

Article

Molecular Survey of *Anaplasmataceae* Agents and *Coxiellaceae* in Non-Hematophagous Bats and Associated Ectoparasites from Brazil

Priscila Ikeda ¹, Jaire Marinho Torres ², Ana Julia Vidal Placa ¹, Victória Valente Califre de Mello ¹, Elizabete Captivo Lourenço ³, Heitor Miraglia Herrera ², Carina Elisei de Oliveira ², Claudia Hemsley ⁴, Richard W. Titball ⁴, Rosângela Zacarias Machado ¹ and Marcos Rogério André ^{1,*}

- ¹ Laboratório de Imunoparasitologia, Departamento de Patologia, Reprodução e Saúde Única, Universidade Estadual “Júlio de Mesquita Filho”, Jaboticabal 14884-900, São Paulo, Brazil; priscila.ikeda@unesp.br or pri Ikeda@gmail.com (P.I.); anajuliaplaca@hotmail.com (A.J.V.P.); vick_vvc@hotmail.com (V.V.C.d.M.); rz.machado@unesp.br (R.Z.M.)
- ² Laboratório de Biologia Parasitária, Programa de Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande 79117-900, Mato Grosso do Sul, Brazil; jairemarinho@hotmail.com (J.M.T.); herrera@ucdb.br (H.M.H.); carinaelisei@ucdb.br (C.E.d.O.)
- ³ Laboratório de Ecologia de Mamíferos, Universidade do Estado do Rio de Janeiro, Rio de Janeiro 20550-013, Rio de Janeiro, Brazil; beteclouren1205@yahoo.com.br
- ⁴ College of Life and Environmental Sciences—Biosciences, University of Exeter, Exeter EX4 4QD, UK; C.Mueller@exeter.ac.uk (C.H.); R.W.Titball@exeter.ac.uk (R.W.T.)
- * Correspondence: mr.andre@unesp.br; Tel.: +55-(16)-3209-7302; Fax: +55-(16)-3202-4275



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Abstract: The *Anaplasmataceae* family (order *Rickettsiales*) encompasses obligately intracellular bacteria of the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia*. Together with *Coxiella burnetii* (*Coxiellaceae* family, order *Legionellales*), these bacteria represent important causative agents of diseases in humans and animals. The scarcity of studies that investigated the occurrence of these agents in bats and their associated ectoparasites, emphasizes the need to achieve a better understanding of the role of these animals in the maintenance of such bacteria. Herein, 418 samples (133 blood, 135 spleen, and 150 ectoparasites) are collected from 135 non-hematophagous bats belonging to 12 species in a periurban area of Campo Grande city, Mato Grosso do Sul state, midwestern Brazil. In the results, 1.65% (7/418), 12.04% (50/418), and 13.63% (57/418) of samples are positive in PCR assays for *Anaplasma* spp. (16S rRNA gene), *Ehrlichia* spp. (*dsb* gene), and *Neorickettsia* spp. (16S rRNA gene), respectively. *Anaplasma* spp. and *Neorickettsia* spp. are detected in one (5.26%) *Ornithodoros hasei* tick larva. *Ehrlichia* spp. is detected in 14% of bat flies (represented by *Megistopoda aranea*, *Trichobius costalimai*, and *Strebla hertigi*), 6% of tick larvae (*O. hasei*), 12% of Spinturnicidae mites (represented by *Periglischrus* sp., *P. torrealbai*, and *P. acutisternus*), and 38% of Macronyssidae mites (*Steatonyssus* sp.). The obtained sequences are observed to be similar to *Anaplasma phagocytophilum* (97.42–97.6% identified), *Ehrlichia minasensis* (96.73–100% identified), *Neorickettsia risticii* (96.7–100% identified), and *Neorickettsia findlayensis* (95.07–100% identified) by BLASTn analyses, and closely related to *Ehrlichia ruminantium* by phylogenetic analyses based on the *gltA* gene. No bat samples (blood/spleen) are positive in the qPCR assay for *C. burnetii* based on the *IS1111* gene. The present work shows, for the first time, the occurrence of *Anaplasmataceae* in bats and associated ectoparasites (ticks, mites, and bat flies) from Brazil.

Keywords: *Anaplasma* spp.; *Ehrlichia* spp.; Chiroptera; *Coxiella burnetii*; *Neorickettsia* spp.

1. Introduction

Approximately 20% of known mammal species belong to the order Chiroptera, which composes the second largest group of mammals in the world [1]. Currently, there are 1400 species described worldwide. From those, 181 bat species are officially registered in

Brazil and distributed in 68 genera and nine families [2]. Even though bats are considered as important reservoirs, hosts, and sources of infection for various zoonotic agents (mainly viruses [3]), little is known about their role in the maintenance of vector-borne bacteria, such as *Ehrlichia* spp., *Anaplasma* spp., *Neorickettsia* spp., and *Coxiella burnetii*.

The *Anaplasmataceae* family (order *Rickettsiales*) comprises mandatory intracellular agents of veterinary and medical importance. The family comprises the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Neoehrlichia*, and *Wolbachia* [4–6]. Among the most common diseases caused by this group of bacteria, human granulocytic anaplasmosis (HGA) and human granulocytic ehrlichiosis (HME) affect humans and cause similar clinical symptoms that usually start after a tick bite event [7–9].

The main *Anaplasmataceae* agents associated with animal diseases are *Ehrlichia canis* and *Anaplasma platys*, which cause canine monocytic ehrlichiosis and canine cyclic thrombocytopenia; *Anaplasma marginale*, which causes bovine anaplasmosis; and *Neorickettsia risticii*, which causes the Potomac horse fever (PHF).

Up to 20 tick species are involved in the transmission of *A. marginale* and *A. phagocytophilum*; the main tick genera are represented by *Ixodes* spp. and *Haemophysalis* spp. (for *A. phagocytophilum*), *Dermacentor* spp. (for *A. phagocytophilum* and *A. marginale*), and *Rhipicephalus* spp. and *Hyalomma* spp. (for *A. marginale*) [9,10]. While *E. canis* is transmitted by the tropical lineage of *Rhipicephalus sanguineus sensu lato* [11] and *Amblyomma* spp., ticks are involved in the transmission of *E. ewingii*, *E. chaffeensis*, and *E. ruminantum* [12,13]. *Ehrlichia minasensis* has been primarily isolated from *Rhipicephalus microplus* and naturally infected cattle [5].

Despite being a sister clade to *Ehrlichia*, '*Candidatus Neoehrlichia* spp.' is not recognized as a new genus, mainly due to the difficulty of isolation [14–17]. Additionally, a plethora of uncultured agents was also described in the literature with the '*Candidatus*' status [18–29].

Neorickettsia genera encompasses obligatory intracellular bacteria that, differently from the other *Anaplasmataceae* agents, are endosymbiotic within digeneans and depend on trematodes for their life cycle [30]. Four diseases are related to these agents: Potomac horse fever (PHF) caused by *N. risticii*; salmon poisoning disease in dogs, which is mainly caused by *N. helminthoeca*; Elokomin fluke fever (EFF) in canids and bears, which is caused by *N. elokominica*; Sennetsu fever in humans caused by *N. sennetsu* agent. Recently, Teymournejad et al. (2020) [31] described a new species of *Neorickettsia* (i.e., *Neorickettsia findlayensis*) in Ontario, Canada, causing PHF disease based on serological evidence, whole genome sequencing, and experimental infection of horses. In Brazil, *N. risticii* was molecularly detected in horses from Rio de Janeiro, which presented with hypoalbuminemia, high levels of creatine kinase, and lactate dehydrogenase [32], as well in equids in the state of Rio Grande do Sul, where outbreaks of equine monocytic ehrlichiosis were reported [33,34].

Studies on the occurrence of *Ehrlichia* spp. and *Anaplasma* spp. in bats are scarce. For instance, *A. phagocytophilum* DNA was already reported in bat fecal samples in France [35] and in *Ixodes simplex* parasitizing bats in Hungary and Romania [36]. The DNA of *A. phagocytophilum* and *Ehrlichia* spp. was found in *Pipistrellus pipistrellus* and *P. kuhlii* bats, respectively, in Russia [37]. *Ehrlichia* spp. was also molecularly detected in bat-related *Argas vespertilionis* ticks in France [38] and *Brachyphylla cavernarum* bats in Saint Kitts Island [39]. *A. vespertilionis* ticks collected from bats in different counties of England were also positive for *Ehrlichia/Anaplasma* [40]. The detection of these agents in bats and associated ectoparasites in human-shared sites [35,38] contributed to an increased concern about the role of these animals as reservoirs and in the spread of potentially zoonotic diseases.

On the other hand, bats species have been implicated as important hosts for *N. risticii*. This agent was detected in bat tissues and/or associated trematodes in the USA, Argentina, Egypt, Philippines, and the Netherlands [41–45]. Additionally, this agent was detected in fecal samples collected from a variety of bats from Europe [46].

Coxiella burnetii (order *Legionellales*), the etiological agent of Q fever in humans, is a Gram-negative bacterium that parasites monocytes and macrophages [47]. It can be found in feces, milk, urine, and abortion products, such as placenta, where it is possible to find

a high concentration of the bacteria [48]. This agent can cause abortion and infertility in cattle and goats [49]. In humans, while the acute phase is characterized by hepatitis or, more unusually, pneumonia, the most frequent clinical manifestation in the chronic phase is endocarditis [47,50]. This agent was already described in bats and related ectoparasites from Algeria, South Africa, Chile, and Brazil [51–53].

Due to the importance of *Anaplasmataceae* agents and *C. burnetii* in animal and human health and the high representativeness of the order Chiroptera in Brazil, the present study aims to molecularly survey these agents in non-hematophagous bats and their ectoparasites sampled in midwestern Brazil.

2. Results

A total of 135 bats were captured and DNA samples obtained from 135 spleen tissues, 133 blood, and 150 ectoparasites (including 64 bat flies, 46 Spinturnicidae mites, 21 Macronyssidae mites and 19 tick larvae) were positive in the PCR assays for the endogenous genes (Supplementary Table S1).

All DNA samples obtained from spleen fragments, whole blood samples, and ectoparasites, totaling 418 samples, were negative for *Ehrlichia* spp. and *Anaplasma* spp. when tested for the *groEL* gene by a multiplex real-time PCR. Seven (1.67%) out of the 418 samples submitted to the protocol, using conventional nested PCR targeting the 16S rRNA gene for *Anaplasma* spp., were considered positive, presenting a single band with low intensity (Supplementary Table S2). Out of these, three samples were represented by spleen fragments from *Platyrrhinus lineatus* individuals, three from blood samples of *Phyllostomus discolor* specimens, and one from the *Ornithodoros hasei* tick larvae, which was collected from a negative *P. discolor* individual. Out of all positive samples, only one amplicon obtained from *O. hasei* was sequenced, due to the low intensity of bands in agarose gel electrophoresis. When analyzed by BLASTn analysis, the obtained sequence (542 bp) showed 97.6% identity (100% query coverage; E-value = 0.0) with the *A. phagocytophilum* detected in a deer (*Hydropotes inermis*) from the Republic of Korea (Accession number: KR611598.1) and 97.42% of identity with *A. phagocytophilum* and *A. bovis* detected in *Haemaphysalis longicornis* (100% of query coverage; E-value = 0.0), sampled in South Korea (Accession numbers: GU046565.1 / GU064901.1).

Out of the seven samples showing positivity in the nested PCR for *Anaplasma* spp., none showed positivity neither when submitted to quantitative real time PCR (qPCR) for *A. phagocytophilum* based on the *msp-2* gene nor in PCR assays targeting ITS 23S-5S and *groEL*. On the other hand, one blood sample obtained from a *P. discolor* individual was positive in the PCR targeting the *gltA* gene and sequenced. However, when tested by BLASTn, the sequence obtained (620 bp) showed to be similar (83.15% of identity) to *Ehrlichia* sp. identified in a tick from China (KJ410275.1) (100% of query coverage; E-value = 4×10^{-155}).

Regarding the PCR targeting the *dsb* gene for *Ehrlichia* spp., 50 (11.96%) out of the 418 samples were positive: ten (20%) blood samples (collected from five *Artibeus planirostris*, four *P. discolor*, and one *Eptesicus furinalis*), eight (16%) spleen fragments (collected from three *A. planirostris*, two *Artibeus lituratus*, two *P. lineatus*, and one *Carollia perspicillata*), seven (14%) bat flies [namely *Megistopoda aranea* (n = 3), *Trichobius costalimai* (n = 2), and *Strebla hertigi* (n = 2)], three (6%) tick larvae (*O. hasei*), six (12%) Spinturnicidae mites (three *Periglischrus* sp., two *Periglischrus torrealbai* and one *Periglischrus acutisternus*), and 19 (38%) Macronyssidae mites (*Steatonyssus* sp.). Six animals had more than one positive sample, while animal #7 (*A. planirostris*) showed positivity in both spleen and blood samples. All the remaining five individuals were represented by *P. discolor* specimens and showed positivity for blood and/or one or more ectoparasites associated. Out of these positive samples, one bat blood sample, two Spinturnicidae mite samples, and nine Macronyssidae mite samples were chosen for sequencing (Supplementary Table S3). The sample obtained from one Macronyssidae mite (#75) (353 bp) showed to be 100% identical to a sequence of *E. minasensis* detected in *R. microplus* (MT135771) from Brazil (100% of query coverage;

E-value = 0.0). The remaining sequences (399–416 bp) were similar (identity ranging from 96.73 to 99.26%) to a sequence of *E. minasensis* detected in *Bradypus variegatus* (MT212414) from Brazil (query coverage ranging from 99–100%; E-value = 0.0). None of the 50 *Ehrlichia* (*dsb*)-positive samples showed to be positive neither in qPCR protocols for *Ehrlichia canis* (*dsb* gene) and *E. chaffeensis* (*vlpt* gene) nor in cPCR protocols for *Ehrlichia* spp. based on the *omp-1*, *sodB*, and *groEL* genes.

Fifty-seven (13.63%) out of 418 samples were positive in the nested PCR assay for *N. risticii*, based on the 16S rRNA gene: 11 (19.3%) were from animals of the species *A. lituratus* (five from spleen fragments and six from whole blood), 11 (19.3%) of the species *P. lineatus* (four from spleen fragments and seven from whole blood), 10 (17.54%) of the species *A. planirostris* (six from spleen fragments and four from whole blood), nine (15.79%) of the species *P. discolor* (two from spleen fragments and seven from whole blood), four (7.01%) of the species *E. furinalis* (one from spleen fragments and three from whole blood), three (5.26%) of the species *C. perspicillata* (two from spleen fragments and one from whole blood), two (3.5%) of the species *Molossus molossus* (one from spleen fragments and one from whole blood), two (3.5%) of the species *Glossophaga soricina* (one from spleen fragments and one from whole blood), and one (1.57%) positive blood sample of the species *Myotis nigricans*, *Molossops temminckii*, *Eumops perotis*, and *Chiroderma villosum*. The only ectoparasite sample that was positive for this agent was an *O. hasei* tick larva collected from a negative specimen of *P. discolor* (Supplementary Table S4). Out of the 34 positive blood samples for the 16S rRNA-based PCR assay, 11 (32.35%) were chosen for sequencing. The samples were chosen for convenience, but in such a way as to represent an individual of each bat species that showed positivity in this protocol. When analyzed by BLASTn, eight sequences (426–524 bp) obtained from blood samples showed similarity (97.9–100%) with *N. risticii* (Accession numbers MK760560 and CP001431.1) (100% of query coverage; E-value = 0.0); three sequences obtained from a tick larvae (304 bp), one bat spleen (421 bp) and one bat blood sample (385 bp) was observed to be 95.07–100% identical to *N. findlayensis* (Accession number MH476282) (100% of query coverage; E-value = 7×10^{-131} to 0.0); finally, two sequences obtained from bat blood samples (212–282 bp) were observed to be similar (96.7–100%) to *N. risticii* and *N. findlayensis* (Accession numbers MK580530 and MH476282) (100% of query coverage; E-value = 1×10^{-143} to 1×10^{-96}). All the positive samples were negative in the additional PCR protocols targeting the *p51kDa*, *groEL*, and near-full 16S rRNA genes.

Moreover, *Ehrlichia* DNA was detected in 7/7 bat flies, 3/3 ticks, 18/19 Macronyssidae, and 4/6 Spinturnicidae mites, whose bat hosts were negative. The ticks that were positive for *Neorickettsia* spp. and *Anaplasma* spp. were also collected from a negative animal.

All the BLASTn results are summarized and presented in the Supplementary Table S5.

A total of 268 DNA samples (133 from whole blood and 135 from spleen fragments) were screened for the presence of *C. burnetii* DNA and none were positive. Unfortunately, the DNA samples from ectoparasites did not yield sufficient volume for this assay.

One phylogenetic tree was constructed based on a 522 bp alignment of the *gltA* gene (Figure 1), using the Maximum Likelihood method and TIM3 + F + I + G4 as the evolutive model. The unique sequence obtained from a blood sample from *P. discolor* (GenBank Accession Number OK338001) was positioned closely to *E. ruminantium*. The remaining sequences were not further analyzed as the final alignment length was not suitable for robust inferences.

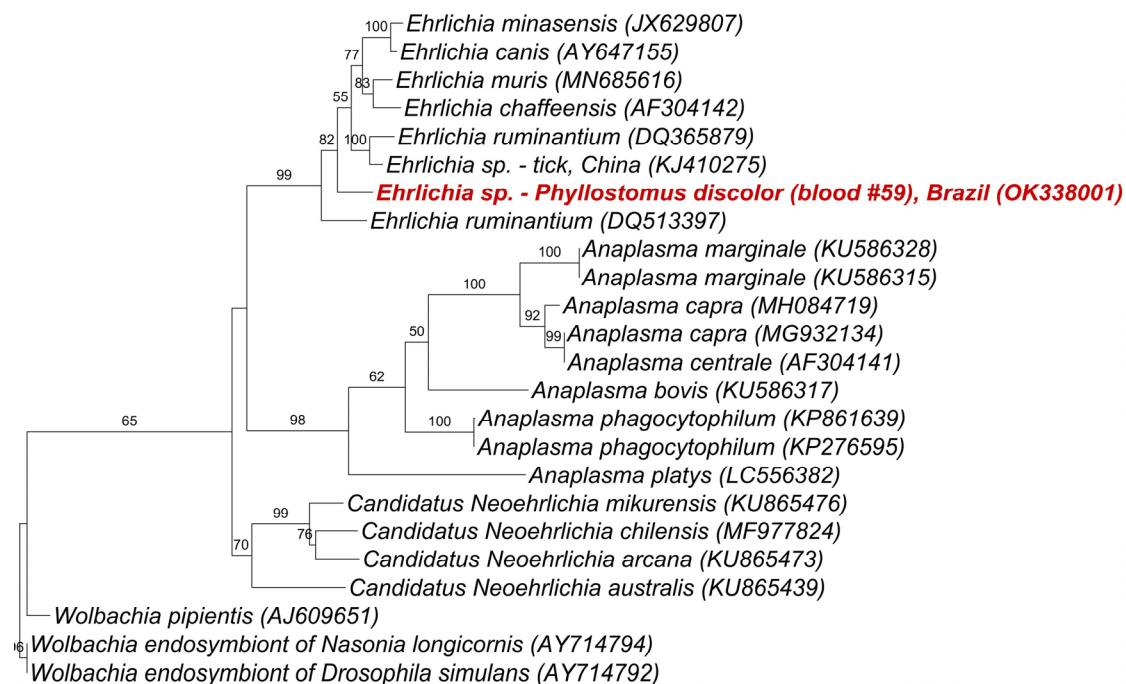


Figure 1. Phylogenetic tree based on an alignment of 522 bp of *Ehrlichia gltA* gene, using Maximum Likelihood method and TIM3 + F + I + G4 as evolutive model. Sequence from the present study is highlighted in red. *Wolbachia* spp. was used as outgroup.

3. Discussion

Among all the seven positive samples for *Anaplasma* spp., only the amplicon obtained from *O. hasei* tick larva was sequenced, obtaining 97.6% similarity with *A. phagocytophilum*, which was previously detected in deer of the species *Hydropotes inermis* from the Republic of Korea. Previously, the occurrence of *A. phagocytophilum* was described in 22.6% (63/278) of fecal samples of *Rhinolophus hipposideros* in France [35], 1.7% (1/59) of blood samples of *Myotis myotis* [54] in Poland, 4.3% (6/138) of *I. simplex* ticks collected from bats from Hungary and Romania [36], and 14.28% (1/7) of *Nyctalus noctula* species from Russia [37]. Despite the high occurrence of *A. phagocytophilum* in bats from France, the remaining abovementioned studies showed low positivity for this agent among bats or associated ectoparasites, corroborating the results found in the present work for *Anaplasma* spp. (1.67%; 7/418). Noteworthy, even though sequences similar to *A. phagocytophilum* have been detected in several wild animals from Brazil, negative results have been reported when a qPCR specific for this agent and based on *msp-2* gene was used [6,55]. Together, these findings suggest that a putative new *Anaplasma* species is circulating among wild animals from Brazil. Indeed, new ‘*Candidatus Anaplasma* spp.’, namely ‘*Candidatus Anaplasma brasiliensis*’ and ‘*Candidatus Anaplasma amazonensis*’ have been recently detected in mammals from the Superorder Xenarthra from Brazil [29]. Future studies aiming at obtaining larger fragments of different molecular markers are needed in order to check the phylogenetic relatedness of bat-associated *Anaplasma* spp. to these newly described ‘*Candidatus Anaplasma* spp.’.

The unique *Anaplasmataceae gltA* sequence obtained was observed to be similar to *Ehrlichia* spp. with low identity obtained when compared to other sequences (<90%). The phylogenetic analysis evidenced that the sequence was closely related to *E. ruminantium* and *Ehrlichia* spp., detected in a tick from China. Even though there are reports of the occurrence of *E. canis*, *E. ruminantium*, and *E. muris*-like in humans [8], these agents represent a major concern in animal health. *E. ruminantium*, which is known to cause heartwater or cowdriosis in wild and domestic ruminants, is mainly transmitted by *Amblyomma* spp. ticks in Africa and the Caribbean [12,56]. Interestingly, a genotype (Strain Jaguar) related to, albeit distantly, called *E. ruminantium*, was detected in jaguars and in associated ticks (*A. triste*,

A. sculptum, and *Amblyomma* spp.) sampled in Pantanal wetland, in midwestern Brazil, and is based on *rrs* and *dsb* sequences [57]. Additionally, a similar genotype (fox-ES-1) was detected in crab-eating foxes (*Cerdocyon thous*) in southeastern Brazil [58].

Herein, 11.96% (50/418) of samples were positive in PCR assays for *Ehrlichia* spp. based on the *dsb* gene. Out of these positive samples, 35 [70% (35/50)] were obtained from ectoparasites. In fact, few reports have shown the occurrence of *Ehrlichia* sp. in bats and associated ectoparasites. Socolosvich et al. (2012) [38] detected *Ehrlichia* DNA in *A. vesper-tilionis*, an Argasidae species often associated with bats and humans in places cohabitated by them. In Saint Kitts Island, Reeves et al. (2016) [39] found 16% (4/25) of positivity for *Ehrlichia* spp. in blood samples collected from bats (*Brachyphylla caverum*). The obtained sequences showed 95% identity with *Ehrlichia* sp. detected in ticks collected from mammals from Asia. More recently, Lv et al. (2018) [40] detected DNA of *Ehrlichia/Anaplasma* in five out of 120 *A. vesper-tilionis* ticks collected from three *P. pipistrellus* bats in England. Zabashta et al. (2019) [37] also detected *Ehrlichia* DNA (without sequencing) in *P. pipistrellus* and *P. kuhlii* bats from Russia.

Interestingly, the obtained *Ehrlichia dsb* sequences were observed to be related to *E. minasensis*. This agent has been described in cattle and *R. microplus* from Brazil [5,59,60], potentially causing disease in cattle [61]. Recently, *Ehrlichia dsb* genotypes closely related to *E. minasensis* were detected in Xenarthra mammals in Brazil [29]. These results suggest that *E. minasensis* may infect a broader range of hosts than previously recognized.

Even though the multiplex qPCR assay, used herein for detecting a fragment of *groEL* gene for *Ehrlichia* and *Anaplasma* species with high sensitivity and specificity [62], was originally designed for amplifying DNA from the main *Ehrlichia* (namely *E. canis*, *E. chaffeensis*, *E. muris*, and *Ehrlichia* sp., Anan strain/IOE agent), and *Anaplasma* (namely *A. phagocytophilum*, *A. platys*, *A. marginale*, *A. centrale*, *A. ovis*, and *A. bovis*) species. Considering that bats and associated ectoparasites likely harbor DNA of genetically diverse *Ehrlichia* and *Anaplasma* species, the used qPCR assays, based on the *groEL* gene, which is less conserved than 16S rRNA gene, were not able to catch these putative new genotypes/species of *Ehrlichia* and *Anaplasma*. This incongruence between the results of the *groEL*-based multiplex qPCR and 16S rRNA-based cPCR assays was already reported previously [29,62,63], when working on the molecular detection of *Ehrlichia* and *Anaplasma* in biological samples from wild animals from Brazil, which emphasizes the occurrence of putative new genotypes or even species of *Anaplasmataceae* in the Brazilian wildlife.

High prevalence of *N. risticii* in bats has been reported in the USA (e.g., 60% (3/5) in *Myotis yumanensis*, 44.7% (17/38) in *Myotis lucifugus*, 40% (6/15) in *Eptesicus fuscus*) [41,42] and in the Netherlands (100% (4/4) in *Myotis dasycneme*) [46]. In South America, while the present work found 38.51% (52/135) of occurrence for *Neorickettsia* spp., Ciccutin et al. [43,44] found 8.2% (5/61)–10% (3/30) prevalence of *N. risticii* in *Tadarida brasiliensis* sampled in Argentina.

N. risticii is known to cause the equine monocytic neorickettsiosis (formerly ehrlichiosis) or Potomac horse fever (PHF) in horses [64], being reported primarily in the USA. The main transmission route occurs through ingestion of aquatic snails that act as intermediary hosts for *Neorickettsia*-infected digenean trematodes [65,66]. Recently, Paulino et al. (2020) [32] detected *N. risticii* (16S rRNA) DNA in horse blood samples, sampled in Rio de Janeiro, Brazil. Previously, an outbreak of equine monocytic neorickettsiosis has also been reported in the state of Rio Grande do Sul [33]. In addition, *N. risticii* DNA has been detected in *Heleobia* snails (*H. piscium*, *H. parchappei*, *H. davisii*) collected in the state of Rio Grande do Sul [34]. Recently, *N. findlayensis* was described as a new species that also causes a disease similar to PHF [31].

It is known that bats not only act as a host for *N. risticii*-infected trematodes but also as a possible natural reservoir for *N. risticii*, as this agent has been detected in tissues (e.g., intestine, lung, liver, and spleen) of naturally infected bats [41–44]. The detection of *Neorickettsia* spp. in digenean trematodes found parasitizing bats [45] emphasized their contribution to the maintenance of these agents. In addition, vertical transmission of *N. risticii* from

adult trematodes (*Acanthatrium oregonense*) to eggs was evidenced when these helminthes were collected from *E. fuscus* bats in the USA [42]. In Europe, Hornok et al. (2018) [46] found 2.96% (4/135) positivity for *N. risticii* in *M. dasycneme* individuals sampled in the Netherlands. Future studies should be performed in order to investigate the bat-associated trematodes involved in the epidemiology of *N. risticii* in Brazil. Considering that it was not possible to confirm which *Neorickettsia* species is circulating among the sampled bats, epidemiological studies should be performed in an attempt to establish a possible correlation between infected bats and the occurrence or neorickettsiosis in horses in Brazil.

Herein, *Ehrlichia* DNA was detected in 14% bat flies (*M. aranea*, *T. costalimai*, and *S. hertigi*), 6% tick larvae (*O. hasei*), 12% Spinturnicidae mites, and 38% Macronyssidae mites. Out of those, *Ehrlichia* DNA was detected in seven bat flies, three ticks, 18 Macronyssidae and four Spinturnicidae mites, collected from bats that showed to be negative in the PCR assay for this agent. Similarly, the *O. hasei* tick larva that was positive for *Neorickettsia* spp. was collected from a specimen of *P. discolor* that showed to be negative for this agent. Moreover, DNA of *Anaplasma* spp. was detected in *O. hasei* larvae collected from a negative *P. discolor* individual. Considering the impossibility that the larval stage had ever fed on another animal, the transovarial transmission of bat-associated *Anaplasma* spp. cannot be ruled out. Indeed, while the transovarial transmission of non-pathogenic *A. phagocytophilum* variants has been already reported in *Dermacentor albipictus* ticks, transovarial transmission of *Ehrlichia* spp. has not been proved [8,67]. Also, a possible explanation for these results might rely on a false-negative result with the bat samples (blood or spleen). It may occur due to the higher concentration of the host blood meal in the arthropod digestive tract, which may have increased the chances of detection of *Ehrlichia* DNA in the ectoparasite sample, as previously reported [68]. Lastly, the efficiency of the PCR assays should also be considered in this case. Nevertheless, the real role of ticks, mites, and bat flies in the transmission of *Anaplasmataceae* agents among bats should be further investigated.

Despite the lack of positivity for *C. burnetii* among bats sampled in the present work, this agent has already been reported in 3.4% (4/119) of bats in the Atlantic Forest in the states of Santa Catarina and Rio de Janeiro, Brazil, by conventional PCR and nested PCR assays based on the *IS1111* gene in which the positive animals belonged to the species *A. lituratus* (3/4) and *A. fimbriatus* (1/4) [53]. Furthermore, this agent was detected in 15.8% (3/19) of *I. verpertilionis* ticks collected from bats in northeastern Algeria using real-time PCR based on the *IS1111* marker [51]. More recently, *C. burnetii* was detected in five individuals (9%) of *T. brasiliensis* from Chile, also using a qPCR targeting the *IS1111* gene [69]. However, it is worth mentioning that *Coxiella*-like endosymbionts are commonly found in ticks and, recently, it was shown that endosymbionts and *C. burnetii* are closely related [70]. The authors also suggested that the lineages might have evolved independently. Therefore, the possible participation of bats and specially their ectoparasites in the transmission of *C. burnetii* should be further investigated, since molecular and serological evidence of infection by *C. burnetii* has been reported in humans from Brazil [71–73].

In conclusion, the present work shows, for the first time, the occurrence of *Ehrlichia* spp. related to *E. minasensis*, *E. ruminantium*, and *Anaplasma* spp., similar to *A. phagocytophilum* and *Neorickettsia* spp., in non-hematophagous bats and associated ectoparasites (ticks, mites, and bat flies) from Brazil.

4. Material and Methods

A total of 135 spleen, 133 blood and 150 ectoparasites samples were collected from 135 non-hematophagous bats captured and sampled in two different sites (“Centro de Educação Ambiental Polonês” and “Centro de Educação Ambiental Florestinha”) situated in the Campo Grande city, Mato Grosso do Sul state, Brazil as described elsewhere [74]. The bats’ capture occurred quarterly between June 2017 and March 2018.

The DNA was purified from 50–200 µL blood samples using the Illustra Blood Mini Kit (GE Healthcare®), Chicago, IL, USA) and from 10 mg of each bat spleen tissue and ectoparasites using the Illustra Tissue Mini Kit (GE Healthcare®), according to manufac-

turer's instructions. The DNA concentration and absorbance ratio (260/280 nm) were measured using a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA, USA). To check the quality of the extracted DNA and avoid the presence of PCR inhibitors, each spleen and blood DNA extracted was tested by a conventional PCR targeting the mammal *gapdh* (Glyceraldehyde 3-phosphate dehydrogenase) gene [75]. For the ectoparasites collected from the animals, two different conventional PCR protocols were used. While a PCR protocol based on cytochrome c oxidase subunit I (*cox-1*) gene [76] was used for DNA samples obtained from bat flies and mites, a protocol based on the 16S rRNA gene [77] was used for DNA samples obtained from tick larvae.

Bat blood and spleen samples positive for the mammals' endogenous gene (*gapdh*) PCR protocol were submitted to PCR assays for *Anaplasmataceae* agents and *C. burnetii*. Ectoparasites positive in PCR assays based on *cox-1* and 16S rRNA genes were only submitted to PCR assays for *Anaplasmataceae* agents, due to the low volume of DNA obtained.

For *Anaplasmataceae* agents, quantitative real-time (q), nested (n), and conventional (c) PCR screening protocols were performed for *Anaplasma* spp. and *Ehrlichia* spp. A multiplex qPCR for *Anaplasma* spp. and *Ehrlichia* spp. based on the *groEL* gene was performed as previously described [62]. Additionally, a nested PCR for *Anaplasma* spp., based on the 16S rRNA gene [78] and a conventional PCR for *Ehrlichia* spp., based on the *dsb* gene [79], were also used as screening protocols. All the positive samples found in the above-mentioned protocols were submitted to qPCR assays for *E. canis* based on the *dsb* gene [79], *E. chaffeensis* based on the *vlpt* gene [80], and *A. phagocytophilum* based on *msp-2* gene [81]. Conventional and nested PCR assays, based on *groEL* [17,82], *gltA* [16], *sodB* [83], and *omp-1* [84] genes as well as based on the intergenic region ITS 23S-5S [85], were performed for molecular characterization.

For molecular detection of *N. risticii*, DNA samples were submitted to a nested PCR based on the 16S rRNA gene, as previously described [86]. Additionally, the positive samples for the 16S rRNA were submitted to PCR assays targeting the nearly full-length 16S rRNA [87], *p51kDa*, and *groEL* [43,63,88] genes.

A quantitative real-time PCR targeting the repetitive element *IS1111* was used for *C. burnetii* detection, as previously described [89].

As positive controls for the PCR assays, DNA samples from *A. phagocytophilum* and *N. risticii* kindly provided by Professor John Stephen Dumler (Uniformed Services University of the Health Sciences, Bethesda, MD, USA), and the *E. canis* Jaboticabal-strain (maintained in DH82 cell culture) were used. The *C. burnetii* DNA positive control was kindly provided by Professor Dr. Renato Arruda Mortara (Universidade Federal de São Paulo, SP, Brazil).

Firstly, the obtained sequences were submitted to a screening test using the Bioedit version 7.0.5.3 [90] to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program [91] was used to analyze the sequences of nucleotides (BLASTn), aiming to compare with sequences from an international database (GenBank) [92]. The consensus sequences obtained in the present study and those retrieved from GenBank were aligned using the ClustalW software [93] via Bioedit version 7.0.5.3 [89]. Phylogenetic inferences were based on Maximum Likelihood (ML). The best evolutionary model was selected by ModelFinder [94] and the trees were constructed using IQTree web server [95] with ultrafast Bootstrap of 1000 replicates [96]. All the trees were examined in Treegraph 2.0 beta software [97].

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/parasitologia1040021/s1>, Table S1: Description of the 418 samples (spleen, blood or ectoparasites) collected from bats in Campo Grande, Mato Grosso do Sul, Brazil, and used in the present work for molecular screening of *Anaplasmataceae* agents and *Coxiella burnetii*, Table S2: Bat samples positive for *Anaplasma* spp. according to PCR assays targeting five different molecular markers, Table S3: Bat samples positive for *Ehrlichia* spp. according to PCR assays targeting six different molecular markers, Table S4: Bat samples positive for *Neorickettsia* spp. according to PCR assays targeting three different

molecular markers, Table S5: BLASTn results obtained after sequencing of amplicons obtained from PCR assays for *Ehrlichia* spp., *Anaplasma* spp. and *Neorickettsia* spp.

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