a sloth (*Bradypus tridactylus*), and could also represent a different *Ehrlichia* species. All these *dsb* haplotypes formed a clade sister to the *Ehrlichia ruminantium* clade. Three distinct 16S rRNA gene haplotypes were generated from a wild guinea pig (*Cavia* sp.), a woolly mouse opossum (*Micoureus demerarae*), and two from robust capuchin monkeys (*Sapajus* sp.). In a phylogenetic analysis inferred from 16S rRNA gene partial sequence, these haplotypes grouped within the *Wolbachia* clade, and are likely to represent *Wolbachia* organisms that were infecting invertebrate metazoarians (e.g., filarids) associated with the sampled mammals. Two deer (*Mazama americana*) samples yielded two distinct 16S rRNA gene sequences, one identical to several sequences of *Anaplasma bovis,* and an unique sequence that grouped in a clade with different *Anaplasma* species. Our results indicate that a variety of genetically distinct Anaplasmataceae organisms, including potentially new *Ehrlichia* species, circulate under natural conditions in the Amazonian wildlife.

1. Introduction

The Anaplasmataceae family includes bacteria of the genera *Anaplasma, Ehrlichia, Neorickettsia*, and *Wolbachia*, from which the first two encompass tick-borne agents of veterinary and public health significance world widely (Dumler et al., 2001). Studies during the last two decades have reported a number of novel Anaplasmataceae agents in the northern hemisphere, where most of investigations have been done (Reeves et al., 2008; Pritt et al., 2011; Sainz et al., 2015; Eisen et al., 2017). In contrast, our knowledge on the diversity of this bacterial family has had relatively little advances during this new century in South America, where most of the studies have been concentrated on agents, such as *Anaplasma marginale* and *Ehrlichia canis* in cattle and domestic dogs, respectively (Ruybal et al., 2009; Vieira et al., 2011; Silva et al., 2015). Noteworthy, novel agents were recently described in Brazil, as for example, *Ehrlichia minasensis* in cattle (Aguiar et al., 2014; Cabezas-Cruz et al., 2016), *Ehrlichia* sp. strain Jaguar infecting jaguars (*Panthera onca*) (Widmer et al., 2011), *Ehlichia* sp. strain fox infecting the crab-eating fox (*Cerdocyon thous*) (Almeida et al., 2012), and *Ehrlichia* sp. strain horse infecting horses (Vieira et al., 2016). In addition, there have had reports of different genotypes of *Anaplasma* and *Ehrlichia* agents infecting domestic dogs, wild carnivores, deer, and birds

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Table 1
PCR primers used in the present study.

Gene/Primers	Target organisms	Primer sequence (5′–3′)[a]	Amplicon size (nt)	Reference
16S rRNA	Anaplasmataceae			
EHR16SD		F-GGTACCYACAGAAGAAGTCC	344 pb	Inokuma et al. (2000)
EHR16SR		R-TAGCACTCATCGTTTACAG		
dsb	*Ehrlichia*			
DSB-330		F-GATGATGTTTGAAGATATSAAACAAAT	401 pb	Doyle et al. (2005)
DSB-720		R-CTATTTTACTTCTTAAAGTTGATAWATC		
DSB-380		F-ATTTTTAGRGATTTTCCAATACTTGG[b]	349 pb	Almeida et al. (2013)
		R-CTATTTTACTTCTTAAAGTTGATAWATC[b]		
flab	*Borrelia*			
FlaLL		F-ACATATTCAGATGCAGACAGAGGT	665 pb	Stromdahl et al. (2003)
FlaRL		R-GCAATCATAGCCATTGCAGATTGT		
FlaLS		F-AACAGCTGAAGAGCTTGGAAT[b]	354 pb	Stromdahl et al. (2003)
FlaRS		R-CTTTGATCACTTATCATTCTAATAGC[b]		

[a] F: forward; R: reverse.
[b] Used in a nested or heminested reaction.

(Santos et al., 2011; André et al., 2012; Machado et al., 2012; Sacchi et al., 2012; Silveira et al., 2012; Mongruel et al., 2017). All these reports were from the Brazilian biomes Atlantic rainforest, Cerrado or Pantanal.

The bacterial genus *Borrelia* comprises a number of tick-borne organisms that infect reptiles, birds, and/or mammals in all continents of the world, including Antarctica (Olsen et al., 1995; Caimano, 2006). While a great number of genospecies of the *Borrelia burgdorferi* sensu lato group have been reported in temperate areas of the world, borrelial organisms of the relapsing fever group have been reported in temperate and tropical areas. Two relapsing fever *Borrelia* species are known to occur in Brazil, *Borrelia anserina* infecting chickens and transmitted by the argasid tick *Argas miniatus* (Ataliba et al., 2007), and *Borrelia theileri* infecting livestock and transmitted by *Rhipicephalus microplus* (Martins et al., 1996; Yparraguirre et al., 2007). While there have been a few reports of DNA of the *Borrelia burgdorferi* sensu lato group in Brazil (Mantovani et al., 2012; Gonçalves et al., 2014), further studies are required to confirm the presence of these agents in the country.

Nearly half of the Brazilian land is occupied by the Amazon biome, which is the most diverse tropical forest of the world, with a great diversity of wildlife (Mittermeier et al., 2003). Incredibly, virtually nothing is known about infection of the Amazonian wildlife by vector-borne bacteria of the Anaplasmataceae family or the genus *Borrelia*. This scenario motivated the present study, which performed a preliminary investigation of the infection of Anaplasmataceae and borrelial agents in different free-ranging vertebrate wild species (reptiles, birds, and mammals) that were obtained (convenience sampling) in two large areas of the Brazilian Amazon.

2. Materials and methods

2.1. Ethical statements

This work was authorized by the Brazilian Institute of Environment and Natural Resources (IBAMA authorization no. 23225-1 and 21526-1) and the Indian National Foundation (FUNAI authorization no. 45/AAEP/10–Process no. 2433/07), and was approved by the Ethical Committee of Animal Use of the Faculty of Veterinary Medicine of the University of São Paulo (protocol no. 1747/2009).

2.2. Samples

Wild animals were sampled in two areas within the Amazon biome of Brazil. In one area in Mato Grosso state (central-western Brazil), samples comprised animals that were hunted by Indians during September 2010 to June 2012 in the Tapirapé Indian Reserve, within Confresa Municipality (10°36′ to 10°52′S; 51°10′ to 51°21′W). In another area in Pará state (northern Brazil), samples comprised road-killed animals during February 2009 to November 2011, alongside the BR163 highway, between Km 50 and 217 within Santarém (02°24′S; 54°42′W) and Rurópolis (04°05′S; 54°54′W) municipalities. A map showing these sampling localities have been reported elsewhere (Soares et al., 2015).

2.3. Molecular tests

From each animal, fragments of internal organs or blood samples were collected and kept frozen at −20 °C until being processed in the laboratory. DNA extraction of blood or fragments of lung or liver was conducted with the DNeasy Tissue and Blood Kit (Qiagen, Chatsworth, CA, USA) according to manufacturer's instructions. Blank tubes containing water were always included as a contamination control during DNA extraction. The concentration of extracted DNA was measured in a spectrophotometer UV (Bio Photometer plus, Eppendorf, Hamburg, Germany). Only samples with at least 20 ng/μl of DNA were subjected to PCR assays. Tissue or blood DNA samples were individually tested by a battery of PCR assays targeting bacteria of the genera *Anaplasma, Ehrlichia*, and *Borrelia*. PCR was performed with family-specific or genus-specific primers shown in Table 1. In each PCR assay, negative controls (non-template water and the blank tubes from the DNA extraction step) and an appropriate positive control sample (DNA of *Ehrlichia canis* or *Borrelia anserina*) were run together with the wild animal DNA samples. PCR products of the expected size for each assay were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced in an automatic sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyser, Foster City, CA, USA) according to the manufacturer's protocol, using the same primers (forward and reverse) used for the PCR. Partial sequences obtained were submitted to BLAST analysis (www.ncbi.nlm.nih.gov/blastwww.ncbi.nlm.nih.gov/blast) to determine the closest similarities in GenBank.

2.4. Phylogenetic analyses

Partial 16S rRNA sequences generated in this study were aligned with corresponding sequences of Anaplasmataceae agents available in GenBank, while partial *dsb* sequences were aligned with corresponding sequences of related genotypes and *Ehrlichia* species from GenBank. The two alignments were performed by using the CLUSTAL X (Thompson et al., 1997) and adjusted manually using GeneDoc (Nicholas and Nicholas, 1997). For phylogenetic analyses, maximum parsimony (MP) method implemented in PAUP version 4.0b10 (Swofford, 2002) was used to obtain the best tree topology. Confidence values for individual branches of the resulting trees were determined with 500 replicates to