UNIVERSIDADE ESTADUAL PAULISTA (UNESP)
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS (FCAV)
CAMPUS DE JABOTICABAL

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR VETORES EM AVES SILVESTRES NO PANTANAL BRASILEIRO

Amir Salvador Alabí Córdova
Médico Veterinario e Zootecnista

2024

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Amir Salvador Alabí Córdova

Orientador: Marcos Rogerio André

Tese apresentada a Faculdade de Ciências Agrárias e Veterinárias – UNESP, Campus de Jaboticabal, como parte das exigências para obtenção do título de Doutor em Medicina Veterinária, área: Patologia Animal.

2024

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR VETORES EM AVES SILVESTRES NO PANTANAL BRASILEIRO

AUTOR: AMIR SALVADOR ALABÍ CORDOVA
ORIENTADOR: MARCOS ROGÉRIO ANDRÉ

Aprovado como parte das exigências para obtenção do Título de Doutor em Medicina Veterinária, área: Patologia Animal pela Comissão Examinadora:

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Jaboticabal, 19 de dezembro de 2024

DADOS CURRICULARES

Amir Salvador Alabí Córdova – Filho de Amir Salvador Alabí Mendoza e Marlene Guadalupe Córdova Coto, nasceu em 29 de julho de 1990, na cidade de Nueva São Salvador, departamento de La Libertad, El Salvador. No ano de 2008 ingressou no curso de Medicina Veterinária e Zootecnia da Universidade Salvadoreña Alberto Masferrer (USAM), San Salvador, El Salvador. Durante a graduação, realizou dois estágios, o primeiro no Laboratorio clínico de medicina veterinária e Zootecnia sob a supervisão da Tecnóloga Veronica Maria Avelar e o segundo estágio voltado para a reabilitação de aves rapinantes de El Salvador, sob a supervisão da Médica veterinária Paola Tinetti e Orlando Jimenez. Em 2017 ingressou no Mestrado em Ciências na área de Saúde Animal pela Faculdade de Ciências Veterinárias da Universidade Austral de Chile, sob orientação da profa. Dra. Ananda Muller, com bolsa AGCID-Chile e defendendo sua dissertação em maio de 2019. Também durante o Mestrado realizou um estágio no Departamento de Patologia Veterinária sob a orientação do Prof. Dr. Marcos Rogerio André. Em agosto de 2020 ingressou no Programa de Pós-graduação em Medicina Veterinária na área de Patologia Veterinária, sob orientação do Prof. Dr. Marcos Rogerio André, na Universidade Estadual Paulista-campus Jaboticabal, inicialmente com bolsa CAPES-PROEX e posteriormente com bolsa FAPESP (#2020/14948-0). Entre dezembro de 2023 a maio 2024, realizou um estágio de pesquisa no exterior com bolsa BEPE-FAPESP (# 2023/08967-0), no UMR-BIPAR, Agência Nacional de Segurança Alimentar, Ambiental e de Saúde Ocupacional (ANSES) em Maison-Alfort, França, sob a supervisão de Dra. Sara Moutailler. Durante esse período teve contato com técnicas de pré-amplificação de DNA e a técnica de PCR em tempo real microfluídica de alto rendimento.

EPÍGRAFE

DUC IN ALTUM

Lc. 5,4

DEDICATORIA

Dedico a quem nunca desistiu, pensou inúmeras vezes em deixar tudo para trás e ainda continuou. Dedico àquele garotinho que sonhava em voar falcões e se perder na natureza

AGRADECIMIENTOS

“Insha'Allah”, “Masha'Allah” e “piolet”, três palavras que merecem ser mencionadas porque marcaram todo esse caminho e vou explicar ao longo deste texto. Insha'Allah que vem do árabe e significa "esperançosamente" porque alguém pode ter a vontade de fazer algo novo, mas para que isso aconteça depende de Deus e Masha'Allah como gratidão pelo que aconteceu porque Ele permitiu que acontecesse. O primeiro que devo agradecer é a Deus por tudo isso.

Não me esqueço de explicar a terceira palavra, “Piolet”, uma ferramenta que é usada no montanhismo ao subir e é assim que começo meus agradecimentos, mencionando meus "machados de gelo" que tornaram o doutorado mais leve.

Agradeço aos meus pais, Amir e Marlene, por estarem sempre atentos, por oferecerem ajuda e apoio da maneira que cada um deles conhece e aprendeu.

Agradeço ao Dr. Jaime Soundy Call e à sua família, porque os seus conselhos, o seu tempo e a sua amizade foram um farol nesta jornada.

Agradeço ao mestre Alex Maeda, por estar sempre atento ao aprimoramento do jiu jitsu dentro e fora do tatame, por sempre buscar fortalecer a mente, ensinar jiu jiu jitsu com tanta paixão e trazer o a disciplina do jiu jitsu para o dia a dia, me ensinando que as batalhas são pessoais, mas sempre haverá pessoas apoiando.

Agradeço ao Prof. Dr. Marcos Rogério André, por ter aceitado me orientar e me integrar à sua equipe de trabalho; agradeço sua paciência, tempo, “puxadas de orelha” e principalmente essa paixão por ensinar e manter essa curiosidade pelo novo e querer transmitir isso aos seus orientados.

Agradeço às Professoras Rosangela Zacarias Machado e Darci Moraes Barros-Battesti pela dedicação ao desenvolvimento da parasitologia no Brasil e paixão por seu trabalho, qualidades que são muito admiradas.

Agradeço a Melissa Sanchez e Alejandro Torres, por sua amizade inestimável e verdadeira, por seus conselhos e tempo compartilhado.

Agradeço aos meus colegas do *Vector-Borne Bioagents Laboratory*, em especial à Ana Calchi, Anna Mongruel, Ana Carolina Santiago, Amanda Garcia, Clara Morato, Ricardo Bassini, Livia Perles e Lizeth Micolta, pelas vezes que me ajudaram nas PCRs, emprestando materiais, pelos cafés preparados e tornando o doutorado mais leve.

Gostaria também de agradecer à Dra. Sara Moutailler e a toda a equipe MiTick, por me receberem em seu grupo de trabalho, permitindo-me aprender novas técnicas, expandindo meus conhecimentos e, especialmente, agradecer a Clemence Galon por sua infinita paciência.

Agradeço a Alan Fecchio, João Pinho e Tiago Ferreira pelas amostras concedidas e disponibilidade para colaborar com a coleta de amostras de aves.

Agradeço à CAPES-PROEX (código 001) pelo benefício da bolsa no início do doutorado, e à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelas bolsas concedidas para o doutorado no Brasil (Processo #2020/14948-0) e o estágio doutoral na França (BEPE-FAPESP # 2023/08967-0).

Agradeço à FAPESP pelos Auxílios à Pesquisa concedidos ao Prof. Marcos R. André que permitiram a realização do presente trabalho (Processos # 2020/12037-0 e 2022/08543-2).

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

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Comissão de ética no uso de animais.

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Detecção e caracterização molecular de agentes Anaplasmataceae, Bartonellaceae e Hepatozoidae em passeriformes no Pantanal Brasileiro"**, protocolo nº 268/21, sob a responsabilidade do Prof. Dr. Marcos Rogério André, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 11 de fevereiro de 2021.

Vigência do Projeto	01/03/2021 a 10/12/2024
Espécie / Linhagem	Aves passeriformes
Nº de animais	500
Peso / Idade	5-25 g
Sexo	Macho e fêmea
Origem	Fazenda localizada no Pantanal (Mato Grosso e Mato Grosso do Sul)

Jaboticabal, 16 de fevereiro de 2021.

Fabiana Pilarski

Profa. Dra. Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
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Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Detecção e caracterização molecular de filarídeos em aves no Pantanal Brasileiro"**, protocolo nº 3241/22, sob a responsabilidade do Prof. Dr. Marcos Rogério André, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 15 de junho de 2022.

Vigência do Projeto	26/06/2022 a 10/12/2024
Espécie / Linhagem	Aves Selvagens
Nº de animais	Aproximadamente 300
Peso / Idade	Variável
Sexo	Machos e fêmeas
Origem	Poconé, Pantanal do estado do Mato Grosso

Jaboticabal, 15 de junho de 2022.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
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Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Registro de Expedição

Licença permanente para coleta de material zoológico

Número: 72548	Data da emissão: 02/04/2019 15:51:39
Número da solicitação: 10698	Data da expedição: 20/04/2019 à 30/04/2019

Dados do titular

Nº de Registro: 266492	Nome: JOAO BATISTA DE PINHO	CPF: 177.363.231-00

Equipe

#	Nome	CPF	Doc. Identificação	Tipo Doc.	Nacionalidade	*Resp.
1	Alan Fecchio	033.149.189-33	2632240 SSP-DF	Identidade	Brasileiro	Sim
2	RAPHAEL IGOR DA SILVA CORRÊA DIAS	000.449.091-60	2632240 SSP-DF	Identidade	Brasileiro	Não

* O titular será o responsável pela equipe caso não designe alguém para exercer tal função.

Locais onde as atividades de campo serão executadas

#	Localidade	UF	Município	Bioma	Caverna
1	diversas	MS	Corumbá	Pantanal	-

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Registro de Expedição

Licença permanente para coleta de material zoológico

Número: 72790	Data da emissão: 12/09/2019 09:15:49
Número da solicitação: 10698	Data da expedição: 19/09/2019 à 30/01/2020

Dados do titular

Nº de Registro: 266492	Nome: JOAO BATISTA DE PINHO	CPF: 177.363.231-00

Anexo para registrar Coletas Imprevistas de Material Biológico

De acordo com a Instrução Normativa nº154/2007
Art. 19. A coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta.
§ 1° O transporte do material biológico ou do substrato a que se refere o caput deste artigo deverá ser acompanhado da autorização ou da licença permanente com a devida anotação.
§ 2° A coleta a que se refere o caput deste artigo deverá ser comunicada no relatório de atividades.
§ 3° O material biológico coletado conforme previsto no caput deverá ser destinado à instituição científica, preferencialmente depositado em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO)

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

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Autorização para atividades com finalidade científica

Número: 83622-1	Data da Emissão: 14/06/2022 08:50:27	Data da Revalidação*: 14/06/2023

De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Marcos Rogério André	CPF: 302.435.148-59
Título do Projeto: Detecção e caracterização molecular de filarídeos em aves no Pantanal brasileiro	
Nome da Instituição: UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta de material	06/2022	07/2022
2	processamento laboratorial	07/2022	12/2023

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Alan Fecchio	coleta de amostra	033.149.189-33	Brasileira
2	JOAO BATISTA DE PINHO	coleta de amostra	177.363.231-00	Brasileira
3	AMANDA GARCIA PEREIRA	coleta de amostra	424.136.898-00	Brasileira
4	Tiago Valadares Ferreira	coleta de amostra	017.487.741-23	Brasileira
5	AMIR SALVADOR ALABI CORDOVA	coleta de amostra	244.676.538-64	Estrangeira

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Outras ressalvas

1	A proporção máxima de redes de neblina é dez redes por pesquisador com experiência no método. O intervalo máximo de revisão de redes deve ser de 20 minutos se a captura ocorrer em local ensolarado e de 45 minutos se ocorrer em local sombreado. O volume de sangue coletado não deve exceder o correspondente a 1% da massa corporal da ave. Em coletas consecutivas, não deve ultrapassar 2% a cada 14 dias.	CEMAVE Cabedelo-PB
2	O pesquisador estrangeiro AMIR SALVADOR ALABI CORDOVA possui vínculo junto a Programa de bolsas ou auxílio à pesquisa patrocinado pela FAPESP. Portanto, está dispensado de autorização do Ministério da Ciência, Tecnologia e Inovação conforme definido no ?item? 56, da Portaria MCT nº 826/2008, e no inciso II, art. 5º da Resolução Normativa nº 20/2017, do Conselho Nacional de Imigração - CNig.	COINF

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Mato grosso	Santo Antônio do Leverger-MT	Pantanal	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de amostras biológicas in situ	Fora de UC Federal
2	Captura de animais silvestres in situ	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Captura de animais silvestres in situ	Coraciiformes	-
2	Captura de animais silvestres in situ	Anseriformes	-
3	Captura de animais silvestres in situ	Apodiformes	-
4	Captura de animais silvestres in situ	Piciformes	-
5	Captura de animais silvestres in situ	Passeriformes	-
6	Coleta/transporte de amostras biológicas in situ	Passeriformes	-
7	Captura de animais silvestres in situ	Falconiformes	-
8	Captura de animais silvestres in situ	Gruiformes	-
9	Captura de animais silvestres in situ	Galliformes	-
10	Captura de animais silvestres in situ	Cuculiformes	-
11	Captura de animais silvestres in situ	Columbiformes	-
12	Captura de animais silvestres in situ	Charadriiformes	-

A quantidade prevista só é obrigatória para atividades do tipo "Coleta/transporte de espécimes da fauna silvestre in situ". Essa quantidade abrange uma porção

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Dados do titular

Nome: Marcos Rogério André	CPF: 302.435.148-59
Título do Projeto: Detecção e caracterização molecular de filarídeos em aves no Pantanal brasileiro	
Nome da Instituição: UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

territorial mínima, que pode ser uma Unidade de Conservação Federal ou um Município.

A quantidade significa: por espécie X localidade X ano.

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Aves)	Sangue, Fragmento de tecido/órgão, Ectoparasita
2	Método de captura/coleta (Aves)	Rede de neblina

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	UNESP JABOTICABAL	Laboratório

Sistema nacional de gestão do patrimônio genético e do conhecimento tradicional associado

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso
Cadastro nº AF30FD1

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AF30FD1**
Usuário:	**Marcos Rogério André**
CPF/CNPJ:	**302.435.148-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Agelaioides badius
Agelasticus cyanopus
Antilophia galeata
Ramphocelus carbo
Saltator coerulescens
Pseudoseisura unirufa
Cercomacra melanaria
Turdus leucomelas
Leptotila verreauxi
Basileuterus flaveolus
Aramides cajanea
Arremon flavirostris
Arundinicola leucocephala

Basileuterus hypoleucus
Brotogeris chiriri
Busarellus nigricollis
Butorides striata
Cacicus cela
Cacicus solitarius
Campylorhynchus turdinus
Cantorchilus leucotis
Certhiaxis cinnamomeus
Chloroceryle aenea
Chloroceryle amazona
Chloroceryle americana
Cnemotriccus fuscatus
Coccycua minuta
Coccyzus melacoryphus
Columbina talpacoti
Coereba flaveola
Coryphospingus cucullatus
Cranioleuca vulpina
Crax fasciolata
Crotophaga ani
Crypturellus undulatus
Cyclarhis gujanensis
Cyanocorax chrysops
Cyanocorax cyanomelas
Dendroplex picus
Donacobius atricapilla
Elaenia albiceps
Elaenia spectabilis
Eucometis penicillata
Formicivora rufa
Fluvicola albiventer

Fluvicola albiventer
Furnarius leucopus
Furnarius rufus
Glaucis hirsutus
Gnorimopsar chopi
Guira guira
Hemitriccus margaritaceiventer
Hemitriccus striaticollis
Herpsilochmus longirostris
Hylocharis chrysura
Hylophilus pectoralis
Hypocnemoides maculicauda
Icterus cayanensis
Icterus croconotus
Jacana jacana
Legatus leucophaius
Lepidocolaptes angustirostris
Machetornis rixosa
Megaceryle torquata
Monasa nigrifrons
Myiarchus ferox
Myiophobus fasciatus
Myiozetetes cayanensis
Paroaria capitata
Phaethornis nattereri
Phaethornis subochraceus
Picumnus albosquamatus
Pipra fasciicauda
Pitangus sulphuratus
Poecilotriccus latirostris
Progne tapera
Pteroglossus castanotis

Sicalis flaveola
Sporophila angolensis
Sporophila caerulescens
Sporophila collaris
Stelgidopteryx ruficollis
Synallaxis albilora
Taraba major
Thamnophilus doliatus
Thraupis palmarum
Thraupis sayaca
Thryothorus genibarbis
Tigrisoma lineatum
Turdus amaurochalinus
Turdus hauxwelli
Turdus rufiventris
Tyrannus melancholicus
Vireo olivaceus
Volatinia jacarina

Título da Atividade:	**Detecção e caracterização molecular de Piroplasmídeos, agentes Anaplasmatacea, Bartonella spp., Hepatozoon spp., Trypanosoma spp. e hemosporídeos em aves de vida livre no Pantanal do Mato Grosso e Mato Grosso do Sul.**

Equipe

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Data do Cadastro:	**24/03/2023 17:27:18**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **18:00** de **30/10/2024**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - **SISGEN**

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão
Cadastro nº AD5EDDB

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**AD5EDDB**
Usuário:	**Marcos Rogério André**
CPF/CNPJ:	**302.435.148-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Arremon flavirostris
Camptostoma obsoletum
Campyloramphus trocholirostris
Campylorhynchus turdinus
Casiornis rufus
Cercomacra melanaria
Certhiaxis cinnamomeus
Cnemotriccus fuscatus
Coccycua minuta
Columbina squammata
Columbina talpacoti
Coryphospingus cucullatus
Cranioleuca vulpina
Crotophaga ani
Dendroplex picus
Elaenia cristata
Eucometis penicillata
Euscarthmus meloryphus
Fluvicola albiventer
Furnarius leucopus
Furnarius rufus
Galbula ruficauda
Hemitriccus margaritaceiventer

Hypocnemoides maculicauda
Icterus pyrrhopterus
Inezia inornata
Leptotila verreauxi
Machetornis rixosa
Momotus momota
Monasa nigrifrons
Myiarchus ferox
Myiopagis gaimardii
Myiophobus fasciatus
Myiothlypis flaveola
Paroaria capitata
Pheugopedius genibarbis
Picumnus albosquamatus
Pipra fasciicauda
Pitangus sulphuratus
Poecilotriccus latirostris
Pseudoseisura unirufa
Ramphocelus carbo
Rupornis magnirostris
Saltator coerulescens
Sicalis flaveola
Sporophila angolensis
Sporophila caerulescens
Sporophila leucoptera
Synallaxis albescens
Synallaxis albilora
Taraba major
Thamnophilus pelzelni
Thraupis palmarum
Tolmomyias sulphurescens
Turdus amaurochalinus
Veniliornis passerinus
Volatinia jacarina

Título da Atividade:	**Detecção e caracterização molecular de Piroplasmídeos, agentes Anaplasmatacea, Bartonella spp., Hepatozoon spp., Trypanosoma spp. e hemosporídeos em aves de vida livre no Pantanal do Mato Grosso.**

Equipe

Marcos Rogério André	**Campus de Jaboticabal**
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Data do Cadastro:	**15/04/2023 13:20:36**
Situação do Cadastro:	**Concluído**

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR VETORES EM AVES SILVESTRES NO PANTANAL BRASILEIRO

Resumo - O presente estudo avaliou, por meio de técnicas moleculares, a ocorrência e a diversidade genética dos agentes Anaplasmataceae, *Bartonella* spp., *Hepatozoon* spp. e *Borrelia* spp. em aves silvestres no Pantanal dos estados de Mato Grosso (MT) e Mato Grosso do Sul (MS). Para tanto, 500 amostras de sangue de aves silvestres capturadas no Pantanal de MT e MS, durante o mês de abril e entre os meses de agosto e novembro de 2019, foram submetidas à extração de DNA e ensaios de PCR em tempo real e convencional baseados nos genes *gro*EL, *glt*A, *dsb*, região intergênica 23S-5S, *omp-1* e *sodB* para agentes Anaplasmataceae, *nuo*G, *glt*A, *rpo*B*, ftsZ*, *pap*31 e *ribC* para *Bartonella* spp., 16S rRNA e *flaB* para *Borrelia* spp., 18S rRNA para *Hepatozoon* spp. e *cox*-1, 12S rRNA e 28S rRNA para filarídeos. Análises filogenéticas foram construídas para posicionar filogeneticamente as sequências obtidas. Como resultado, 37/500 (7,4%) aves foram positivas para *Anaplasma* spp. e 4 (0,8%) para *Ehrlichia* spp. no ensaio qPCR (*gro*EL); além disso, 13 (2,6%) foram positivos na PCR para agentes Anaplasmataceae com base no gene 16S rRNA. As sequências de 16S rRNA de *Ehrlichia* spp. detectadas foram posicionadas próximas a *Ehrlichia* sp. Magellanica. As sequências *dsb* de *Ehrlichia* detectadas agruparam-se com *Ehrlichia minasensis*. Os genótipos 16S rRNA de *Anaplasma* spp. detectados foram agrupados com *'Candidatus* Allocryptoplasma spp.'. Os genótipos 23S-5S detectados foram relacionados a *Anaplasma phagocytophilum*. Dezenove (3,8%) das 500 amostras de sangue de aves foram positivas em ensaio de qPCR para *Bartonella* spp. com base no gene *nuo*G. Com base nos resultados do BLASTn, enquanto 1 sequência *nuoG*, 2 *rib*C e 2 ITS (16S-23S rRNA) apresentaram alta identidade com *Bartonella henselae*, uma 16S rRNA e 2 ITS apresentaram alta identidade com *Bartonella machadoae*. Três amostras (0,6%) foram positivas na PCR para filarídeos. Enquanto uma das sequências de *cox*-1 detectadas agrupou-se com *Cardiofilaria* sp., a outra posicionou-se basal a *Splendidofilaria* spp. Além disso, protocolos previamente estabelecidos de qPCR microfluídica em tempo real foram padronizados para a detecção de agentes transmitidos por vetores em 282 amostras de sangue de aves coletadas no Pantanal do estado de Mato Grosso em 2022, visando amplificar os genes 16S rRNA para *Anaplasma* spp., *ssr*A para *Bartonella* spp., *cyt*B para hemosporídeos, e LSU rRNA para filariídeos. Os resultados foram validados por ensaios de PCR baseados no gene 16S rRNA e região intergênica 23S-5S para *Anaplasma* spp., espaçador intergênico 16-23S rRNA, e genes *nuo*G, *gltA*, *pap*31, *rib*C, *fts*Z, *gro*EL e *rpo*B para *Bartonella* spp., *cox-1,* 12S rRNA e 18S rRNA para filarídeos e gene *cyt*B para hemosporídeos. As seguintes taxas de positividade foram encontradas na qPCR microfluídica de alto rendimento: 18.2% para *Anaplasma* spp., 6.5% para Onchocercidae, 6.2% para *Plasmodium* spp., 5.09% para *Haemoproteus* spp. e 0.36% para *Bartonella* spp. DNA de *Bartonella machadoae*, *Cardiofilaria* spp., *Aproctella* spp. e filarídeo posicionado basalmente ao clado de *Eufilaria* spp. foi detectado por esta técnica de qPCR. Em conclusão, novos genótipos de *Anaplasma* foram detectados em aves do Pantanal. *Bartonella* spp. relacionadas a *B. machadoae* e *B. henselae* foram detectadas, pela primeira vez, em aves silvestres do Pantanal brasileiro. Este é o primeiro relato molecular de filariídeos Onchocercidae (*Aproctella* spp., *Cardiofilaria* spp., e filarídeos basais a *Eufilaria* spp. e *Splendidofilaria* spp.) em aves do Pantanal brasileiro. A qPCR microfluídica em tempo real de alto rendimento mostrou-se eficiente na triagem de agentes transmitidos por vetores em aves silvestres no Brasil, desde que sejam desenhados primers específicos para variantes genotípicas circulantes no território nacional.

Palavras-chave: Anaplasmataceae, *Bartonella* spp., *Hepatozoon* spp., *Borrelia* spp., Haemosporida, Onchocercidae, aves tropicais

MOLECULAR DETECTION AND CHARACTERIZATION OF VECTOR-BORNE AGENTS IN WILD BIRDS IN THE BRAZILIAN PANTANAL

Abstract - The present study evaluated, by means of molecular techniques, the occurrence and genetic diversity of agents Anaplasmataceae, *Bartonella* spp., *Hepatozoon* spp., and *Borrelia* spp. in wild birds in the Pantanal of the states of Mato Grosso (MT) and Mato Grosso do Sul (MS). To this end, 500 blood samples from wild birds captured in the Pantanal of MT and MS, during the month of April and between the months of August and November 2019, were subjected to DNA extraction and real-time and conventional PCR assays based on the *gro*EL, *glt*A, *dsb*, intergenic region 23S-5S, *omp*-1 and *sod*B genes for *Anaplasmataceae* agents, *nuoG*, *gltA*, *rpoB*, *fts*Z, *pap*31 and *rib*-C for *Bartonella* spp., 16S rRNA and *flaB* for *Borrelia* spp., 18S rRNA for *Hepatozoon* spp., and *cox*-1, 12S rRNA and 28S rRNA genes for filariids. Phylogenetic analyses were constructed to phylogenetically position the sequences obtained. As a result, 37/500 (7.4%) birds were positive for *Anaplasma* spp. and 4 (0.8%) for *Ehrlichia* spp. in the qPCR assay (*groEL*); in addition, 13 (2.6%) were positive in the PCR for *Anaplasmataceae* agents based on the 16S rRNA gene. The *Ehrlichia* 16S rRNA sequences detected were positioned near *Ehrlichia* sp. Magellanica. The *Ehrlichia dsb* sequences detected clustered with *Ehrlichia minasensis*. The *Anaplasma* spp. 16S rRNA genotypes detected clustered with *'Candidatus* Allocryptoplasma spp.'. The 23S-5S genotypes detected in were related to *Anaplasma phagocytophilum*. Nineteen (3.8%) out of the 500 blood samples from birds were positive in a qPCR assay for *Bartonella* spp. based on the *nuo*G gene. Based on the BLASTn results, while 1 *nuo*G, 2 *rib*C and 2 ITS (16S-23S rRNA) sequences showed high identity with *Bartonella henselae*, one 16S rRNA and 2 ITS showed high identity with *Bartonella machadoae*. Three samples (0.6%) were positive in the PCR for filarids. While one *cox*-1 sequence detected clustered with *Cardiofilaria* sp., another one sequence was positioned basal to *Splendidofilaria* spp. In addition, previously established protocols of new microfluidic real-time PCR assays were standardized for the detection of vector-borne agents in 282 bird blood samples collected in the Pantanal of the state of Mato Grosso in 2022, aiming to amplify the 16S rRNA genes for *Anaplasmataceae*, *ssr*A gene for *Bartonella* spp., *cyt*B for hemosporidians, and LSU rRNA for filariids. The results were validated by PCR assays based on the 16S rRNA gene and 23S-5S intergenic region for *Anaplasma* spp., 16-23S rRNA intergenic spacer, *nuo*G, *glt*A, *pap*31, *rib*C, *fts*Z, *gro*EL and *rpo*B genes for *Bartonella* spp., *cox*-1 for filariids and *cytB* gene for haemosporidians. The following positivity rates were found in high-throughput microfluidic qPCR: 18.2% for *Anaplasma* spp., 6.5% for Onchocercidae, 6.2% for *Plasmodium* spp., 5.09% for *Haemoproteus* spp. and 0.36% for *Bartonella* spp.. DNA from *Bartonella machadoae*, *Cardiofilaria* spp., *Aproctella* spp., and a filariid ancestral to *Eufilaria* spp. clade was detected by this new qPCR technique. In conclusion, novel genotypes of *Anaplasma*, *Ehrlichia*, and *'Candidatus* Allocryptoplasma' were detected in birds from the Pantanal wetland. *Bartonella* spp. related to *B. henselae* and *B. machadoae* were detected, for the first time, in wild birds from the Brazilian Pantanal. This is the first molecular report of *Borrelia* spp. and onchocercid filariids (*Aproctella* spp., *Cardiofilaria* spp., and onchocercid filariids basal to *Eufilaria* spp. and *Splendidofilaria* spp. clade) in birds from the Brazilian Pantanal. High-throughput real-time microfluidic qPCR proved to be efficient in the screening of vector-borne agents in wild birds in Brazil as long as specific primers are designed for genotypic variants circulating in the national territory.

Key-words: Anaplasmataceae, Bartonellaceae, Hepatozoideae, Borreliaceae, Haemosporida, Onchocercidae, Tropical birds

Chapter 1 – General Considerations

1 Birds

Birds comprehend the most diverse lineage, representing more than 10,000 living species and presenting the most diverse morphologic, ecologic and behavior features (Gill & Prum, 2019; Prum et al., 2015). The diversification of modern birds (Neornithes) was influenced by considerable factors, including mass extinctions (Feduccia, 1995; Prum et al., 2015), geologic events (Claramunt & Cracraft, 2015; Moyle et al., 2016), and climate change (Claramunt & Cracraft, 2015; Jetz et al., 2012). Previously, molecular analyses of genomes resulted in a new phylogenetic resolution to the initial branching in the Neoaves, describing five major clades (Prum et al., 2015).

Birds show a considerable capability to adapt to different ecological niches (Schmitt & Edwards, 2022), favoring the dispersion of vector-borne agents, viruses and parasites through migratory movements (Fecchio et al., 2020a; Reed et al., 2003). These features make birds of great interest to Public Health (Reed et al., 2003).

In the bird migration season, it is estimated that 200 species of birds migrate from North America during autumn to Central and South America, as well as from Eastern Europe to Africa (Gill & Prum, 2019). In South America, the Pantanal is used by several migratory routes throughout the year (Batista et al., 2017). During these movements, birds can transport and spread infected ticks with vector-borne and zoonotic pathogens (Comstedt et al., 2006; Elfving et al., 2010; S. A. Hamer et al., 2012; Waldenström et al., 2007).

2 Vector-borne agents in birds

2.1 Anaplasmataceae agents

Anaplasmataceae agents are amongst the most prominent vector-borne pathogens (Cutler et al., 2010). The Anaplasmataceae family, belonging to the order Rickettsiales, comprises small (0.5 - 1.5 µm) obligate intracellular Gram-negative cocci (Brouqui & Matsumoto, 2007). Until now, four genera have been described, namely *Anaplasma, Ehrlichia, Wolbachia* and *Neorickettsia* and four putative *Candidatus* ('*Candidatus* Neoehrlichia', '*Candidatus* Allocryptoplasma', '*Candidatus* Xenohaliothis' and '*Candidatus* Xenolissoclisum') (Dumler et al., 2001; Eshoo et al., 2015; Friedman et al., 2000; Kwan and Schmidt, 2013; Nicholson, 2018; Ouass et al., 2023). The genus *Anaplasma* comprises eight species, namely *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma bovis*, *Anaplasma ovis*, *Anaplasma platys* (Cabezas-Cruz et al., 2023), *Anaplasma capra* (Nicholson, 2018), and *Anaplasma odocoilei* (Tate et al., 2013). The genus *Ehrlichia* genus comprises six recognized species, namely *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Ehrlichia ruminantium* (Dumler et al., 2001), and *Ehrlichia minasensis* (Cabezas-Cruz et al., 2016). The genus *Neorickettsia* comprises *Neorickettsia risticcii*, *N. findylandensis* (Budachetri et al., 2022), and *Neorickettsia sennetsu* (Dumler et al., 2001). '*Candidatus* Neoehrlichia' comprises four species, namely '*Candidatus* Neoerhlichia mikurensis', '*Candidatus* Neoerhlichia lotoris', '*Candidatus* Neoerhlichia arcana' and '*Candidatus* Neoerhlichia chilensis' (Gofton et al., 2016; Li et al., 2012; Müller, Monti, et al., 2018; Silaghi et al., 2016). '*Candidatus* Allocrytoplasma' comprises, so far, only of one species, namely '*Candidatus* Allocryptoplasma californiensis' (Eshoo et al., 2015). '*Candidatus* Xenohaliothis' and '*Candidatus* Xenolissoclisum' consist of one species each, namely '*Candidatus* Xenohaliothis californiensis' (Friedman et al., 2000) and '*Candidatus* Xenolissoclisum pacificiensis' (Kwan and Schmidt, 2013).

Anaplasmataceae agents are mainly transmitted by vectors or mechanically, depending on the species (Kocan, K.M., De La Fuente, J., Cabezas-Cruz, 2015). For instance, transmission dynamics is more complex for *Anaplasma marginale,* due to mechanical transmission, than for *A*. *phagocytophilum* (Kocan, K.M., De La Fuente, J., Cabezas-Cruz, 2015) and *Ehrlichia* species (Fourie et al., 2013). *Anaplasma* species can be transmitted interstadially (Kocan, K.M., De La Fuente, J., Cabezas-Cruz, 2015) and transovarial in *Dermacentor albipictus* ticks (Baldridge et al., 2009). *Anaplasma* spp. (*A. bovis*, *A*. *marginale*, *A. centrale*, *A*. *ovis*, *A*. *phagocytophilum*) can be biologically transmitted by ticks (*Dermacentor*, *Haemphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus). Ehrlichia* spp. can be transmitted by *Amblyomma*, *Ixodes* and *Rhipicephalus* ticks (Nicholson, 2018; Thirumalapura & Walker, 2014)*. 'Candidatus* Neoehrlichia spp.) are transmitted by *Ixodes ricinus* ticks in Europe and *Ixodes persulcatus* in Asia (Nicholson, 2018). On the other hand, *Neorickettsia* spp. are transmitted by ingestion of infected immature stages of trematodes (Dittrich et al., 2015; Nicholson, 2018). The role of birds in the epidemiology of tick-borne Anaplasmataceae agents is unclear (Johnston et al., 2013). This difficulty unraveling the epidemiologic role of birds could be due to their natural resistance to *A*. *phagocytophilum* infection. Indeed, the normal avian temperature is quite distinct from the optimal temperature for *A. phagocytophilum* growth in vitro (Borjesson, 2008; Johnston et al., 2013; Møller, 2010).

In the American continent, the Anaplasmataceae agents detected are: *Aegytianella pullorum* from experimentally infected white turkeys (*Meleagris gallopavo*) and according to (Rikihisa et al., 2003) when analysing 16S rRNA and *groEL* genes, *Aegyptianella pullorum* grouped within the clade *Anaplasma* at an almost equal distance from *A*. *marginale* and *A*. *phagocytophilum*.

In Brazil, Machado et al. (2012) detected *Anaplasma* spp and *Ehrlichia* spp. in birds of prey, an *Anaplasma* spp. sequence closely related to *A. phagocytophilum* and *Ehrlichia* spp. closely related to *E*. *chaffeensis* based on a phylogenetic analysis inferred with the 16S rRNA gene. The phylogenetic

analysis based on the *omp-1* gene, the sequences detected were was closely related to other *Ehrlichia* spp. sequences from Brazilian wild felids.

Werther et al. (2017) detected three *Anaplasma* spp. and 24 *Ehrlichia* spp. positive samples from Orinoco geese by means of a nested PCR targeting the 16S rRNA gene. The *Anaplasma* spp. sequences grouped with *Anaplasma* sp. previously detected in a vulture, in the clade of *A*. *phagocytophilum*. On the other hand, the detected *Ehrlichia* sequences were separated into four different genotypes. While genotypes #1 and #2 clustered into the clade of *E*. *canis* along with sequences previously detected in wild felids from Brazil and in a tick (*Rhipicephalus sanguineus* sensu lato*)*, genotypes #3 and #4 grouped with *E. chaffeensis*. In addition, a sequence obtained from a positive sample from a nested PCR based on the *omp*-1 gene; was positioned in a separate clade together with the sequences of *Ehrlichia* sp. detected in wild felids and in an Orinoco goose from Brazil.

Mongruel et al. (2017) detected Anaplasma spp. on a single specimen of *P. obscura* in a nested PCR for *Anaplasma* spp. based on the 16S rRNA gene. The sequence was positioned into the clade of *A*. *phagocytophilum*, close to the sequence of *Anaplasma* sp. detected in domestic cats in Brazil.

Muñoz-Leal et al. (2019) detected a putative novel *Ehrlichia* sp. (named *Ehrlichia* sp. strain Magellanica) in three penguins and in non-engorged *Ixodes uriae* ticks from Chile, based on a conventional PCR for the 16S rRNA gene and nested PCR targeting the *groEL* and *dsb* genes. Phylogenetic analyses based on the 16S *rRNA gene showed that Ehrlichia sp. Magellanica formed a polytomic clade with Ehrlichia sp. BAT detected in Argas vespertilionis in France and Ehrlichia sp. Natal detected in an opossum (Didelphis albiventris) from* Brazil. The phylogenetic inferences based on the non-rooted *dsb* and *gro*EL genes showed that the sequences of *Ehrlichia* sp. Magellanica were positioned in well-supported branches. While *Ehrlichia* sp. strain Magellanica was positioned in a separate clade, but close to *Ehrlichia* sp. Natal in the phylogenetic tree based on the *dsb* gene, the detected *groEL* sequences were closely related to *'Candidatus* Ehrlichia shimanensis'.

Sacchi et al. (2021) detected *Anaplasma* spp. and *Ehrlichia* spp. in birds of prey (Cathartiformes, Accipitriformes, Falconiformes, and Strigiformes) collected in Sao Paulo and Rio de Janeiro states. For that purpose, 121 DNA blood samples were submitted to qPCR assays targeting the *groEL* (*Anaplasma* spp. and *Ehrlichia* spp.), *msp*-2 (*Anaplasma phagocytophilum*) and *vlpt* (*Ehrlichia chaffeensis*) genes and nested PCR assays targeting the *rrs* (Anaplasmataceae), *omp*-1 (*Ehrlichia* spp.), and *gro*ESL (*Anaplasma* spp.) genes, and a conventional PCR assay targeting the *dsb* gene (*Ehrlichia*). As a result, the phylogenetic analyses positioned the *rrs* sequences in two different clades: while the first clade was related to *E. chaffeensis* and contained the sequences obtained from a *Speotyto cunicularia*, *Rupornis magnirostris*, *Tyto alba,* and *Megascops choliba*, the second clade was related with *Ehrlichia* spp. and *E. canis* and contained sequences obtained from an *Asio clamator* and *R*. *magnirostris*.

In French Guyana, a total of 290 blood samples from 26 avian species collected in 16 localities along the coastline from 2012-2018. Avian blood samples were submitted to a 16S rRNA gene barcoding and individual PCR assays targeting the 16S rRNA gene for *Ehrlichia* spp., *Anaplasma* spp., and '*Candidatus* Allocryptoplasma spp.' to confirm results. Only an *Ehrlichia* spp. genovariant #1 was detected in five out of 26 avian species (19.23%) (Buysse et al., 2024).

In Europe, Sándor et al. (2015) collected 180 heart tissue samples and 459 ticks from 120 birds belonging to 37 different species in Romania. Two cardiac tissue samples (1.7%) from a European thrush (*Erithacus rubecula*) and a thrush (*Turdus philomelos*) and eight ticks (1.7% [*Haemaphysalis concinna* larvae, *Ixodes arboricola* larvae, and *Ixodes ricinus* larvae and nymphs]) were positive for *Anaplasma* spp. in a conventional PCR based on the 16S rRNA gene.

Hornok et al. (2020) detected new genotypes of *Neorickettsia* and *Ehrlichia* sp. in Hungary. The *Neorickettsia* genotype detected in a common teal (*Anas crecca*) was positioned together with *N*. *helminthoeca* isolates related to

fish and dogs from the USA. The *Ehrlichia* genotype detected in a thrush (*Turdus philomelo*) was positioned in a clade with sequences of *E*. *chaffeensis* from Asia and America.

Recently, Defaye et al. (2023) detected *Ehrlichia* spp. in an *Erithacus rubecula* when targeting the gene 16S rRNA using a microfluidic real-time PCR as screening. To confirm the microfluidic real time PCR results, the same sample was submitted to a conventional PCR targeting the 16S rRNA gene. As a result, a genotype closely related to a sequence of *E*. *chaffeensis* from a goat from China was detected.

In *Ille-de-France,* Rouxel et al. (2024) detected two sequences showing 100% identity to *A*. *phagocytophilum* were obtained from a blackbird (*Turdus merula*) when targeting the *groEL* gene. When targeting the *ank*A gene three sequences were obtained showing a 99.9-100% similarity with *A. phagocytophilum.*

2.2 *Bartonella* spp.

The genus *Bartonella*, belonging to the order Hyphomicrobiales and family Bartonellaceae, comprises fastidious facultative intracellular Alphaproteobacteria that primarily infect erythrocytes and endothelial cells (Birtles & Raoult, 1996; Breitschwerdt et al., 2010). These bacteria are short, pleomorphic, Gram-negative rods, culture-demanding, oxidase-negative, and present aerobic metabolism (Jacomo et al., 2002). The transmission of bartonellae is mainly mediated by arthropod vectors, such as fleas (Billeter et al., 2011; Chomel et al., 1996; Klangthong et al., 2015; Müller, Rodríguez, et al., 2018; Nasereddin et al., 2014; Nziza et al., 2019; Salas et al., 2019), lice (Anstead, 2016; Faccini-Martínez et al., 2017; Louni et al., 2018), sand flies (Minnick et al., 2014), and potentially by ticks (Billeter et al., 2009; Cotté et al., 2008; Kang et al., 2013; Reis et al., 2011), as well as by bites, scratches, and body fluids from infected animals (Breitschwerdt et al., 2000, 2010). Currently, there are 39 recognized species (https:// www. bacte rio. net/ genus/ bartonella- accessed on August, 11th 2024), from which 17 showing zoonotic potential

(Breitschwerdt et al., 2010; Buffet et al., 2013; Chomel et al., 2009; Kosoy & Goodrich, 2019; Maggi et al., 2011).

Reports of *Bartonella* in birds and associated ectoparasites are limited and scarce. For instance, by means of a quantitative PCR assay targeting the *gltA* gene in 386 ticks (*Hyalomma* spp [359 ticks] *Hyalomma rufipes* [nine ticks] and *Hyalomma marginatum* [one tick]), *Ixodes* spp. (seven ticks), *Amblyomma* spp. (two ticks), *Haemaphysalis* spp. (two ticks) and six unidentified ticks collected from birds from Italy and Greece, *Bartonella* DNA was not detected (Molin et al., 2011).

In Hong Do Island, Korea, Kang et al. (2013) sampled 212 ticks, totaling 108 pools (*Ixodes turdus*, *Ixodes nipponensis, Haemaphysalis longicornis*, and *Haemaphysalis ornithophila*) collected from 65 birds (3%) from a total of 2,161 migratory birds. *Bartonella grahamii* was detected by nested PCR assay based on the 16-23S rRNA intergenic region (ITS) in an *Ixodes turdus* specimen (0.92% [1 pool/108 pools]) collected from a White's thrush (*Zoothera aurea*). The sequence was positioned closely to *Bartonella grahamii*.

In Morehead City, North Carolina, USA, Mascarelli et al. (2014), amplified *Bartonella* DNA from the northern mockingbird (*Mimus polyglottos*) and the red-winged thrush (*Agelaius phoeniceus*), sharing a 99.6% similarity with *B. henselae*. *Bartonella koehlerae* DNA was amplified from a red-bellied woodpecker (*Melanerpes carolinus*) and a Common loon (*Gavia immer*).

In the summer of 2014 at Karrak Lake, Nunavut, Canada, Buhler et al. (2020) sampled 827 fleas (*Ceratophyllus vagabundus vagabundus* [n=251 fleas]) from goose nests and 86 fleas from local burrows. In addition, fleas were collected from 48 Ross geese (*Chen rossii*) and 54 lesser snow geese (*Chen caerulescens caerulescens*) that were euthanized. As a result, 43% and 40% of flea samples pooled from individual nests and from individual burrows were positive in cPCR assays for *Bartonella* spp. based on the ITS region. The sequences obtained from fleas from nests and burrows were identified as *B. henselae* and showed 100% identity with *B*. *henselae* sample SA2 (San Antonio 2). Fleas collected from nine Ross geese and 11 smaller snow geese were

positive for *Bartonella* spp. showing 100% identity with *Bartonella vinsonii berkhoffii*.

Williams and Dittmar (2020) investigated the presence of *Bartonella* spp. DNA in blood samples from 49 blue swallows (*Progne subis*), six bluebirds (*Sialia sialis*), six swallows (*Tachycineta bicolor*), and in hematophagous ectoparasites (69 *Dermanyssus prognephilus*, 76 *Ceratophyllus idus*, and 12 *Protocalliphora sialia* larvae) collected from bird nests in the Iro Area of National Wild Refugee and Tonawanda Wildlife Management, Genesee County, New York State, USA. The occurrence of *Bartonella* spp. was assessed by 16S rRNA- metagenomics and conventional PCR based on the *gltA* gene. When targeting the 16S rRNA gene, 33% (2/6) bluebirds, 39% (19/49) purple martins, and 83% (5/6) swallows were positive for *Bartonella* spp. Regarding the blood-sucking ectoparasites, *Bartonella* spp. DNA was detected in 13-25% of *P*. *sialia*, 9-36% of *C*. *idius* and 13-100% in *D*. *prognephilus* based on the16S rRNA-metagenomics. To confirm the results, positive samples were subjected to a PCR based on the *glt*A gene. The *glt*A sequences obtained were distributed in two subclades in the phylogenetic analysis. The sequences detected in mites collected from tree swallows were positioned in a clade related to *Bartonella* spp. sequences, while the sequences obtained in blue swallow mites were related to *Bartonella tamiae*.

Bertelloni et al. (2024) investigated the presence of *Bartonella* DNA in spleen samples from 300 birds belonging to 24 avian species collected in the Tuscany region, Italy. The ocurrence of *Bartonella* spp. was determined by conventional PCR based on the 16S rRNA gene. One sample (0.33%) from a magpie (*Pica pica*) was positive to *Bartonella* spp. on agarose gel electrophoresis, albeit without sequencing.

2.3 *Borrelia* spp.

The genus *Borrelia*, belonging to order Spirochetales, comprises vector-borne spirochetes, measuring 0.2-0.5 mm wide and 3 to 30 mm length (Barbour & Hayes, 1986). Phylogenetically, the *Borrelia* genus is divided into three groups, namely: (i.) Lyme borreliosis (LB) group, which is mainly transmitted by Ixodidae ticks; (ii.) Relapsing fever (RF) group, which is transmitted by Argasidae ticks; (iii.) *Borrelia* related to reptiles and echidna, which are mainly transmitted by ticks of the genus *Amblyomma*, *Bothriocroton* and *Hyalomma* (Binetruy et al., 2020; Margos et al., 2018)

Avian-related *Borrelia* spp. detected in avian-associated ticks are allocated in three groups: (i) LB group: *Borrelia garinii*, *Borrelia burgdorferi*, *Borrelia afzelli*, *Borrelia valaisiana* (Comstedt et al., 2006) and *Borrelia turdi* (Kang et al., 2013; Margos et al., 2019), (ii) RF group: *Borrelia miyamotoi* detected in ixodid ticks collected from migratory birds (Comstedt et al., 2006) and *Borrelia anserina* (Elelu, 2018)*;* (iii) equidna-reptilian group or third group: *'Candidatus* Borrelia mahuryensis' (Binetruy et al., 2020)*.*

The molecular detection of *Borrelia* spp. in birds and avian-associated arthropods has extended our comprehension on the role of sea birds (Olsen et al., 1995) and passerines (Comstedt et al., 2006) as spreaders of *Borrelia* spp. (Jordan et al., 2009; Kang et al., 2013; Lilly et al., 2022).

In Poland, 1,254 passerines (belonging to 42 species) had blood samples collected along three years (1996 to 1998). When targeting the histone protein B gene (*hbb*), 53 (4.2%) out of the 1,254 passerines were positive to *B. burgdorferi* s.l. (Gryczyńska et al., 2004)

In Germany, different *Borrelia* species were detected in passerines-associated ticks, when using an heminested PCR targeting the *osp*A gene. In total, 190 *Ixodes ricinus* ticks (56 larvae, 134 nymphs and one adult) were collected from 99 ground-foraging passerines. *Borrelia* spp. DNA was detected in 14.1% (27/191) ticks (11[20%] larvae and 16 [11.9%] nymphs. Five sequences were identified as *Borrelia garinii osp*A type-6. *Borrelia afzelii osp*A

type-2 was detected in 11 samples (6 larvae and 5 nymphs); *B. garinii osp*A type-6 was detected in two larvae and 2 nymphs, and *B*. *burgdorferi* was detected in one larva and one nymph. Coinfection by *B*. *garinii osp*A types 6 and 8 was detected in an *I*. *ricinus* nymph (Franke et al., 2010).

In Portugal, 102 ixodid ticks were sampled from 66 birds representing 10 species. As a result, 17 (16.66%) ticks were positive to *Borrelia turdi* after sequencing the *flaB* gene. Isolates of *B. turdi* cultured in Barbour-Stonner-Kelly-II (BSK-II) were obtained from ixodid ticks (*I. frontalis* and *I. ricinus*) collected from *P. major*, *T*. *merula*, *T*. *philomelos*, *Turdus iliacus*, and *Troglodytes troglodytes*. On the phylogenetic analyses, the isolates of *B*. *turdi* were closely positioned with other *B*. *turdi* sequences when inferring with 5S-23S rRNA (Intergenic spacer region) and MLST (Multi-locus Sequencing Typing) (based on the *clp*X, *nif*S, *pep*X, *pyr*G, *rec*G, *rpl*B and *uvr*A genes) (Norte et al., 2015).

In Belgium, Norte et al. (2020) demonstrated the tropism of *Borrelia valaisiana* and *Borrelia turdi* to skin in bird models (Blackbirds [*Turdus merula*] and Great tits [*Parus major*]) when compared to other tissues (foot joint, liver, kidney, brain, heart, spleen and lungs), using qPCR assays targeting the *fla*B and *osp*A genes to detect *Borrelia* spp. DNA, as well as a nested PCR targeting the *fla*B gene and 5S-23S intergenic spacer region.

Bertelloni et al. (2024) detected *Borrelia burgdorferi* sensu lato (s.l.) in spleen samples from nine corvids (*Pica pica* (6/45 [13.33%]) and *Corvus cornix* (3/25 [12%]) and a pheasant (*Phasianus colchinus* 2/40 [5%]) in Italy, when performing a conventional PCR targeting the 16S rRNA gene.

In Mendocino County, northwestern California, United States of America (USA), 623 birds were captured and had blood samples and 286 ticks (284 *Ixodes pacificus* and two *Haemaphysalis leporispalustris*) collected. *Borrelia burgdorferi* s.l. DNA was detected in 57 out of 623 birds (9.14%) when targeting the 16S-23S intergenic spacer (ITS) in conventional PCR assays. When it comes to *I. pacificus* larvae and nymphs, 25 (13%) out of 192 larvae and 21 out of 85 nymphs (24.7%) were positive to *Borrelia* spp. DNA. Different *Borrelia* species were detected in avian blood samples: *B. burgdorferi* s.l., *B*. *burgdorferi*

s.s., and *B. burgdorferi bissettii.* Meanwhile, *B. burgdorferi* s.s, *B. bissettii* were detected in nymphs and *B. burgdorferi* s.l., whereas *B. burgdorferi* s.s. and *B. bissettii* were detected in larvae (Newman et al., 2015).

In Cook County, southwestern Chicago, USA, 357 ticks were sampled from 97 birds. A total of 120 ticks were subjected to PCR assays for *Borrelia* spp. based on the 16S rRNA and *p44* genes and sequence typing targeting the 16S-23S intergenic spacer (ITS) and *osp*C gene. As a result, 3 out of 6 *Ixodes scapularis* adults (50%), 1 out of 22 *I. scapularis* nymphs (4.5%) and 1 out of 34 *Haemaphysalis leporispalustris* nymphs (2.9%) were positive for *Borrelia* spp. when targeting the 16S-23S intergenic spacer (IGS). *Borrelia burgdorferi* sequences were obtained from all three *Ixodes scapularis* nymphs and were represented three ITS ribotypes (2,28,14) and *ospC* genotypes (H, T and A3) (Hamer et al., 2012).

In the state of Tennessee, USA, blood samples were collected from 163 migratory Anseriformes birds, belonging to six species (*Anas rubripes*, *Branta canadensis*, *Anas platyrhynchos*, *Anas acuta*, *Aythya collaris,* and *Aix sponsa*) and 226 wild turkeys (*Meleagris gallopavo silvestris*). *Borrelia burgdorferi* and *Borrelia lonestari* DNA amplification was performed using a nested PCR and dot-blot hybridization targeting the *flaB* gene. From the 389 blood samples collected, 91 (23%) were positive to *B. burgdorferi* or *B. lonestari*. The confirmation of positivity for both *B. burgdorferi and B. lonestari* was performed with specie-specific hybridization probes in dot-blot assays (Jordan et al., 2009).

In French Guyana, out of 290 sampled *Amblyomma* spp. ticks collected from 78 birds belonging to 26 avian species, *Borrelia* spp. DNA was detected in 20 specimens (6.9%). Based on the *fla*B gene, the *Borrelia* detected was 100% identical to *Borrelia* spp. clone 2T (MN064675) from an *Amblyomma longirostre* collected from passerines in Brazil. The phylogenetic analyses based on *fla*B genes positioned the new *Borrelia* spp. detected in French Guiana in the *Borrelia* reptile-echidna group (Binetruy et al., 2020).

In Argentina, Cicuttin et al. (2019) collected 127 ticks from birds (*Ixodes auritulus* [56 larvae, 44 nymphs and 8 females] and *Ixodes aureolatum* [1 larva and 11 nymphs]) and 1,091 ticks were collected from the vegetation (larvae, nymphs and adults of *Amblyomma aureolatum*, *Amblyomma triste* and *Amblyomma auritulus*). The ticks' DNA was submitted to a nested PCR assay targeting the *flaB* gene for *Borrelia* spp. Out of 1,091 ticks collected from vegetation, nine nymphs of *A*. *aureolatum* (0.8%) and four nymphs of *I*. *auritulus* (0.36%) were positive for *Borrelia* spp. Out of 127 ticks collected from birds, only 100 ticks were submitted to a nested PCR targeting the *fla*B gene for *Borrelia* spp.; out of those ticks, 5 (5%) out of 22 pools of *I. auritulus* larvae were positive for *Borrelia* spp. and the overall percentage of infected ticks was of 24% (24/100). The *Borrelia* spp. *flaB* sequences obtained from *A*. *aureolatum* were positioned together with *Borrelia turcica* from Egypt. The *flaB* gene sequences obtained from *I*. *auritulus* formed an independent clade within the *B*. *burgdorferi* sensu lato, being closely related to *Borrelia* spp. detected in Canada and Uruguay. The 16S rRNA sequences obtained from *I*. *auritulus* showed 99.2-100% identity with sequences of the *Borrelia* LB group, and a sequence obtained from an *A*. *aureolatum* showed 99% identity with *B*. *turcica* from Jordan.

In South Korea, a total 3,816 birds were trapped to collect ticks. As a result, the *Borrelia* spp. 16S rRNA sequence showed 99.9% identity with *Borrelia tanuki* from Japan, and the *B*. *turdi* 16S rRNA obtained sequence showed 99.99% identity to *B*. *turdi* from Japan. When targeting the *groEL* gene, one *Borrelia* spp. and one *B*. *turdi* sequences were obtained from *Ixodes* spp. ticks parasitizing migratory birds.The *Borrelia* spp. sequence showed identity of 99.4% with *B*. *turdi* (AF517972) and 99% with *B*. *tanuki* (AF517973), respectively. The obtained *B*. *turdi* sequence showed 100% identity with *B*. *turdi* (AF517972) and 99.7% identity with *B*. *tanuki* (AF517973) (Kang et al., 2013).

In Brazil, *Borrelia anserina* was isolated from *Argas* (*Persicargas*) *miniatus* collected from a chicken coop. The chickens infested by these ticks presented clinical signs as fever, depression, diarrhea, and spirochetes were found in blood smears and in dark field microscopy (Labruna et al., 1999).

(Ataliba et al., 2007) characterized *Borrelia anserina* strain PL previously isolated by (Labruna et al., 1999) targeting both the *rrs* and *fla*B genes. In the phylogenetic analyses using *fla*B gene, *Borrelia anserina* strain PL was positioned in the same clade with *B. anserina* from the USA.

In Rio de Janeiro, 22 nymphs of *Amblyomma longirostre* ticks were collected from birds captured in Serra dos Orgãos National Park and Guapimirim. DNA from ticks was submitted to a PCR targeting the *fla*B gene, and only one (4.54%) *A. longirostre* was positive to *Borrelia* spp. The sequences obtained showed 91% of identity with *B*. *turcica* from Turkey, and 99% similarity with *Borrelia* sp. from the USA (Pacheco et al., 2019).

2.4 *Hepatozoon* spp.

The genus *Hepatozoon* comprises Apicomplexan protozoa belonging to the suborder Adeleorina and family Hepatozoidae (Baneth et al., 2007). The life cycle of these agents includes invertebrates, such as ticks, fleas, flies, mosquitoes or lice, as definitive hosts (Lainson et al., 2003; Rubini et al., 2006; Watkins & Nowell, 2003) and intermediate hosts represented by amphibians, reptiles, mammals, and birds. *Hepatozoon* spp. are transmitted by ingestion of the definitive hosts containing mature oocysts by the intermediate hosts (Smith, 1996), vertical transmission (Murata et al., 1993), and predation (Johnson et al., 2009).

The description of *Hepatozoon* spp. in birds is predominantly based on morphometric features, as it was performed by (Bennett & Peirce, 1989) for the identification of *Hepatozoon parus*. Additional information on the life cycle of *Hepatozoon* parasites from birds is limited. *Ornithodorus peringueyi* ticks and *Xenopsylla trispinis* fleas are the only arthropods described harboring avian hemogregarinids (*Hepatozoon atticorae*), suggesting that birds become infected when they ingest arthropods (Bennett et al., 1992b). Infections by *Hepatozoon* spp. in birds are usually misclassified as *Haemogregarina* or *Atoxoplasma*, contributing to the overlook of possible infections (Bennett et al., 1992). Recently, (bani and Mancianti (2022) listed avian *Hepatozoon* species, namely

Hepatozoon estrildus, Hepatozoon lanis, Hepatozoon malacotinus, Hepatozoon sylvae, Hepatozoon numidis, Hepatozoon pittae, Hepatozoon zosteropis (Bennett et al., 1992), *Hepatozoon atticorae* (Bennett & Peirce, 1989), *Hepatozoon passeris* (Ebani and Mancianti, 2022), *Hepatozoon prionopis* (Bennett et al., 1995), *Hepatozoon apodis* (Barraclough et al., 2008), and *Hepatozoon peircei* (Merino et al., 2014). *Hepatozoon parus* (Bennett and Peirce, 1989) and *Hepatozoon kabeeni* (Biedrzycka et al., 2013) are more related to Lankesterellids due to its phylogenetic position (Biedrzycka et al., 2013; Merino et al., 2006). The phylogenetic inferences of *Hepatozoon* spp. revealed two main clades: i.) Clade I includes *Hepatozoon* spp. related to small mammals, birds and herpetozoa and ii.) Clade II comprises *Hepatozoon* spp. related to carnivores (Thomas et al., 2024). The taxonomic position of *Hepatozoon* species associated with birds should be revised. In fact, a greater number of *Hepatozoon* DNA sequences must be obtained from birds (Biedrzycka et al., 2013).

Merino et al. (2006) performed blood smears from 189 Eurasian blue tits (*Cyanistes caeruleus*) at a site near Valsaín, Segovia, Spain. *Hepatozoon* gametocytes with morphometry similar to that of *H. parus* were found in 31.2% (59/189) of the blood smears. Later, cPCR assays based on the 18S RNA gene were performed on six blood samples from birds positive in the blood smears. Phylogenetic analyses showed that the sequences detected were closely related to *Lankesterella minima*.

In Poland, Biedrzycka et al. (2013) investigated the occurrence of *Hepatozoon* spp. in 131 blood samples of adult Reed Warbler (*Acrocephalus schoenobaenus*). As a result, 47.3% (69/147) of the samples were positive for Apicomplexa in a conventional PCR protocol directed to 18S rRNA. In the phylogenetic analysis, two of the Apicomplexa haplotypes were closely related to *Lankesterella minima* and *Lankesterella valsainensis*. The third haplotype was closely related to *Caryospora* and *Eimeria*. None of the Apicomplexa haplotypes were phylogenetically related to *Hepatozoon* sequences of reptiles and mammals.

2.5 Hemosporidians

Avian haemosporidians comprise three protozoa genera: *Haemoproteus*, (containing two subgenera, *Haemoproteus* and *Parahaemoproteus*), *Leucocytozoon,* and *Plasmodium* (Valkiunas, 2004), which are transmitted by blood sucking dipterans (Atkinson et al., 2009; Valkiunas, 2004). The distribution of haemosporidians is described all around the world except in the Antarctica (Friend & Franson, 1999). Microscopic identification of these agents requires great expertise to morphologically identify the species (Valkiunas, 2004), since they show high diversity, including more than 5,133 unique genetic lineages (obtained from: http://130.235.244.92/bcgi/malaviReport.cgi?report8=Database+Summary+Report, accessed on September, 23rd 2024.(BENSCH et al., 2009).

The members of the genus *Haemoproteus* are defined by their intraerythrocytic development and production of golden-brown or black pigment (hemozoin). Phylogenetically, they are divided into two clades (Martinsen et al., 2008) or subgenera based on the vector. Those belonging to sub-genus *Haemoproteus* infect Columbiformes and are vectored by hippoboscid flies. On the other hand, the diverse sub-genus *Parahaemoproteus* is transmitted by biting midges and occurs in Passeriformes(Atkinson et al., 2009; Martinsen et al., 2008; Valkiunas, 2004).

The avian-associated *Plasmodium* species are cosmopolitan and found in almost all zoogeographic regions (Valkiunas, 2004). At present, 1,446 distinct lineages of *Plasmodium* have been described in avian hosts (Martinsen et al., 2008). *Plasmodium* infections are reported in a wide variety of avian orders, and exhibit the highest diversity in Columbiformes, Galliformes and Passeriformes (Valkiunas, 2004). Avian malaria caused by *Plasmodium* spp. is primarily a hematogenous disease, but also a disease of the reticuloendothelial system. The clinical signs are proportional to parasitemia levels (van Riper et al., 1986).

Leucocytozoon has a wide variety of species but only some are known to cause disease in their hosts (Bennett et al., 1993; Valkiunas, 2004). Although

a hotspot for haemosporidians is not stable over the time, *Leucocytozoon* is spread throughout the world (Valkiunas, 2004), with its major occurrence in the Afrotropical, Nearctic and Palearctic regions, where Passeriformes showed the highest prevalence of infection (Fecchio et al., 2021). These haemosporidians have been described in 113 out of the 204 avian families and 30% of avian species, with the major occurrence in Passeriformes (Valkiunas, 2004).

Several studies have been performed on haemosporidians in birds from different Brazilian geographic regions. For instance, in the São Paulo Zoo, a prevalence of 18% (24/133) was found for hemosporidians among free-living birds using PCR assays targeting the *cytB* gene. Four and 20 out of 133 free-living birds from the Sao Paulo Zoo were positive for *Plasmodium* spp. (3%) and *Haemoproteus* spp. (15%), respectively. Four *Haemoproteus* lineages and three *Plasmodium* spp. lineages were described and their sequences were deposited in MalAvi: HAECL 1, COQUI05 and COLIV03 (Chagas et al., 2016).

In the state of Minas Gerais, a study conducted in the “Sabiá Municipal Park”, Uberlândia, and based on analyses of blood smears of 46 captive birds (19 species) showed an overall prevalence of 6.5% (3/46). *Plasmodium* spp. was found in the blood from two *Pavo cristata* and one *Tyto furcata* (De Aguiar and Júnior, 2021).

Also in Minas Gerais, specifically in the Triângulo Mineiro region, (Ribeiro et al., 2020) found a prevalence of 18.8% (103/548) for *Plasmodium* spp. and 2.37% (13/548) for *Haemoproteus* spp. by blood smear analysis of captive birds. Out of the 56 avian species that were analyzed, 33 (59%) were infected with *Plasmodium* spp. and 6 avian species (10.7%) were also infected with *Haemoproteus* spp. The positivity for hemosporidians was directly proportional to the size of the sampled birds (Ribeiro et al., 2020).

In the city of Palmas, Lajeado (LSP) and Cantão State Park (CSP), state of Tocantins, comprising Cerrado and Amazon rainforest biomes, respectively, the prevalence of infection with *Plasmodium/ Haemoproteus* was 49% (331/676) using PCR assays based on the *cyt*B gene. Based on a combination of PCR and blood smear analyses, *Plasmodium* spp. and *Haemoproteus* spp.

infections were found in 26 avian families (89.7%) and 93 avian host species (97%), and included 11 *Plasmodium* spp. lineages (TOC-11, TOC-21, TOC-28, TOC-32 [urban area]; TOC-19 and TOC-14 [in LSP and CSP respectively]); TOC-4, TOC-9, TOC-16, TOC-24 and TOC-15 [in LSP, CSP and urban area]) and 10 *Haemoproteus* spp. lineages (TOC-7, TOC-26, and TOC-29 [in LSP]; TOC-22 [in CSP]; TOC-1, TOC-2, TOC-3, TOC-13 and TOC-20 [in LSP, CSP and urban area]) (Belo et al., 2011).

In the Pantanal region (municipalities of Corumbá, Poconé, Cáceres, and Santo Antonio de Leverger, state of Mato Grosso), a study performed by (Fecchio et al., 2022), and based on PCR assays targeting the *cytB* gene, an overall prevalence of 11.2% (72/642) was found for haemosporidians, including 6.3% prevalence for *Plasmodium* spp., 3.9% prevalence for *Haemoproteus* (*Parahaemoproteus*), and 1.3% prevalence for *Haemoproteus* (*Haemoproteus*). The families that had the highest prevalence were Ardeidae (33%), Thraupidae (22.6%) and Columbidae (21.6%). According to the authors, birds that used multiple strata were at higher risk of acquiring the infection when compared to those birds that foraged on water or on the ground (Fecchio et al., 2022).

2.6 Filariids

Filariids of the subfamilies Dirofilariinae, Onchocercinae, Splendidofilariinae and Lemdaniinae, belonging to family Onchocercidae, are mostly hosted by birds and present a cosmopolitan distribution (Adamson and Anderson, 1993; Atkinson et al., 2009).

The morphological characterization of microfilariae can provide useful clues for identification up to the genus level, but morphological analysis of adult nematodes is imperative for a correct taxonomic identification (Rodríguez and Matta, 2001). The diversity of species of filariid parasites of birds is in fact unknown. The last description of new species of filariids in birds was performed

by Bartlett (Bartlett, 1992) and Binkienė (Binkienė et al., 2021). The last literature review on avian filariids was published in 2009 (Atkinson et al., 2009).

Avian filariids are mainly transmitted by dipterans from the Simuliidae, Culicidae and Ceratopogonidae families or lice (order Phthiraptera) (Atkinson et al., 2009). These nematodes may have implications in Public Health (Sanchez-Godoy et al., 2020). For instance, *Pelecitus* spp. has been confirmed to parasitize birds and mammals, including humans (Jiménez-Ruiz et al., 2004; Xie et al., 1994)

The knowledge of avian filariids is limited. In Canada, Iceland and the USA, 636 birds belonging to 32 species and 4 families were captured. As a result, 88 (13.8%) were parasitized by adult nematodes of *Eulimdana* spp. (*E. sonini, E. andersoni, E. metcalforum, E. florencae, E. juventarum, E wogae, E. baine*, and *E. aspertum*); on the other hand, 46 (52.3%) birds had microfilaremia. *Eulimdana* spp. microfilariae were found in blood smears (Bartlett, 1992). In Colombia, 26 (8.2%) out of 315 birds presented microfilariae in blood smear analysis. The species could not be identified due the lack of adult filariids (Rodríguez and Matta, 2001). In Costa Rica, in Hitoy Cerere Biological Reserve and Barbilla National Park, 24 (6.6%) out of 362 birds showed microfilaremia in blood smears, with description of 9 morphotypes (Benedikt et al., 2009).

In Czech Republic, the occurrence of microfilaremia in passerine birds was assessed during post-nesting and pre-nesting periods. In the course of the post-nesting period of 2005, birds showed microfilaremia (*Eufilaria delicata*) in blood smears. During the pre-nesting period of 2007, three specimens of *Turdus merula* were infected with *Eufilaria delicata* and one showed a coinfection by *E. delicata* and *Ornithofilaria mavis*. Two *Turdus philomelos* were infected with *Eufilaria delicata*, whereas one *T. philomelos* presented a coinfection by *E. delicata* and *O. mavis.* One *T. philomelos* was infected by non-identified microfilariae. Two specimens of *Erithacus rubecula* were infected (while one presented a coinfection by *E. delicata* and *O. mavis,* another one was

parasitized by an unidentified microfilariae). One *Poecile montanus* presented microfilaremia with an unspecified filariid (Haas et al., 2011).

In the USA, during a period of 2 years (2010-2011), 70 turdus (*Turdus migratorius*) had blood samples collected during the daytime. Only 1 (1.4%) had microfilaremia on the blood smear; however, out of 63 turdus (*Turdus migratorius*) captured at night, 11.1% (7) had microfilaremia on the blood smear. In the necropsy, one male and one female adult filariids of the species *Chandlerella quiscali* and one male and one adult female of the species *Splendidofilaria* were collected, identified and deposited in the United States National Parasite collection, Maryland, U.S.A. *Chandlerella quiscali* specimens were recovered from brain tissues of a robin (*T. migratorius*) and *Splendidofilaria* spp. specimens were recovered from the heart from juvenile and adult house sparrows (*Passer domesticus*). Using molecular techniques using the 18S rRNA gene as the target gene, the obtained sequences were positioned in 2 clades in the phylogeny: a putative one for *Chandlerella quiscali,* with 98% bootstrap, and another one formed by *Splendidofilaria,* with 79% bootstrap (Hamer et al., 2013).

In Mexico City, five *Ramphastos sulfuratus* and 1 *Aulacorhyncus prasimus* were sent to the Research and Diagnostics Laboratory for Avian Diseases (College of Veterinary Medicine, UNAM) for diagnosis of avian diseases. The necropsy showed gross lesions (branchiocephalic arteries and truncus of aorta were thickened, hardeness and reduced lumen, hyperthrophy of left cardiac ventricule and hepatomegaly) associated with filariids. The presence of microfilariae was confirmed in histopathology. In molecular analyses, DNA extracted from parafined embedded tissues from the aorta with intralesional filariae were submitted to a conventional PCR using the 12S rRNA and 18S rRNA genes as the target genes. A 12S rRNA gene sequence of the genus *Filaroidea* was obtained (Sanchez-Godoy et al., 2020).

In Lithuania, a total of 206 passerines belonging to the genera *Acrocephalus* (n= 137) and *Sylvia* (n=69) were captured. Microfilaremia was observed in two common reed warblers (*Acrocephalus scirpaceus*), one great reed warbler (*Acrocephalus arundinaceus*), one Eurasian blackcap (*Sylvia atricapilla*), one garden warbler (*Sylvia borin*) and one lesser white throat (*Sylvia curruca*). At necropsy, adult filariids of the genus *Eufilaria* were identified in the subcutaneous tissue in two common reed warblers (*Acrocephalus scirpaceus)* and one garden warbler (*Sylvia borin*), while a *Splendidofilaria* spp. was obtained from the finger joints of one Eurasian blackcap (*Sylvia atricapilla*). Based on the analysis of blood smears and molecular techniques targeting the 28S rRNA and *cox1* genes, three new species were proposed, namely: *Eufilaria acrocephalusi*, *Eufilaria sylviae* and *Splendidofilaria bartletti* (Binkienė et al., 2021).

In the USA, *C. quiscali* microfilariae was detected in 3 out of 6 birds of the species *Turdus migratorius,* as well as in 5 of the 9 *Quiscalus quiscala* (Vaughan et al., 2021).

In the Lajeado State Park, Tocantins, 11 out of 166 wild birds belonging to the families Thamnophilidae, Parulidae, and Picidae, 6.6% presented unidentified microfilaremia (Silveira et al., 2010). In addition, when 925 birds' blood samples belonging to 11 families collected in the Atlantic Forest in the state of Minas Gerais were analyzed, the presence of microfilariae was detected in 2.1% of the individuals blood smears (Sebaio et al., 2012). Silva et al. (2014) described the presence of two nematodes identified by morphologic features as *Pelecitus* spp. in cervical air sacs and lungs in an *Athene cunicularia* collected by environmental authorities. More recently, in the Cerrado region of the state of Minas Gerais, one out of seven birds of the species *Antophilia galeata* had microfilaremia in the blood smear. Based on morphological features, *Eufilaria* spp. was identified (Vitor et al., 2020).

3 Avian related ticks in Brazil

Birds host several tick species (Luz & Faccini, 2020) and are also responsible for spreading ticks due to their high mobility and migrational behaviour (Hamer et al., 2012; Ogden et al., 2008)

In Brazil, 64 species were reported in birds according to (Dantas-Torres et al., 2009), reporting species from several Ixodidae ticks, namely *Amblyomma*, *Ixodes*, *Haemaphysalis*, and *Rhipicephalus*. When it comes to *Argasidae* ticks, the following genus were described: *Argas miniatus* has been detected only in domestic chicken or bird species closely associated with chickens, and *Ornithodoros capensis* in marine birds (Ribeiro et al., 2013).

The genus *Amblyomma*, *Ixodes*, *Haemaphysalis* and *Rhipicephalus* stand out the most when parasitizing passerine birds and the genus *Amblyomma* and *Ixodes* when parasitizing non-passerine birds (Dantas-Torres et al., 2009).

In the Pantanal biome, several tick species were reported in wild birds, namely *Amblyomma longirostre*, *Amblyomma cajennense*, *Amblyomma calcaratum*, *Amblyomma nodosum*, *Amblyomma* ovale*, Amblyomma triste*, *Amblyomma sculptum, Ornithodoros mimon* (Ramos et al., 2015; Fecchio et al., 2020b, 2023). In the Cerrado biome, wild birds were found parasitized by *A. cajennense, A. nodosum, A. calcaratum, A. longirostre, Amblyomma* spp*.,* and *Ornithodoros mimon* (Ramos et al., 2015; Pascoal et al., 2013; Witter et al., 2016). *Amblyomma nodosum*, *A*. *cajennense*). *Amblyomma aureolatum*, *Amblyomma romarioi*, *Amblyomma parkeri, Amblyomma coelebs*, *A*. *longirostre*, *A*. *nodosum*, *A*. *calcaratum, A. cajennense Amblyomma parkeri* and *Haemaphysalis leporispalirustris* were reported parasitizing wild birds in the Atlantic rainforest (Ogrzewalska et al., 2011; Ramirez et al., 2020; Rocha et al., 2021; Santolin et al., 2012; Zeringóta et al., 2017).

4 Vector-borne pathogens and Public Health

Amongst the most known arthropod-borne zoonotic agents that can also infect birds, we can name: *Borrelia burgdorferi s.l., Borrelia afzelii, Borrelia garinii, E. chaffeensis*, *A. phagocytophilum* (Baneth, 2014; Gage et al., 2008)*, Bartonella* species (*B*. *henselae*, *B*. *vinsonii berkhoffii*) (Breitschwerdt et al., 2000; Chian et al., 2002; Fontalvo et al., 2017), and filariids, namely *Pelecitus* spp. (Bain et al., 2011) and *Chandlerella quiscali* (Law et al., 1993).

5 OBJECTIVES

5.1 General

The present study aimed to investigate the occurrence and genetic diversity of Anaplasmataceae, Bartonellaceae, Borreliaceae, Hepatozoidae, and Onchocercidae in birds sampled in the Pantanal, in the states of Mato Grosso and Mato Grosso do Sul, central-western Brazil.

5.2 Specific

1. To investigate, by means of molecular methods, the occurrence and genetic diversity of Anaplasmataceae (*Ehrlichia* spp. and *Anaplasma* spp.), *Bartonella* spp., *Borrellia* spp., *Hepatozoon* spp., and Onchocercidae filariids in blood samples of wild birds in the Pantanal, in the states of Mato Grosso and Mato Grosso do Sul.
2. To standardize and validate high throughput microfluidic real-time PCR protocols, aiming at applying this technique in the molecular detection of Anaplasmataceae, Bartonellaceae, Borreliaceae, Hepatozoidae, Haemosporidians, and Filariidae agents in blood samples from birds sampled in the Pantanal of Mato Grosso state;
3. To infer the phylogenetic positioning of the detected genotypes based on distinct molecular markers.

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Chapter 2. Molecular evidence of *Bartonella* spp. in tropical wild birds from the Brazilian Pantanal, the largest wetland in South America

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Resumo: Apesar da ocorrência mundial de bartonelas em uma ampla gama de espécies de mamíferos, nas quais geralmente causam uma bacteremia eritrocítica de longa duração, poucos estudos relataram Bartonella spp. em hospedeiros aviários. O presente trabalho teve como objetivo investigar a ocorrência e a identidade molecular de aves infectadas de *Bartonella* spp. no Pantanal, Centro-oeste do Brasil, utilizando uma abordagem multigênica. Para tanto, foram coletadas amostras de sangue de 517 indivíduos de 13 ordens aviárias nos estados de Mato Grosso e Mato Groso do Sul. O DNA foi extraído do sangue aviário e 500/517 (96,7%) amostras foram positivas em uma PCR convencional direcionada ao gene da β-actina aviária. Dezenove (3,8%) das 500 amostras de sangue de aves foram positivas em um ensaio de qPCR para Bartonella spp. com base no gene nuoG. Entre 19 amostras de DNA de sangue

de aves positivas na qPCR para *Bartonella* spp., 12 também foram positivas na qPCR para Bartonella com base na região intergênica 16S-23S RNA (ITS). Nos ensaios de PCR realizados para caracterização molecular, foram obtidas uma sequência de rRNA 16S, três de *rib*C e uma de *nuo*G. Com base nos resultados do BLASTn, enquanto uma sequência *nuo*G, dois *rib*C e dois ITS apresentaram alta identidade para *Bartonella henselae*, uma 16S rRNA e dois ITS mostraram alta similaridade com *Bartonella machadoae* nas aves amostradas. Bartonella spp. relacionadas a *B*. *henselae* e *B*. *machadoae* foram detectadas, pela primeira vez, em aves silvestres do Pantanal brasileiro.

Palavras-chaves: Bartonellosis; Birds; *Bartonella henselae*; *Bartonella machadoae*

Abstract: Despite the worldwide occurrence of bartonellae in a broad range of mammal species, in which they usually cause a long- lasting erythrocytic bacteremia, few studies reported *Bartonella* spp. in avian hosts. The present work aimed to investigate the occurrence and molecular identity of *Bartonella* spp. infecting birds in the Pantanal wetland, central-western Brazil using a multigene approach. For this purpose, blood samples were collected from 517 individuals from 13 avian orders in the states of Mato Grosso and Mato Grosso do Sul. DNA was extracted from avian blood and 500/517 (96.7%) samples were positive in a conventional PCR targeting the avian β-actin gene. Nineteen (3.8%) out of 500 avian blood samples were positive in a qPCR assay for Bartonella spp. based on the nuoG gene. Among 19 avian blood DNA samples positive in the qPCR for Bartonella spp., 12 were also positive in the qPCR for *Bartonella* based on the 16S-23S RNA Intergenic region (ITS). In the PCR assays performed for molecular characterization, one 16S rRNA, three ribC, and one nuoG sequences were obtained. Based on BLASTn results, while 1 nuoG, 2 ribC, and 2 ITS sequences showed high identity to Bartonella henselae, one 16S rRNA and 2 ITS showed high similarity to *Bartonella machadoae* in the sampled birds. *Bartonella* spp. related to *B. henselae* and *B*. *machadoae* were detected, for the first time, in wild birds from the Brazilian Pantanal.

Keywords: Bartonellosis; Birds; *Bartonella henselae*; *Bartonella machadoae*

7 Introduction

The genus *Bartonella* (Hyphomicrobiales: Bartonellaceae) encompasses fastidious, facultative intracellular Gram-negative alpha-proteobacteria that primarily infect erythrocytes and endothelial cells (Birtles and Raoult 1996; Breitschwerdt et al. 2010). The transmission of bartonellae is mainly mediated by arthropod vectors, such as fleas (Billeter et al. 2011; Nasereddin et al. 2014; Müller et al. 2018a; Nziza et al. 2019; Salas et al. 2019), lice (Billeter et al. 2009, 2011; Kang et al. 2013; Nasereddin et al. 2014; Klangthong et al. 2015; Anstead 2016; Faccini-Martínez et al. 2017; Louni et al. 2018; Müller et al. 2018a; Salas et al. 2019), sandflies (Battisti et al. 2015), ticks (Król et al. 2021) as well as scratches, bodily fluids and bites (Breitschwerdt et al. 2000, 2010). Currently, there are 39 recognized species (https://www.bacterio.net/genus/bartonella - accessed on December, 29th 2023), with 17 presenting zoonotic potential (Okaro et al. 2017; Kosoy and Goodrich 2019).

Despite the worldwide occurrence of bartonellae in different mammal species, in which they usually cause a long-lasting erythrocytic bacteremia (Chomel et al. 2009), such as rodents (Buffet et al. 2013; Gonçalves et al. 2016), bats (Stuckey et al. 2017; André et al. 2019b), wild carnivores (Kosoy and Goodrich 2019), dogs (Álvarez-Fernández et al. 2018; Müller et al. 2018b; André et al. 2019a), and cats (Álvarez-Fernández et al. 2018; Dias et al. 2023), few studies reported Bartonella spp. in birds. For instance, *Bartonella* spp. (*B*. *henselae*, *B*. *koehlerae*) were detected in wild birds (Northern mockingbird [*Mimus polyglottos*], red-winged blackbird [*Agelaius phoeniceus*], Red-bellied woodpecker [*Melanerpes carolinus*], Common loon [*Gavia immer*], purple martin [*Progne subis*], Tree swallow [*Tachycineta bicolor*] and eastern bluebird [*Sialia sialis*]) in the USA (Mascarelli et al. 2014; Williams and Dittmar 2020). *Bartonella vinsonii berkhoffii* was detected in one Ross's geese (*Anser rossii*) in Canada (Buhler et al. 2022).

A deeper comprehension on the role of avian hosts in the eco-epidemiology of vector-borne agents (Woolhouse and Gowtage-Sequeria 2005) may help to prevent future spillover of emergent zoonotic pathogens (Reed et al. 2003). Birds are known to be carriers of infected ticks and contribute to the spreading of zoonotic pathogens, such as spirochetes (Comstedt et al. 2006; Hamer et al. 2012), tick-borne encephalitis virus (Waldenström et al. 2007), and rickettsiae (Elfving et al. 2010). Conversely, the

role of avian hosts in the epidemiology of Bartonella sp. has been scarcely studied (Buhler et al. 2020, 2022). Recently, it's been hypothesized that the interspecies transmission of Bartonella sp. among Ross's geese (*Anser rossii*), red-backed voles (*Myodes rutylus*) and artic foxes (*Vulpes lagopus*) could be mediated by fleas; if this is the case, geese might play a role as carriers of bartonellae across long distances (Buhler et al. 2022).

The Brazilian Pantanal is recognized as one of the main wetlands for its vast territory (Harris et al. 2005), multiple ecosystems and biodiversity (Mitsch et al. 2015), harboring a wide diversity of avian species (De Pinho et al. 2017; Fecchio et al. 2020). Despite Passeriformes being the most speciose order of birds, representing around 60% of all bird taxa (Sibley and Monroe 1991), only two works reported the occurrence of Bartonella spp. in members of this avian order (Mascarelli et al. 2014; Williams and Dittmar 2020).

Taking into account the huge diversity of Passeriformes and other avian orders in the Brazilian Pantanal and the lack of evidence of Bartonella spp. infecting this group of birds in Brazil, the present work aimed to investigate the occurrence and molecular identity of Bartonella in birds in the Pantanal of the states of Mato Grosso and Mato Grosso do Sul, central-western Brazil.

8 Material and methods

8.1 Sampling and studied area

During the months of April and August-November 2019, birds were captured with 20 mist nets (36 mm mesh, 12 m long and 2.5 m height) installed along the trails in four locali- ties in the Pantanal region, namely: Nossa Senhora do Livra- mento (99 samples) [16°21′46.8"S 56°17′24.0"W], Poconé (100) [16°29′56.4"S 56°24′46.8"W] and Santo Antonio de Leverger (200 samples)[16°44′34.8"S 55°33′10.8"W], state of Mato Grosso and Corumbá (101 samples)[19°34′37.2"S 57°01′08.4"W], state of Mato Grosso do Sul (Fig. 1). Each site was sampled during five consecutive days. Nets were opened immediately after sunrise and monitored every 30 min for bird removal. The nets were closed at sunset.

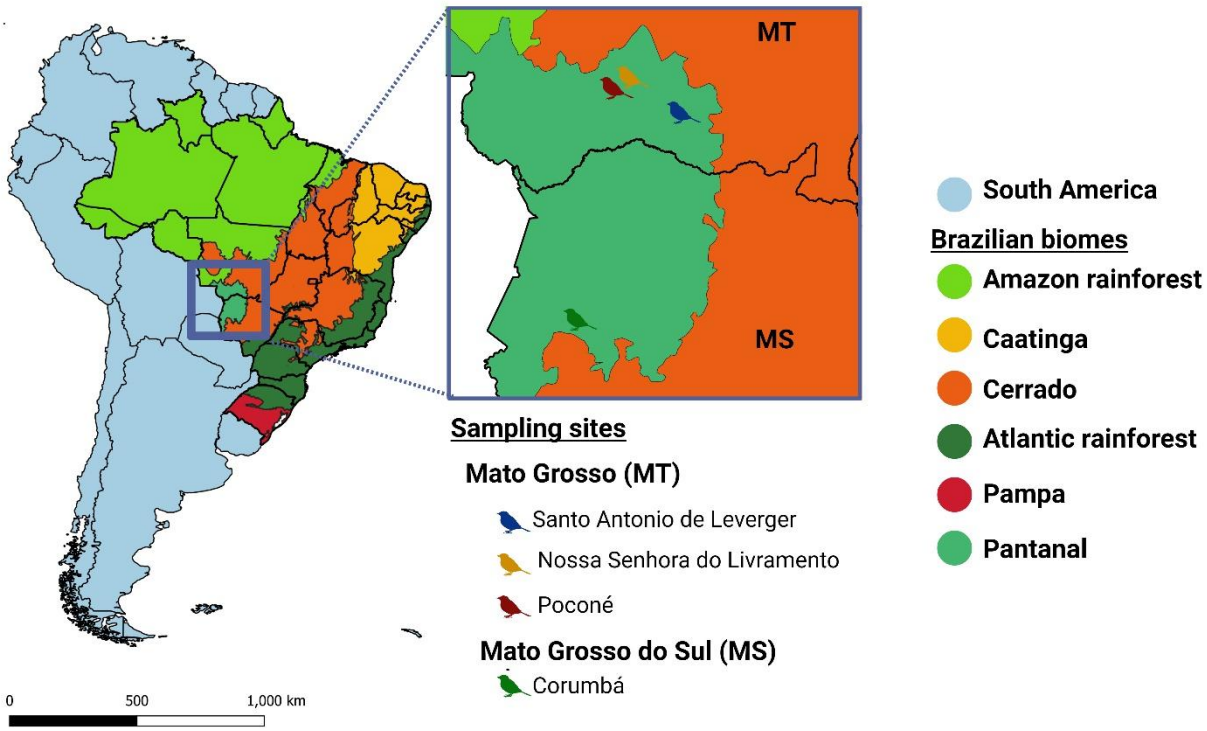

Figure 1. Map showing Brazilian biomes and the sampling sites in the Pantanal wetland in the states of Mato Grosso (MT) and Mato Grosso do Sul (MS).

The birds were identified to species, marked with non-toxic nail-polish and then released after blood collection. In total, 517 blood samples were collected from the following species: *Agelaioides badius* (Grayish baywing) (n=5), *Agelasticus cyanopus* (Unicolored blackbird) (n=3), *Antilophia galeata* (Helmeted manakin) (n=1), *Arremon flavirostris* (Saffron-billed sparrow) (n=7), *Arundinicola leucocephala* (White-headed marsh tyrant) (n=4), *Basileuterus flaveolus* (Flavescent warbler) (n=23), *Basileuterus hypoleucus* (White-bellied warbler) (n=4), *Cacicus cela* (Yellow-rumped cacique) (n=10), *Cacicus solitarius* (Solitary cacique) (n=2), *Campylorhynchus turdinus* (Thrush-like wren) (n=2), *Cantorchilus leucotis* (Buff-breasted wren) (n=6), *Casiornis rufus* (Rufous casiornis) (n=1), *Cercomacra melanaria* (Mato grosso antbird) (n=9), *Certhiaxis cinnamomeus* (Yellow-chinned spine tail) (n=18), *Cnemotriccus fuscatus* (Fuscous flycatcher)(n=5), *Coereba* flaveola (Bananaquit) (n=4), *Conirostrum speciosum* (Chesnut-vented conebill) (n=1), *Coryphospingus cucullatus* (Red-crested finch) (n=1), *Cranioleuca vulpina* (Rusty-backed spinetail) (n=3), *Cyanocorax chrysops*

(Plush-crested jay) (n=1), *Cyanocorax cyanomelas* (Purplish jay) (n=3), *Cyclarhis gujanensis* (Rufous-browed peppershrike) (n=1)*, Dendroplex picus* (Straight-billed woodcreeper) (n=6), *Donacobius atricapilla* (Black-capped donacobius) (n=5), *Elaenia albiceps* (White-crested elaenia) (n=2), *Elaenia spectabilis* (Large elaenia) (n=1), *Eucometis penicillata* (Gray-headed tanager) (n=5), *Euscarthmus meloryphus* (Fulvous-crowned scrub tyrant) (n=3), *Fluvicola albiventer* (Black-backed water tyrant) (n=8), *Furnarius leucopus* (Pale-legged hornero) (n=13), *Furnarius rufus* (Rufous hornero) (n=20), *Hemitriccus margaritaceiventer* (Pearly-vented Tody-tyrant) (n=1), *Hemitriccus striaticollis* (Stripe-necked Tody-tyrant) (n=5), *Herpsilochmus longirostris* (Large-billed antwren) (n=1), *Hylophilus pectoralis* (Ashy-headed greenlet) (n=3), *Hypocnemoides maculicauda* (Band-tailed antbird) (n=7), *Icterus cayanensis* (Epaulet oriole) (n=2), *Icterus croconotus* (Orange-backed troupial) (n=1), *Legatus leucophaius* (Piratic flycatcher) (n=4), *Lepidocolaptes angustirostris* (Narrow-billed woodcreeper) (n=1), *Machetornis rixosa* (Cattle tyrant) (n=5), *Molothrus oryzivorus* (Giant cowbird) (n=1), *Myiarchus ferox* (Short-crested flycatcher) (n=6), *Myiophobus fasciatus* (Bran-colored flycatcher) (n=3), *Myiozetetes cayanensis* (Rusty-margined flycatchter) (n=5), *Paroaria capitata* (Yellow-billed cardinal) (n=36), *Pipra fasciicauda* (Band-tailed manakin) (n=7), *Pitangus sulphuratus* (Great kiskadee) (n=25), *Poecilotriccus latirostris* (Rusty-fronted Tody-flycatcher) (n=8), *Progne tapera* (Brown-chested martin) (n=1), *Pseudoseisura* unirufa (Grey-crested cacholote) (n=8), *Ramphocelus carbo* (Silver-beaked tanager) (n=101), *Saltator coerulescens* (Bluish-gray saltator) (n=18), *Sicalis flaveola* (Saffron finch) (n=2), *Sporophila angolensis* (Chesnut-belliend finch) (n=9), *Sporophila coerulescens* (Double-collared seedeater) (n=1), *Sporophila collaris* (Rusty-collared seedeater) (n=11), *Sporophila lineola* (Lined seedeater) (n=1), *Stelgidopteryx ruficollis* (Southern roughed-wing swallow) (n=3), *Synallaxis albilora* (White-lored spinetail) (n=10), *Taraba major* (Great antshrike) (n=4), *Thraupis palmarum* (Palm tanager) (n=1), *Thraupis sayaca* (Sayaca tanager) (n=3), *Thryothorus genibarbis* (Moustached wren) (n=1), *Todirostrum cinereum* (Common-Tody flycatcher) (n=2), *Turdus amaurochalinus* (Creamy-bellied thrush) (n=3), *Turdus hauxwelli* (Hauwell's thrush) (n=1), *Turdus leucomelas* (Pale-breasted thrush) (n=8), *Turdus rufiventris* (Rufous-bellied thrush) *(*n=7), *Tyrannus melancholicus* (Tropical

kingbird) (n=3), *Vireo olivaceus* (red-eyed vireo) (n=1), *Volatinia jacarina* (Blue-black grassquit) (n=3).

8.2 DNA extraction

DNA extraction from 517 avian blood samples from 13 avian orders (1 Accipritiformes, 4 Apodiformes, 3 Chradriiformes, 14 Columbiformes, 12 Coraciiformes, 12 Cuculiformes, 1 Galliformes, 2 Gruiformes, 452 Passeriformes, 4 Pelecani- formes, 5 Piciformes, 5 Psittaciformes e 1 Tinamiformes) were performed using the Biopur Mini Spin Plus kit (Mobius Life Science, Paraná, Brazil), according to the manufacturer' s recommendations, with a previous pre-treatment of the samples in Whatman® FTA card (de Sena Oliveira et al. 2018). Avian DNA concentration and 260/280 ratio were determined with a spectrophotometer (NanoDrop ND-1000 Thermo Scientific©, Waltham, MA, USA).

8.3 PCR for avian endogenous gene (ß-actin)

To confirm that the DNA extraction was well succeeded, DNA samples extracted from avian blood stored in What- man® FTA cards were subjected to a conventional PCR tar- geting the avian β-actin gene (700 bp) (Hatai et al. 2008). Each reaction used a total volume of 25 µL, containing a 5 µL DNA, 0.2 mM of each deoxynucleotide, 0.4 µM of each primer (β-actin F 5'-ATC TCG TCT TGT TTT ATG CG-3' and β-actin R 5'-TAT CCG TAA GGA TCT GTA TG-3'), 3.0 mM of MgCl2, 1.25 U of Taq Platinum DNA Polymerase (Life Technologies®, Carlsbad, California, United States of America), PCR buffer 10 X and sterilized ultra-pure water (Invitrogen®, Carlsbad, California, United States of Amer- ica). The amplification was carried out in a T100 thermal cycler (BioRad®, Hercules, California, United States), under the following thermal conditions: 95 °C for 5 min for initial amplification and 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min and a final extension 72ºC for 5 min (Hatai et al. 2008).

8.4 Quantitative real-time PCR (qPCR) for *Bartonella* spp.

DNA blood samples that were positive in the PCR targeting the avian β-actin gene were subjected to a qPCR assay to detect and quantify a fragment of 83 bp of the *nuo*G gene of *Bartonella* spp. (André et al. 2016) (number of copies/ µL). The qPCR assay was performed using a mixture containing 1.2 µM of each primer, 2 × Master Mix buffer (GoTaq ™ Probe qPCR Master Mix, Promega Corporation, Madison, USA) and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madison, USA) q.s.p. 9 µL and 1 µL of DNA sample, reaching a final volume of 10 µL. The F-Bart (5' CAA TCT TCT TTT GCT TCA CC 3') and R-Bart (5' TCA GGG CTT TAT GTG AAT AC 3') and the hydrolysis probe (TexasRed-5'-TTY GTC ATT TGA ACA CG-3'[BHQ2a- Q] -3') were used. The amplification was carried out in a CFX96 thermal cycler (BioRad®, Hercules, California, United States) with the following thermal conditions: 95 °C for 30 s for amplification and 40 cycles of 95 °C for 10 s and 52.8 °C for 30 s (André et al. 2016). Samples were evaluated in duplicates. When only one of the replicates turned out positive or the Cq differences between the replicates were higher than 0.5, the sample was re-tested in triplicate. The number of copies was determined by the following formula: (XG / µL DNA / [Plasmid size (bp) × 660]) × 6, 22 × 1023 × plasmid copies / µL. All analyzes were performed according to the standards established by MIQE (“Minimum Information for Publication of Quantitative real-time PCR Experiments”). Efficiency was calculated with the follow- ing formula: $E = 10^{-1/slope}$ (Bustin et al. 2009). As a positive control was used a G-block containing a fragment of 83 bp of the nuoG gene (IDTS- MART; Integrated DNA Technolo- gies, Coralville, IA, USA) and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madison, USA) q.s.p was used as negative control.

8.5 Molecular characterization by quantitative PCR assay for the *Bartonella* ITS (16S-23S rRNA) region

The positive samples to the *nuo*G-based qPCR assay were subjected to a quantitative PCR (qPCR) assay targeting the *Bartonella* spp. ITS (16S-23S rRNA) region for molecular characterization (Oteo et al. 2017; Maggi et al. 2020). The qPCR assay was performed with some modifications using a mixture containing 1.2 µM of each primer, 2 × Master Mix buffer (GoTaq ™ Probe qPCR Master Mix, Promega Corporation, Madison, USA) and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madison, USA) q.s.p. 20 µL and 5 µL of DNA sample, reaching a final volume of 25 µL. Primers used were BsppIRS325s (5′ CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG 3′) Forward, BsppITS543as (5′ CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG 3′) Reverse and BsppITS500 (5′ FAM- GTT AGA GCG CGC GCT TGA TAAG -IABkFQ) Probe. *Bartonella henselae* DNA (Dias et al. 2023) and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madi- son, USA) q.s.p were used as positive and negative controls, respectively. The qPCR assays whose amplicons showed identity to *B. henselae* and *B. machadoae* were re-run after 10 days to double-check the results. In each batch of PCR assays, different positive controls were used, aiming at ruling out the occurrence of cross-contamination. In case of an amplicon turned out to correspond to *B. henselae* after sequencing, we repeated such a PCR using *B. machadoae* and *B. harrusi* as positive controls after 10 and 20 days, then sequencing again the obtained PCR products. The same strategy was used for those PCR whose amplicons showed identity to B. machadoae after sequencing, but using *B. henselae* as a positive control. To avoid cross-contamination, the avian DNA samples and negative controls were pipetted first into the microtubes containing the reaction mix, closed and placed in the thermocycler. Afterwards, the positive controls were pipetted and placed on different lanes in the same thermocycler. The amplicons were sequenced for a second and third times and showed the same results.

8.6 Conventional PCR assays for *Bartonella* molecular characterization

The positive samples in the nuoG-based qPCR assay were subjected to additional PCR assays for molecular characterization targeting 8 genes, namely: *glt*A (Norman et al. 1995; Billeter et al. 2011), 16S rRNA, *rpo*B (Paziewska et al. 2010), *fts*Z, *gro*EL (Iredell et al. 2003), *rib*C (Johnson et al. 2003), *pap*-31 (Maggi and Breitschwerdt 2005) and *nuo*G (Colborn et al. 2010). The PCR assays were performed using a mixture containing 1.25 U de Go Taq hot start polymerase (Promega®, Wisconsin, United States of America), Green Buffer PCR 5X (Promega®, Wisconsin, United States of America), 0.2 mM of each deoxynucleotide (dATP, dTTP, dCTP e dGTP) (Invitrogen®, Carlsbad, California, United States of America), 1.5 mM of MgCl2 (Promega®, Wis- consin, United States of America), 0.5 µM of each primer (Sigma-Aldrich Co, Missouri, United States of America), 3 µL of DNA template, and ultra-pure sterilized water (Inv- itrogen®, Carlsbad, California, United States of America) q.s.p. 25 µL. *Bartonella henselae* DNA (Dias et al. 2023), *Bartonella machadoae* (do Amaral et al. 2022b) and *Bartonella harrusi* (do Amaral et al. 2022a) DNA samples and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madison, USA) q.s.p were used as positive and negative controls, respectively. The strategy adopted to avoid cross-contamination and confirm results for qPCR was implemented when sequencing the amplicons targeting other molecular markers in conventional PCR assays.

8.7 Agarose gel electrophoresis and PCR product purification

The amplified PCR products in the PCR assays were subjected to horizontal electrophoreses in a 1% agarose gel stained with Ethidium bromide (0.5 µL/mL) in TBE buffer (pH 8.0). A 100 base pair marker was used to determine the size of the PCR products (Kasvi, Campinas, São José- dos Pinhais, Paraná, Brazil). Results were visualized and analyzed with an ultra-violet transilluminator Chemidoc (BioRad®, Hercules, California, USA) and image analyzer Image Lab (BioRad®, Hercules, California, USA). Purification of the PCR/ ITS-qPCR products was performed using the

Wizard SV Gel and PCR cleanup System kit (Promega, Wisconsin, USA), following instructions of the fabricant.

8.8 Sequencing

The purified PCR products were sequenced by dideoxy- nucleotide chain termination method (Sanger et al. 1977). The sequencing was performed on the ABI PRISM 3700 DNA Analyzer (Applied Biosystems) at “Centro de Recur- sos Biológicos e Biologia Genômica” (CREBIO -FCAV -UNESP, Jaboticabal, SP, Brazil).

8.9 BLASTn analysis

The amplified sequences were edited and the consensus sequences were constructed using BioEdit v7.2.5 software (Hall 1999). The consensus sequences were subjected to BLASTn analysis to compare the molecular identity with previous deposited sequences in the GenBank database (Benson et al. 2018).

9 Results

9.1 PCR for avian endogenous gene (ß-actin)

Five hundred out of 517 (96.71%) samples were positive in the PCR assays for the endogenous gene. Extracted DNA samples showed a mean concentration of 120.99 ng/ µL ± 0.88 (IC = 120.11 ng/µL—121.81 ng/µL), and SD 260/280 ratio of 2.20 ± 0.88 (IC 1.32–3.08).

9.2 Quantitative real-time PCR (qPCR) for *Bartonella* spp. based on the *nuo*G gene

Out of 500 avian DNA blood samples submitted to qPCR for *Bartonella* spp. (number of copies/µL), 19 (3.8%) samples were positive. Of these, only 2 positive samples were quantified, due to the Montecarlo effect (Bustin et al. 2009): BAP122 (12) from a *Formicivora rufa* from Poconé (Mato Grosso) with an average of Cq 30.45 and average quantification of 1.56 × 10–1 copies/ µL, and BEP353 (108) from a

Chloroceryle americana from Corumbá (Mato Grosso do Sul) with a Cq 38.2 and an average quantification of 1.278 × 10 0 copies/ µL.

9.3 Conventional PCR assays for molecular characterization of *Bartonella* spp.

Among 19 avian blood DNA samples positive in the qPCR for *Bartonella* spp. based on the nuoG gene, 1 avian blood DNA sample (#12 [BAP122 *Formicivora rufa*] Accession number: OR809206) was positive in the PCR based on the 16S rRNA gene, 3 avian blood DNA samples were positive in the PCR based on the ribC gene (#102 [BEP299 *Furnarius rufus*] Accession number: OR834127, #103 [BEP290 *Paroaria capitata*] Accession number: OR834128, and #376 [BAP259 *Progne tapera*] Accession number: OR834129), and 1 sample was positive in the PCR based on the nuoG gene (#376 [BAP259 *Progne tapera*] Accession number: OR834130) (Table 1).

A 16S rRNA sequence obtained from #12 (BAP122 *Formicivora rufa*) from Poconé, Mato Grosso state, showed 100% identity with *Bartonella machadoae* (CP087114) isolated from a *Trichomys fosteri* sampled in Mato Grosso do Sul, Brazil, and 99.7% to *Bartonella vinsonii arupensis* (NR104902) detected in a human in the USA. A nuoG sequence obtained from (#376 [BAP259 *Progne tapera*]) from Nossa Senhora do Livra- mento, Mato Grosso state, showed 100% identity to *Bartonella henselae* strain Houston I (CP020742) detected in a human from Germany. ribC sequences obtained from #102 [BEP299 *Furnarius rufus*] and #103 [BEP290 *Paroaria capitata*] from Corumbá, Mato Grosso do Sul state, showed 99.06% with *Bartonella henselae* strain Houston I (CP020742) detected in a human from Germany; the ribC sequence obtained from 376 [BAP259 *Progne tapera*) from Nossa Senhora do Livramento, Mato Grosso state, showed 100% identity with *Bartonella henselae* strain Houston I (CP020742) detected in a human from Gemany. Interesting, sample 103 [BEP290] from Corumbá pre- sented co-positivity for *B*. *henselae* (ribC) and *B*. *machadoae* (ITS). On the other hand, sample 376 [BAP259] from Nossa Senhora do Livramento showed positivity for *B*. *henselae* based on two molecular markers (nuoG and ribC).

9.4 Molecular characterization by quantitative PCR assay for the *Bartonella* 16S—23S rRNA Intergenic region (ITS)

From the 19 avian blood DNA samples that were positive in the qPCR based on the nuoG gene, 12 (63.15%) were also positive in the qPCR for *Bartonella* based on the 16S-23S RNA. ITS sequences showing good quality were obtained from four samples. The ITS sequence obtained from #103 (BEP290 [*Paroaria capitata*] Accession number: OR840546) from Corumbá, Mato Grosso do Sul state, showed 98.7% identity with *Bartonella machadoae* (CP087114) isolated from a *Trichomys fosteri* sampled in Mato Grosso do Sul, Brazil; #394 (BAP313 [*Thamnophilus doliatus*] Accession number: OR852822) from Nossa senhora do Livramento, Mato Grosso state, showed 99.54% identity with *Bartonella henselae* (CP072904) detected in a human from France; #114 (BEP335 [*Tyrannus* melancholicus*]* Accession number: OR840547) showed 97.6% identity with *Bartonella machadoae* (CP087114) (Table 1).

Table 1. BLASTn results from *Bartonella* nucleotide sequences (16S rRNA, nuoG, ribC and 16S - 23S rRNA) obtained from wild passerine sampled in the Brazilian Pantanal.

Sample ID Locality	Avian host species	Gene	Sequence size (bp)	Query cover (%)	E-value	Identity (%)	Best match	Host	GenBank sequence	Country
12 BAP 122 Poconé	*Formicivora rufa*	16S rRNA	392	100 100	0 0	100 99.7	*Bartonella machadoae* *Bartonella vinsonii arupensis*	*Trychomis fosteri* Human	CP087114 NR104902	Brazil USA
376 BAP259 Nossa Senhora do Livramento	*Progne tapera*	*nuo*G	332	100 100	8.00 E^{-167} 8.00 E^{-167}	100 100	*B. henselae* *B. henselae*	Human *Acrocephalus scirpaceus*	CP020742 OQ123424	Germany Turkey
102 BEP299 Corumbá	*Furnarius rufus*	*rib*C	381	100 100	6.00 E^{-150} 6.00 E^{-150}	100 100	*B. henselae* *B. henselae*	Human *Felis catus*	CP020742 OR487100	Germany Ecuador
103 BEP290* Corumbá	*Paroaria capitata*	*rib*C	393	100 100	6.00 E^{-125} 6.00 E^{-125}	100 100	*B. henselae* *B. henselae*	Human Human	CP020742 OR487100	Germany Ecuador

376 BAP259 Nossa Senhora do Livramento	*Progne tapera*	*rib*C	275 100	100 100	6.00 E^{-125} 6.00 E^{-125}	100 100	*B. henselae* *B. henselae*	Human Human	CP020742 OR487100	Germany Guatemala
103 BEP290 Corumbá	*Paroaria capitata*	ITS	313 313	99 54%	2 E $^{-155}$ 9.00 E^{-79}	98.7 98.8	*B. machadoae* *B. machadoae*	*T. fosteri* *T. fosteri*	CP087114 OL423158	Brazil Brazil
394 BAP313 Nossa Senhora do Livramento	*Thamnophilus doliatus*	ITS	338	64	2.00 E^{-106}	99.54	*B. henselae*	Human	CP072904	France
114 BEP335 Corumbá	*Tyrannus melancholicus*	ITS	333	100	1.00 E^{-162}	97.6	*B. machadoae*	*T. fosteri*	CP087114	Brazil

"*" stands for sample 103 that shows a coinfection with *B. henselae (ribC* gene) and *B. machadoae* (ITS region)

10 Discussion

The present study showed the first detection of *Bartonella* spp. in wild birds of the families Thamnophilidae, Tyrannidae, Thraupide, Furnariidae, Hirundinidae, and Emberezidae. In addition, it reports, for the first time, the presence of *Bartonella* spp. in avian hosts from Brazil. Previously, *Bartonella* spp. was molecularly detected in spleen samples from birds of the family Anatidae (Buhler et al. 2022) in Canada, in blood samples from Hirundinidae and Turdidae (Williams and Dittmar 2020), Mimidae, Icteridae, Picidae and Gaviidae (Mascarelli et al. 2014) families in the USA, as well as in spleen, liver and brain samples from birds belonging to Turdidae, Muscicapidae, Fringillidae, Sylviidae, Scolopacidae and Phylloscopacidae families in Russia (Korobitsyn et al. 2021).

Herein, *B. machadoae* was detected in the blood sample (#12 [BAP145]) from *Formicivora rufa* from Pocone, Mato Grosso state. *Bartonella machadoae* was recently isolated and described in rodents (*Trichomys fosteri*) from the Brazilian Pantanal, state of Mato Grosso do Sul (do Amaral et al. 2022b). Interestingly, *B. machadoae* was also detected in *Amblyomma sculptum* nymphs collected from wild boars (*Sus scrofa*) in the state of Sao Paulo, southeastern Brazil (Santana et al. 2022), as well as in coati (*Nasua nasua*) blood samples from Iguacu National Park, southern Brazil. *Bartonella* spp. closely related to *B. machadoae* was also detected in *Oligoryzomys longicaudatus* rodents from Chile (Sepulveda-Garcia et al. 2023). These findings suggest that the geographical distribution and host specificity of *B. machadoae* might be broader than previously expected.

Regarding the *Bartonella* species previously detected in birds, ITS sequences from *Bartonella berkhoffii* (novel denomination proposed by do Amaral et al. 2022a; Goncalves- Oliveira et al. 2023−formerly *B. vinsonii* subsp. *berkhoffii*) was detected in 1/42 Ross geese (*Anser rossii*) in Canada (Buhler et al. 2022). In the USA, *Bartonella* 16S rRNA sequences were detected in 24/55 swallows (*Tachycineta bicolor* and *Progne subis*) and in 2/6 eastern bluebird (*Sialia sialis*) (Williams and Dittmar 2020). The obtained sequences showed of 99‑100% with *Bartonella vinsonii* 96.96% with *Bartonella elizabethae*. *Bartonella henselae* ITS sequences were detected in 2/2 Northern mockingbird (*Mimus polyglottos*) and 1/1 Red-winged blackbird (*Agelaius*

phoeniceus) in the USA. On the other hand, *B. koehlerae* ITS sequences were detected in 1/1 woodpeckers (*Melanerpes carolinus*) and in 1/1 great loon (*Gavia immer*) (Mascarelli et al. 2014). In the present study, *B. henselae* sequences were detected in Brown-chested martin (*Progne tapera*) for the *nuo*G gene, Rufus hornero (*Furnarius rufus*), Yellow-billed cardinal (*Paroaria capitata*) and Brown-chested martin (*Progne tapera*) for the *rib*C gene and Barred-antshrike (*Thamnophilus doliatus*) and Silverbeaked tanager (*Ramphocelus carbo*) for ITS region. In Russia, *Bartonella* spp. (*pap-31*) was detected in 5/22 Fieldfare (*Turdus pilaris*), 1/13 Blyth´s reed warbler (*Acrocephalus dumetorum*), 1/4 Common redstart (*Pheonicurus phoenicurus*), 2/12 Commom chaffinch (Fringilla coelebs), 2/4 European pied flycatcher (*Ficedula hypoleuca*), 1/4 Lesser whitethroat (*Sylvia curruca*), 2/4 Redwing (*Turdus illiacus*), 1/4 Common woodcock (*Scolopax rusticola*), and 1/1 Pallas warbler (*Phylloscopus proregulus*) (Korobitsyn et al. 2021).

In the present study the Yellow-beaked cardinal (*Paroaria capitata*) 103 [BEP290] from Corumba showed to be coinfected by *B. henselae* and *B. machadoae.* On the other hand, based on two molecular markers, *B. henselae* closely related to a genotype previously detected in cats [64] was detected in Brown-chested martin (*Progne tapera*) from Nossa Senhora do Livramento.

Although sequences obtained in the present study showed identity with *B. machadoae* (when 16S rRNA and ITS were used as molecular markers), *B. henselae* (when *nuo*G, *rib*C and ITS were used as molecular markers), these results should be interpreted with caution. Since the 16S rRNA and *nuo*G genes are highly conserved, more studies are needed in order to investigate the real molecular identity of these *Bartonella* genotypes circulating in birds in Brazil. Although La Scola (La Scola et al. 2003) criterion based on percentages of identity by BLASTn for different molecular markers has been used for such a long time to discriminate *Bartonella* species, do Amaral et al. (2022a) demonstrated that it is not robust enough for distinguishing phylogenetically closely related species, such as *Bartonella harrusi* and *B. machadoae* [53, 60].

The putative vectors involved in the transmission of bartonellae among birds or cross species transmission between mammals and birds are unknown. The cat flea (*Ctenocephalides felis felis*), the main vector of *B. henselae* among cats (Chomel et al. 1996), has been rarely found infesting birds. There are reports of this flea species in nests of white storks (*Ciconia ciconia*) (Mammeria et al. 2014) in Algeria and in Strigiformes from Brazil (Linardi and Costa 2016). On the other hand, *Bartonella* sp. has already been detected in *Cerathophylus* spp., the most common fleas found parasitizing birds, in the USA (Williams and Dittmar 2020). *Bartonella berkhoffii* and *B. henselae* were detected in *Cerathophylus* spp. in Canada (Buhler et al. 2020; 2022).

Although *Rhipicephalus sanguineus* sensu lato and *Ixodes ricinus* ticks showed to be competent vectors of *B. henselae* in experimental settings (Cotte et al. 2008; Wechtaisong et al. 2021), the vectorial capacity of ticks in the transmission of bartonellae in nature is still unknown. *Bartonella machadoae* was molecularly detected in *A. sculptum* nymphs collected from *Bartonella*-negative wild boars in Brazil (Santana et al. 2022). *Bartonella* spp. closely related to *Bartonella vinsonii* complex was detected in *Polygenis (Polygenis) bohlsi bohlsi* fleas collected from rodents in the Brazilian Pantanal (de Sousa et al. 2018). In the Brazilian Pantanal, Passeriformes are frequently parasitized with ticks of the *Amblyomma* genus (*Amblyomma nudosum*, *Amblyomma longirostre*, *Amblyomma triste,* and *Amblyomma* spp.). On the other hand, Tinamiformes and Cuculiformes were also parasitized by ticks in a less extent (Fecchio et al. 2020). Bird life history, behavior and seasons of the year may influence the probability of tick infestation in birds (Fecchio et al. 2020).

Recently, *Bartonella* spp. (genotype A ‑ putative endosymbiont; genotype B− acquired from blood meal) were detected in the microbiome of *Dermanyssus gallinae* mites collected from *Gallus gallus domesticus* in poultry farms from Czechia (Hubert et al. 2017) and Japan (Nishide et al. 2022). The role of mites in the transmission of bartonellae among birds should be further investigated.

11 Conclusion

Bartonella spp. related to *B. henselae* and *B. machadoae* were detected in wild birds from the Brazilian Pantanal. Future studies aiming at isolating and fully characterizing *Bartonella* from birds are needed to shed some light on the role of wild birds in the dispersion of bartonellae.

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Author contributions

All authors contributed significantly to the conception of the research. Methodology, Validation, Investigation, Data curation, Writing- Original Draft, Visualization, Writing review and editing was performed by Amir Salvador Alabi Cordova; Resources, Methodology, Writing review and editing performed by Alan Fecchio; Investigation, Writing review and editing performed by Ana Claudia Calchi; Investigation, Writing review and editing performed by Clara Morato Dias; Writing review and editing performed by Rosangela Zacarias Machado; Project administration, Funding acquisition, Supervision, Conceptualization, Writing- Original Draft, Writing review and editing, Resources performed by Marcos Rogerio Andre.

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Data availability

The sequences generated during the study were submitted in the NCBI Genbank (https:// www. Ncbi. Nlm. Nih. Gov/ genba nk/). Sequences can be accessed by the following accession numbers: OR809206, OR834130, OR834127, OR834128, OR834129, OR840546, OR852822 and OR840547.

Declarations

Competing interests All authors contributed significantly to the conception of the research. Methodology, Validation, Investigation, Data curation, Writing- Original Draft, Visualization, Writing review and editing was performed by **Amir Salvador Alabí Córdova**; Resources, Methodology, Writing review and editing performed by **Alan Fecchio**; Investigation, Writing review and editing performed by **Ana Cláudia Calchi**; Investigation, Writing review and editing performed by **Clara Morato Dias**; Writing review and editing performed by **Rosangela Zacarias Machado**; Project administration, Funding acquisition, Supervision, Conceptualization, Writing- Original Draft, Writing review and editing, Resources performed by **Marcos Rogério André**.

Ethics approval Sampling procedures involving wild birds were approved by IBAMA (72548 e 72790), the “Comissao de Etica no Uso de Animais” of the Faculdade de Ciencias Agrarias e Veterinarias (FCAV/UNESP) (CEUA 268/21) and SISGEN (AF30FD1).

Consent to participate

All authors agreed to participate in this research.

Consent for publication

All authors agreed to publish this research.

Conflicts of interest

The authors declare there is no conflict of interest that could have influenced the results obtained and reported in this research.

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Chapter 3. Novel tick-borne Anaplasmataceae genotypes in tropical birds from the Brazilian Pantanal wetland.

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Resumo:

Apesar dos inúmeros relatos de agentes de Anaplasmataceae em mamíferos em todo o mundo, poucos estudos investigaram sua ocorrência em aves. O presente estudo teve como objetivo investigar a ocorrência e a identidade molecular de agentes Anaplasmataceae em aves do Pantanal, Brasil. Amostras de sangue foram coletadas de 93 espécies diferentes. Após a extração do DNA, as amostras positivas para o gene da β-actina aviária foram submetidas a uma PCR quantitativa multiplex em tempo real (q) para *Anaplasma* e *Ehrlichia* direcionada ao gene groEL e a uma PCR convencional para agentes Anaplasmataceae direcionada ao gene 16S rRNA. Como resultado, 37 (7,4%) aves foram positivas para *Anaplasma* spp. e 4 (0,8%) para *Ehrlichia* spp. no ensaio qPCR; além disso, 13 (2,6%) foram positivos para agentes Anaplasmataceae na PCR direcionada ao gene 16S rRNA. As sequências de rRNA de *Ehrlichia* 16S detectadas em *Arundinicola leucocephala*, *Ramphocelus carbo* e *Elaenia albiceps* foram posicionadas próximas a *Ehrlichia* sp. Sequências de *Ehrlichia dsb* detectadas em *Agelasticus cyanopus* e *Basileuterus flaveolus* agrupadas com *Ehrlichia minasensis*. Genótipos de rRNA 16S detectados em *Crax fasciolata*,

Pitangus sulphuratus e *Furnarius leucopus* agrupados com *'Candidatus* Allocryptoplasma'. Os genótipos 23S-5S detectados em *C*. *fasciolata*, *Basileuterus flaveolus* e *Saltator coerulescens* foram relacionados a *Anaplasma phagocytophilum*. Em conclusão, novos genótipos de *Anaplasma*, *Ehrlichia* e *'Candidatus* Allocryptoplasma' foram detectados em aves do Pantanal.

Palavras-chave: *Ehrlichia* spp.; *Anaplasma* spp.; '*Candidatus* Allocrytoplasma spp.'; avian hosts; South America; tick-borne diseases

Abstract:

Despite numerous reports of Anaplasmataceae agents in mammals worldwide, few studies have investigated their occurrence in birds. The present study aimed to investigate the occurrence and molecular identity of Anaplasmataceae agents in birds from the Pantanal wetland, Brazil. Blood samples were collected from 93 different species. After DNA extraction, positive samples to the avian β-actin gene were subjected to both a multiplex quantitative real time (q)PCR for *Anaplasma* and *Ehrlichia* targeting the *groEL* gene and to a conventional PCR for Anaplasmataceae agents targeting the 16S rRNA gene. As a result, 37 (7.4%) birds were positive for *Anaplasma* spp. and 4 (0.8%) for *Ehrlichia* spp. in the qPCR assay; additionally, 13 (2.6%) were positive for Anaplasmataceae agents in the PCR targeting the 16S rRNA gene. The *Ehrlichia* 16S rRNA sequences detected in *Arundinicola leucocephala*, *Ramphocelus carbo* and *Elaenia albiceps* were positioned closely to *Ehrlichia* sp. Magellanica. *Ehrlichia dsb* sequences detected in *Agelasticus cyanopus* and *Basileuterus flaveolus* grouped with *Ehrlichia minasensis*. 16S rRNA genotypes detected in *Crax fasciolata, Pitangus sulphuratus* and *Furnarius leucopus* grouped with *'Candidatus* Allocryptoplasma'. The 23S-5S genotypes detected in *C. fasciolata*, *Basileuterus flaveolus*, and *Saltator coerulescens* were related to *Anaplasma phagocytophilum*. In conclusion, novel genotypes of *Anaplasma*, *Ehrlichia* and '*Candidatus* Allocryptoplasma' were detected in birds from the Pantanal wetland.

Keywords: *Ehrlichia* spp.; *Anaplasma* spp.; '*Candidatus* Allocrytoplasma spp.'; avian hosts; South America; tick-borne diseases

1. Introduction

Anaplasmataceae agents, which comprise intracellular obligate α-proteobacteria that infect mainly blood and endothelial cells of vertebrates, stands out amongst the vector borne-agents due to their global distribution [1,2]. The Anaplasmataceae family is composed by four genera (*Anaplasma*, *Ehrlichia*, *Wolbachia*, *Neorickettsia*) and four putative '*Candidatus'* ('*Candidatus* Neoerhlichia', '*Candidatus* Allocryptoplasma', '*Candidatus* Xenohaliotis' and '*Candidatus* Xenolissoclinum) [2–6]. There are several reports describing different *Ehrlichia* species capable of infecting humans, namely *Ehrlichia chaffeensis* [7,8], *Ehrlichia ewingii*, *Ehrlichia muris muris*, *Ehrlichia muris eauclairensis*, *Ehrlichia canis* and *Ehrlichia ruminantium* [2,7]. Regarding *Anaplasma* species, *Anaplasma phagocytophilum* [9], *Anaplasma platys*, *Anaplasma ovis* and *Anaplasma capra* [7] are capable of causing illness in humans. *Neorickettsia sennetsu* [10] and '*Candidatus* Neoehrlichia *mikurensis'* [11] are also known to infect humans. This broad capability to infect humans turned them of great importance to public health [12,13].

Despite several reports of these agents in mammals around the world [14], few studies have investigated the occurrence of Anaplasmataceae agents in birds. For instance, *Anaplasma phagocytophilum* has been detected in Ixodes ticks collected from birds in Canada [15], Greece [16], Lithuania, Norway [17], France [18], and Latvia [19], emphasizing that avian hosts can disperse ticks infected with zoonotic Anaplasma species. When it comes to detection of these agents in birds, *A*. *phagocytophilum* was detected in blood samples from thrushes (*Zoothera aurea*, *Turdus cardis*, and *Turdus palidus*) in South Korea [20], in liver samples from collared doves (*Streptopelia decaocto*) and Eurasian eagle owls (*Bubo bubo*) in China [21], as well as in tissue samples (heart, liver, spleen and kidney) in a robin (*Erithacus rubecula*) and a song thrush (*Turdus philomelos*) in Romania [22]. '*Candidatus* Anaplasma sphenisci' was detected in blood samples from penguins (*Spheniscus demersus*) in South Africa [23]. In Chile, a putative novel Ehrlichia sp. was detected in spleen samples from penguins (*Spheniscus magellanicus*) [24]. An *Ehrlichia* genotype closely related to *Ehrlichia chaffeensis* was detected in blood and spleen samples from a song thrush (*Turdus philomelos*) in Hungary [25] and in liver samples from a common pheasant (*Phasianus colchicus*) from China [21].

In Brazil, previous studies reported the occurrence of Anaplasma 16S rRNA genotypes closely related to *Anaplasma phagocytophilum* in blood samples from black vultures (*Coragyps atratus*), caracaras (*Caracara plancus*) [26], Dusky-legged guan (*Penelope obscura*) [27], Orinoco geese (*Neochen jubata*) [28], burrowing owl (Athene cunicularia), tropical screech owl (*Megascops choliba*), roadside hawk (*Rupornis magnirostris*), barn owl (*Tyto alba*), and striped owl (*Asio clamator*) and aplomado falcon (*Falco femoralis*) [29]. *Ehrlichia* 16S rRNA genotypes closely related to *Ehrlichia chaffeensis* were detected in blood samples from an american kestrel (*Falco sparverius*) [26], Orinoco goose [28], burrowing owl, tropical screech owl, roadside hawk (*Rupornis magnirostris*), and barn owl (*Tyto alba*) [29]. Ehrlichia 16S rRNA genotypes closely related to *E*. *canis* were detected in blood samples from black vulture, Orinoco goose [26,28] roadside hawk and striped owl [29].

Comprehending the role of avian hosts in the eco-epidemiology of vector-borne agents [30] is needed to hamper potential spill-over of emergent zoonotic pathogens [31]. Birds play an important role as carriers of ticks, contributing to the dispersal of zoonotic agents, such as Rickettsiales agents [32] and are responsible for harboring several vector-borne agents, such as Anaplasmataceae agents [20,22,25,26].

Taking into account the huge diversity of resident and migratory birds in the Pantanal wetland [33,34], the multiple ecosystems that provide preservation of the biodiversity [35], the feasibility of the transmission of vector-borne pathogens among a diverse avian population and its influence on the genetic richness of vector-borne pathogens, the current study aimed to investigate the occurrence and genetic diversity of tick-borne Anaplasmataceae agents in birds in the Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul, central-western Brazil.

2. Materials and Methods

2.1 Study area and bird sampling

The sampling period comprised the months of April and August to November 2019. The birds were caught with 20 mist nets with dimensions of 36 mm mesh, 12 m long and 2.5 m height, allocated next to the tracks in four sampling sites in the region of Pantanal, namely Nossa Senhora do Livramento (99 samples) [16°21'46.8"S

56°17'24.0"W], Poconé (100 samples) [16°29'56.4"S56°24'46.8"W] and Santo Antonio de Leverger (200 samples)[16°44'34.8"S 55°33'10.8"W], state of Mato Grosso, and Corumbá (101 samples)[19°34'37.2"S 57°01'08.4"W], state of Mato Grosso do Sul (Figure 1). In each locality, the sampling was performed over a five-day period. The mist nets were opened after dawn and checked periodically every 30 minutes.

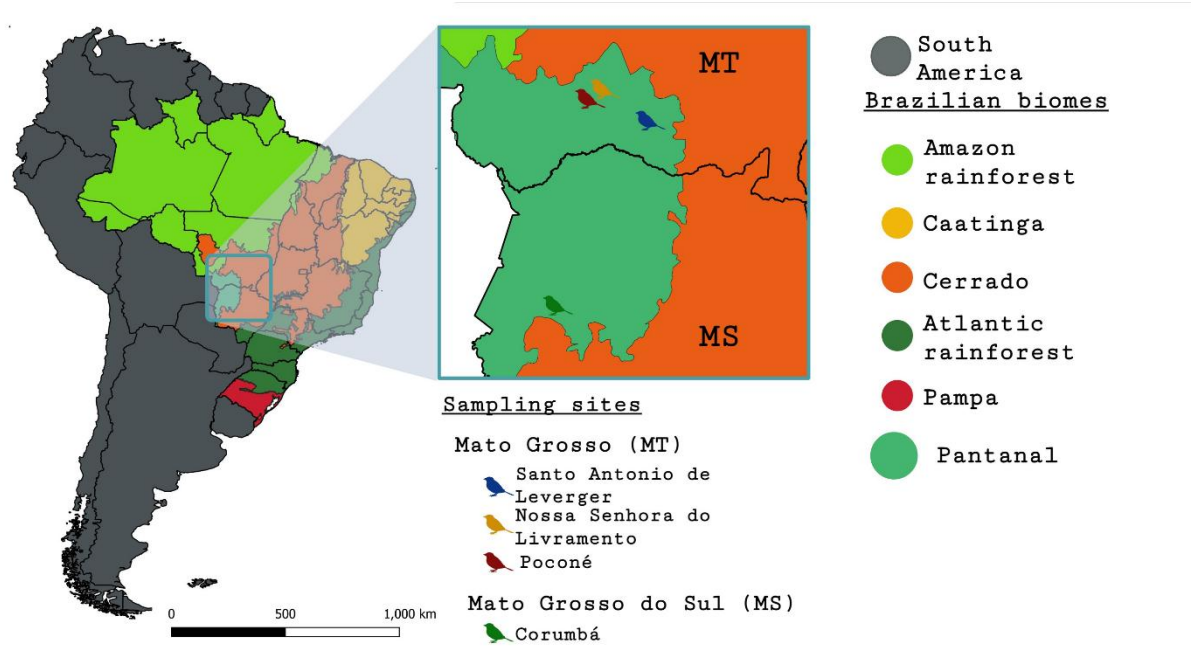

Figure 1. Brazilian biomes and sampling sites in the Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul.

Bird species were identified as previously mentioned (Alabí Córdova et al., 2024). A total of 517 blood samples were collected from the following orders: 1 Accipritiformes, 4 Apodiformes, 3 Chradriiformes, 14 Columbiformes, 12 Coraciiformes, 12 Cuculiformes, 1 Galliformes, 2 Gruiformes, 452 Passeriformes, 4 Pelecaniformes, 5 Piciformes, 5 Psittaciformes and 1 Tinamiformes. the samples are from a previous study (Alabí Córdova et al., 2024) **(Supplementary Table S1)**.

The identification of birds was performed by an skilled ornithologist using several field guides. Bird taxonomy follows Birdtree project [see https://birdtree.org/ taxonomy] [37]. Representative bird specimens were deposited in the Bird Collection

of UFMT, Cuiabá, Brazil. All birds and blood samples were collected under appropriate permits in Brazil"

Sampling procedures involving wild birds were approved by IBAMA (72548 e 72790), the "Comissão de Ética no Uso de Animais" of the Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP) (CEUA 268/21) and SISGEN (AF30FD1).

2.2. Molecular assays

2.2.1 DNA extraction and PCR for avian endogenous gene (*β-actin*)

DNA extraction and PCR for avian *β-actin* were previously processed (Alabí Córdova et al., 2024) **(Supplementary Table S2)**.

2.2.2 Molecular screening for *Anaplasma* spp. and *Ehrlichia* spp. targeting the groEL gene by a multiplex quantitative real-time (q)PCR

DNA avian blood samples that were positive to the endogenous avian β-actin gene were then screened for *Anaplasma* spp. and *Ehrlichia* spp. using a multiplex qPCR targeting the *gro*EL gene as previously described [39,40]. The reactions consisted of 1 µL of sample DNA, 0.2 µM of each oligonucleotide primer and hydrolysis probe, Master Mix 2x (GoTaq ™ Probe qPCR Master Mix, Promega Corporation, Madison, USA) and sterilized ultra-pure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) to complete a final volume of 10 µL. Amplification reactions were carried out in a CFX96 Thermal Cycler (BioRad®, Hercules, California, USA) [39] (Supplementary Table S2). Samples were tested in duplicates.

Quantification of the number of target DNA copies/µL was performed using G-Blocks (G-Blocks, Integrated DNA Technologies®) containing the target sequences. Serial dilutions were made to construct standards with different concentrations of G-Blocks DNA containing the target sequence (2.0 x 107 copies/µL to 2.0 x 100 copies/µL), in order to obtain the efficiency and correlation coefficient of reactions. The number of target gene copies was determined according to the formula (Xg/ µL DNA/ [plasmid size (bp) x 660]) x 6.022 x1023 x plasmid copies/µL. All analyzes were carried out in accordance with standards established by MIQE ("Minimum Information for Publication of Quantitative real-time PCR Experiments") [41]. Quantification cycles (Cq) with a difference of no more 0.5 were considered positive, otherwise they were

repeated in triplicates. Cq is considered as the fraction of a PCR cycle where the target in the samples is quantified [42].

2.2.3 Molecular screening for Anaplasmataceae agents targeting the 16S rRNA gene by conventional PCR

Avian DNA samples that were positive to the endogenous avian β-actin gene were also screened for Anaplasmataceae agents using previously described PCR targeting the 16S rRNA gene [43]. Oligonucleotides sequences and thermal conditions are shown in Supplementary Table S2.

2.2.4 PCR assays for molecular characterization

Avian DNA samples that were positive for the 16S rRNA gene for Anaplasmataceae agents in the screening PCR [43] and/or to the *gro*EL gene in the multiplex qPCR for *Anaplasma* spp. and *Ehrlichia* spp. [39, 40] were subjected to additional previously described conventional PCR assays for *Ehrlichia* spp. based on the *dsb* [44], *glt*A [45,46], *sod*B [47], *omp*-1 [48], *rpo*B, *gro*EL and *fts*Z [45,49] genes.

For Anaplasma molecular characterization, positive samples in the screening PCR assays were subjected to additional previously described conventional PCR assays based on the 16S rRNA gene [20,43,50–54] and the intergenic fragment 23S-5S (ITS) [55], as well as to a nested PCR based on the groEL gene [56]. Samples in which sequences related to '*Candidatus* Allocryptoplasma spp.' were detected were subjected to additional previously described conventional PCR assays targeting the *gro*EL and *suc*A genes [4]. The primer oligonucleotides and thermal sequences used in PCR assays are presented in Supplementary Table S2.

Each reaction had a total volume of 25 µL, 1.25 U Go Taq Hot Start Polymerase (Promega®, Wisconsin, USA), PCR buffer 10 X (Promega®, Wisconsin, USA) and sterilized ultra-pure water (Invitrogen®, Carlsbad, California, USA), 0.2 mM of each deoxynucleotide, 0.4 µM of each oligonucleotide, 3.0 mM of MgCl2 and 5 µL DNA template.

Ehrlichia canis (Jaboticabal strain) DNA (obtained from infected cells DH82 infected [57]) and *Anaplasma* sp. DNA (obtained from cattle from Mozambique [58]) were used as positive controls in all conventional and nested PCR assays for *Ehrlichia*

spp. and *Anaplasma* spp., respectively. Sterilized ultra-pure water (Invitrogen®, Carlsbad, California, USA) was used as a negative control in all PCR assays.

2.3. Gel electrophoresis and amplicon purification

PCR products were separated in a 1% agarose gel stained with Ethidium bromide (0.5 µL/mL) in TBE buffer (pH 8). A molecular weight size marker of 100 pb was employed to verify the size of the PCR products (Kasvi, Campina, São José dos Pinhais, Paraná, Brazil). Agarose gels were visualized under an ultra-violet transilluminator Chemidoc (BioRad®, Hercules, California, USA) and image analyzer software, Image Lab (BioRad®, Hercules, California, USA). Amplicons were purified with the Wizard SV Gel and PCR cleanup system kit (Promega, Wisconsin, USA), following manufacturer's recommendations.

2.4 Sequencing and nBLAST analysis

After purification, PCR products were submitted to sequencing by dideoxynucleotide chain termination method [59]. Sequencing was performed in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) at "Centro de Recursos Biológicos e Biologia Genômica" (CREBIO -FCAV -UNESP, Jaboticabal, SP, Brazil).

The retrieved sequences were trimmed and the consensus sequences were assembled utilizing BioEdit v7.2.5 software [60]. The consensus sequences were compared in nBLAST [61] with homologous sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) [62].

Sequences can be accessed by the following accession numbers: PP326052, PP326053, PP326054, PP346669, PP346773, PP373675, PP373692, PP373702, PP417932, PP417933, PP417934, PP417935 and PP417936.

2.5 Phylogenetic analyses

The obtained sequences were aligned with homologous sequences obtained from the Genbank database, using the Clustal/W software (Thompson et al., 1994) via Bioedit v. 7.0.5.3 [60]. Alignments saved in "FASTA" mode were used to infer the evolutionary model and the Maximum likelihood analyzes performed using the online version of IQtree version (http://iqtree.cibiv.univie.ac.at/) [63]. Clade supports for Maximum Likelihood (ML) analyzes were assessed using bootstrap analyzes [64] of

100 repetitions. Editing of phylogenetic trees as well as the outgroup was performed using Figtree V1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/) [65]

3. Results

3.1 Molecular screening for endogenous gene avian ß-actin

The values of mean concentration and 260/280 ratio of the DNA extracted samples can be found in a previously published work (Alabí Córdova et al., 2024). Individual DNA samples ratio and concentration were estimated, those samples that exceeded 100 ng/µL were diluted to a final concentration 50 ng/µL with sterilized ultra-pure water (Invitrogen®, Carlsbad, California, USA). The 17 negative samples to avian β-actin gene were #13 (SL068) *Coccycua minuta*, #412 (SL48) *Picumnus albosquamatus*, #500 (SL55) *P. albosquamatus* and #505 (SL56) *Hemitriccus striaticollis* from Santo Antonio de Leverger, #99 (BAP048) *Ramphocelus carbo*, #138 (BEP259) *Donacobius atricapilla*, #146 (BEP268)*D. atricapilla*, #257 (BAP52) *Cacicus cela*, #269 (BAP85) *C. cela*, #295 (BAP33) *Cnemotriccus fuscatus*, #296 (BAP70) *Cercomacra melanaria* from Poconé; #308 (BAP144) *Aramides cajanea*, #364 (BAP187) *P*. *albosquamatus*, #385 (BAP266) *P. albosquamatus*, #390 (BAP308) *Thraupis palmarum* from Nossa Senhora do Livramento, and #96 (BEP424) *Cyanocorax cyanomelas*, #99 (BAP048) *Ramphocelus carbo*, #138 (BEP259) *D. atricapilla*, #146 (BEP268) *D. atricapilla* from Corumbá.

3.2 Molecular screening for *Anaplasma* spp. and *Ehrlichia* spp. by a quantitative real-time PCR based on the *gro*EL gene

Out of 500 avian DNA samples that were submitted to a multiplex qPCR for *Anaplasma* spp. and *Ehrlichia* spp. based on the *groEL* gene, 37 (7.4%) were positive for *Anaplasma* spp. and 4 (0.8%) were positive for *Ehrlichia* spp. Co-positivity for *Anaplasma* spp. and *Ehrlichia* spp. was found in two samples (0.4%) (**Supplementary Table S3**). Positivity for *Anaplasma* spp. by avian species was 2/14 (14.28%) *Agelasticus cyanopus*, 1/20 (5%) *Basileuterus flaveolus*, 1/1 (100%) *Busarellus nigricollis*, 1/9 (11.11%) *Cacicus cela*, 1/5 (20%) *Cantorchilus leucotis*, 1/12 (8.33%)

Certhiaxis cinnamomeus, 1/5 (20%) *Chloroceryle americana*, 1/5 (20%) *Cranioleuca vulpina*, 1/4 (25%) *Dendroplex picus*, 1/6 (16.66%) *Eucometis penicillata*, 1/2 (50%) *Guira guira*, 1/3 (33.33%) *Icterus cayanensis*, 1/4 (25%) *Legatus leucophaius*, 3/10 (30%) *Leptotila verreauxi*, 1/26 (3.84%) *Paroaria capitata*, 1/1 (100%) *Phaetornis nattereri*, 1/7 (14.28%) *Pipra fasciicauda*, 2/21 (9.52%) *P. sulphuratus*, 9/73 (12.32%) *R*. *carbo*, 1/16 (6.25%) *S*. *coerulescens*, 1/8 (12.5%) *Sporophila angolensis*, 1/5 (20%) *Sporophila collaris*, 1/3 (33.33%) *Thraupis sayaca*, 1/3 (33.33%) *Tigrisoma lineatum* and 1/5 (20%) *Tyrannus melancholicus.* Positivity for *Ehrlichia* spp. by avian species was 1/12 (8.33%) *C. cinnamomeus,* 1/4 (25%) *D. picus* (coinfection with *Anaplasma* sp.)*,* 1/16 (6.25%) *Furnarius rufus,* 1/*73* (1.36%) and *R. carbo* (coinfection with *Anaplasma* sp.). According to locality, the higher positivity was found among birds in Poconé, with 30/100 (30%) birds positive, followed by the locality of Nossa Senhora do Livramento with 12/99 (12.1%) infected birds, Santo Antonio de Leverger presented 15/200 (7.5%) infected birds, and the locality which presented the lowest infection frequency was Corumbá with 3/101 (3%) birds.

While the Cq values of *Anaplasma*-positive samples ranged from 28.94 to 39.24, *Ehrlichia*-positive samples showed Cq values ranging from 33.24 to 37.89. Even after re-testing the positive samples in triplicates, the estimated quantification was possible to be achieved in only one positive sample for *Anaplasma* spp. probably due to Monte Carlo effect [41]: BAP87 (232) from a specimen of *Legatus leucophaius* from Poconé, with an average Cq of 37.96 and an average quantification of 5.845 x 10^{-1} copies/ µL. All samples positive in the qPCR were negative in additional conventional PCR assays based on the 16S rRNA, *dsb*, *glt*A, *sodB*, *omp*-1, *rpoB*, *groEL* and *fts*Z genes and 23S-5S (ITS) intergenic region mostly likely due the low bacteremia at the time of blood sampling.

3.3 Molecular screening for Anaplasmataceae agents by a cPCR based on the 16S rRNA gene and molecular characterization

Thirteen avian DNA samples were positive in the screening for Anaplasmataceae agents targeting the gene 16S rRNA (345 bp) [43]: *Ehrlichia* sp. detected in one *R*. *carbo* from Poconé (MT); Ehrlichia sp. was detected in one E*. albiceps* from Poconé (MT); *Ehrlichia* sp. was detected in one *R*. *carbo* from Santo Antonio de Leverger (MT). *Anaplasma* sp. was detected in one *P*. *sulphuratus* from Santo Antonio de Leverger (MT). '*Candidatus* Allocryptoplasma spp.' was detected in one *C. fasciolata* from Nossa Senhora do Livramento (MT). The BLAST results regarding the five 16S rRNA sequences (ranging from 179- 281 bp) obtained in the Parola et al. (2000)'s protocol [43] is showed in Supplementary Table S4.

When combining the results obtained by the multiplex qPCR (*gro*EL) and cPCR (16S rRNA gene) assays and comparing the positivity for *Anaplasma* spp./'Candidatus *Allocryptoplasma* spp.' and *Ehrlichia* spp., a higher positivity for *Anaplasma* spp./*'Candidatus* Allocryptoplasma spp.' (9.7%; 37/382) and *Ehrlichia* spp. (2.3%; 9/382) was found among birds sampled in the state of Mato Grosso. On the other hand, positivity of 2.5% (3/118) for *Anaplasma* spp. was found among birds sampled in the state of Mato Grosso do Sul.

When performing conventional PCR assays to characterize Anaplasmataceae agents with the 16S rRNA [53], *dsb* gene [44] and 23S-5S rRNA intergenic region (ITS) [55], three sequences (ranging from 832-884 bp) were obtained in the semi-nested PCR protocol targeting a fragment of 800 bp of the 16S rRNA gene [53]. The positive samples were obtained from one *C*. *fasciolata*, one *F*. *leucopus*, and one *A*. *leucocephala* from Nossa Senhora do Livramento.

Two sequences (ranging from 401- 402 bp) were obtained in the PCR protocol targeting the *dsb* gene. The two positive samples were obtained from one *A*. *cyanopus* from Nossa Senhora do Livramento (MT), and one *B*. *flaveolus* from Santo Antonio de Leverger (MT).

Three sequences (ranging from 832-884 bp) were obtained in the PCR protocol targeting the 23S-5S rRNA intergenic region (ITS). The positive samples were

obtained from one *C*. *fasciolata* from Nossa Senhora do Livramento (MT), one S. coerulescens and one *B*. *flaveolus* from Santo Antonio de Leverger (MT). The BLAST results for the obtained 16S rRNA, dsb and ITS sequences are shown in Supplementary Table S4.

Sequences showing high identity to '*Candidatus* Allocryptoplasma sp.' and obtained in the PCR protocols described by Parola [43]. or Eberhardt [53] targeting the 16S rRNA gene were submitted to additional PCR assays based on the *suc*A and *gro*EL genes [4]. As a result, one *C*. *fasciolata* from Nossa Senhora do Livramento (MT) showed to be positive in the PCR targeting the *gro*EL gene, but due to low intensity of the obtained band was not possible to retrieve a readable sequence.

3.4 Phylogenetic analyses

The phylogenetic analysis based on an alignment of 1,439 bp positioned the sequences detected herein in a clade exclusively formed by '*Candidatus* Allocryptoplasma spp.' previously detected in *Ixodes ricinus* ticks (OQ724839) from French Guiana, in a green lizard (*Lacerta viridis*) (MG924904) from Slovakia, in *Ixodes ricinus* ticks (GU734325, AY672415, AY672416, AY672417, MT829288, MT829287) from France, Tunisia and Italy, *'Candidatus* Allocryptoplasma californiense' (KP276585-KP276587) detected in *Ixodes pacificus* ticks from the USA, *'Candidatus* Allocryptoplasma sp.' detected in *Amblyomma tholloni* ticks (OQ724840-OQ724842) from Uganda, in *Amblyomma coelebs* tick (OQ724839) from French Guiana, and *Haemaphysalis longicornis* ticks from South Korea (GU075701-GU075704). The consensus sequence of '*Candidatus* Allocryptoplasma spp.' detected in a *Crax fasciolata* from Nossa Senhora do Livramento, Mato Grosso #330 BAP146] [PP373675 and PP326053] was closely related to the sequence of '*Candidatus* Allocryptoplasma sp.' detected in an *Amblyomma coelebs* tick (OQ724839) from French Guiana; while the sequences detected in *P. sulphuratus (*#441 [F10]) [PP326054] from Santo Antonio de Leverger and in a *F. leucopus [*#341 BAP428] [PP373692] from Nossa Senhora do Livramento) were closely related to '*Candidatus*

Allocryptoplasma sp.' sequences detected in *Haemaphysalis longicornis* from South Korea (GU075701-GU075704) **(Figure 2).**

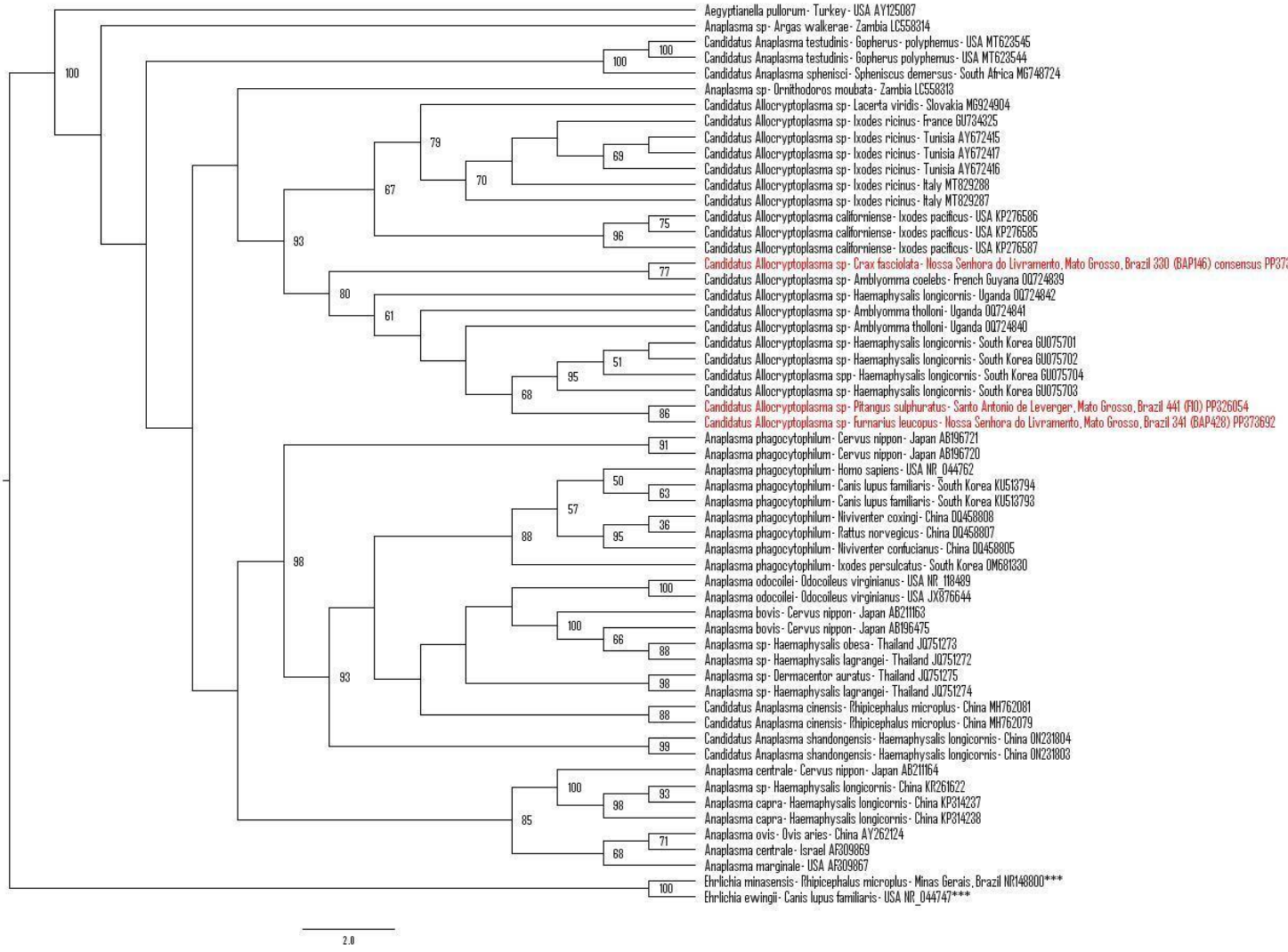

Figure 2 Phylogenetic analysis generated by the Maximum Likelihood method and TIM2+F+I+G4 evolutionary model based on an alignment of 1,439 bp of 16S rRNA gene sequences from Anaplasmataceae agents, containing 57 homologous sequences for the 16S rRNA gene from the genera *Anaplasma*, *'Candidatus* Allocryptoplasma sp.' and *Aegyptianella* sp. *Ehrlichia minasensis* and *Ehrlichia ewingii* sequences were used as outgroups (NR148800 and NR_044747) and were indicated with (***). The sequences obtained in this project were highlighted in red. Bootstraps lower than 50 were not shown.

The phylogenetic analysis based on an alignment of 1,339 bp positioned the obtained sequences in three different clades. The first clade grouped the *Ehrlichia* sequence obtained from an *Aurindinicola leucocephala* from Nossa Senhora do Livramento, Mato Grosso, Brazil [#345 BAP267] [PP373702] with *Ehrlichia* sp. Magellanica detected in a Magellanic penguin (*Spheniscus magellanicus*) from Chile (MK049840). The second clade was exclusively formed by sequences detected in present study and were obtained from an *Elaenia albiceps* from Poconé, Mato Grosso, Brazil [#292 BAP72] [PP326052] and a *Ramphocelus carbo* from Santo Antonio de Leverger [#444 F68] [PP346773]. The third clade is formed by a sequence of *Ehrlichia* sp. detected in a *R.carbo* from Poconé, Mato Grosso, Brazil [#264 BAP123] [PP346669] (**Figure 3**).

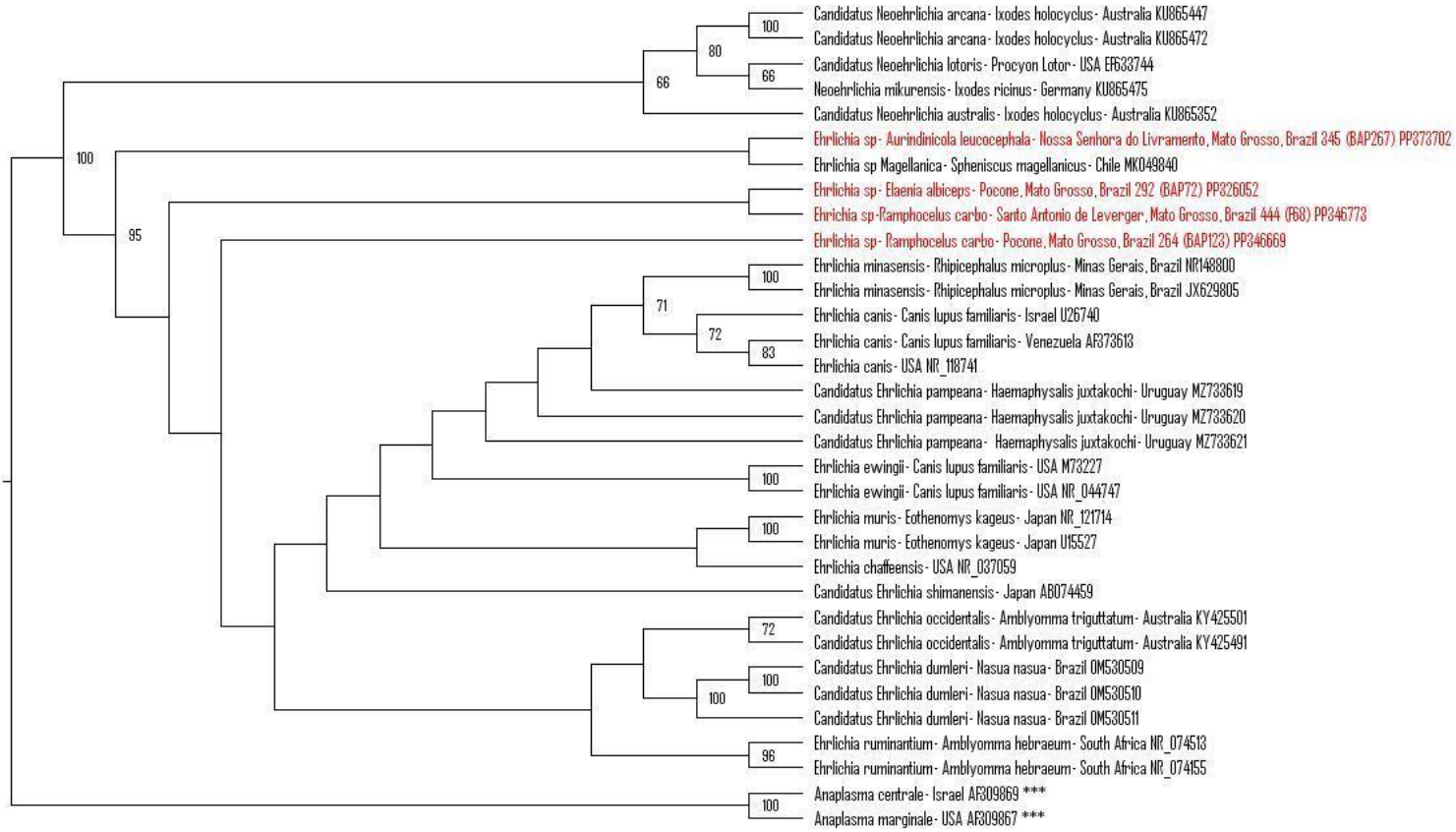

Figure 3 Phylogenetic analysis generated by the Maximum Likelihood method and GTR+I+G4+F evolutionary model based on an alignment of 1,339 bp of 16S rRNA gene sequences from *Ehrlichia* spp. and *Neoehrlichia* spp. containing 33 homologous sequences. *Anaplasma centrale* (AF309869) and *Anaplasma marginale* (AF309867. sequences were used as outgroups and indicated with (***). The sequences obtained in this project were highlighted in red. Bootstraps lower than 50 were not shown.

The phylogenetic analysis based on an alignment of 409 bp of the *dsb* gene positioned the two sequences obtained in the present study into the same clade: the sequences obtained from *A. cyanopus* (#389 [BAP289]) [PP417932] from Nossa Senhora do Livramento and from *B. flaveolus* (#418 [F54]) [PP417933] from Poconé were closely related to *Ehrlichia minasensis* obtained from a common sloth (*Bradypus variegatus*) (MH212419) from Brazil, from cattle from the Philippines (LC641910) and Colombia (ON209405), from *Rhipicephalus microplus* ticks from Brazil (JX629808) and Colombia (KM015219) and from a *Rhipicephalus sanguineus* ticks from Brazil (MT135769) (**Figure 4**).

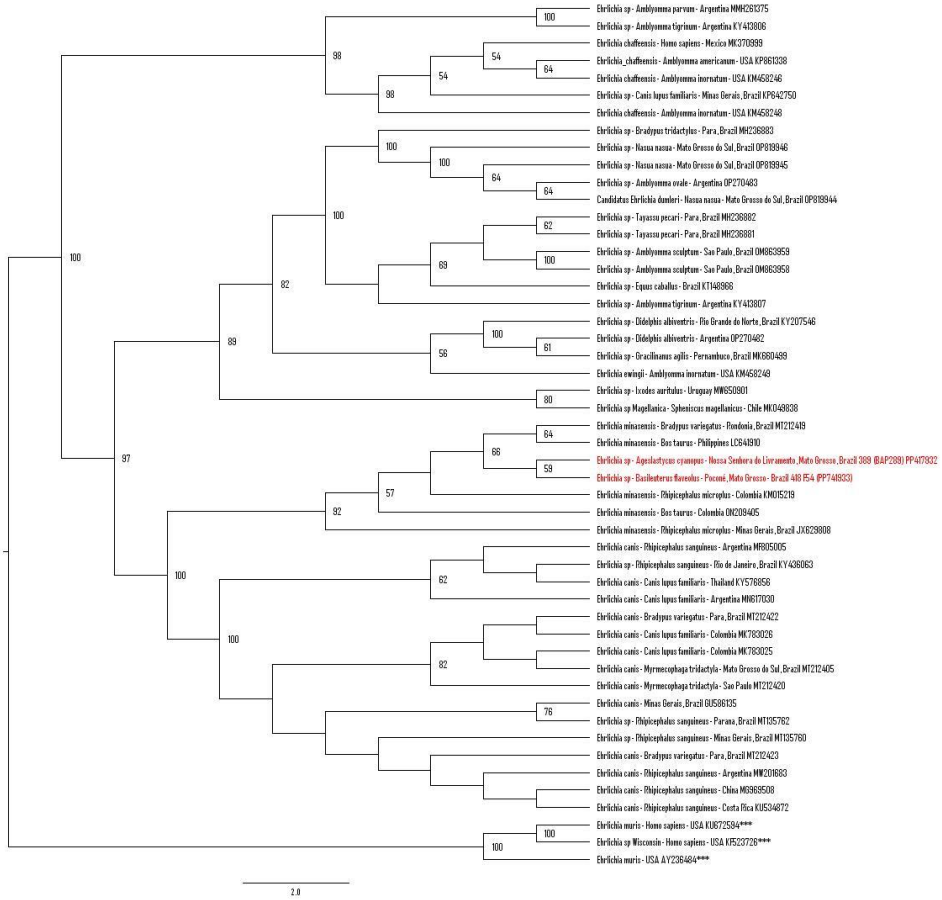

Figure 4. Phylogenetic analysis generated by the Maximum Likelihood method and TIM3+I+G4+F evolutionary model based on an alignment of 409 bp of *dsb* gene sequences, containing 53 homologous sequences. *Ehrlichia muris* and *Ehrlichia* sp. were used as outgroups (KU672594, KF523726 AY236484) and indicated with (***). The sequences obtained in this project were highlighted in red. Bootstraps lower than 50 were not shown.

The phylogenetic analysis based on an alignment of 430 bp of the 23S-5S intergenic region (ITS) positioned the sequences obtained herein (*C. fasciolata* [#330 BAP146] [PP417934] and *B. flaveolus* [#507 F12] [PP417936] from Nossa Senhora do Livramento, and *S. coerulescens* [#497 F58] [PP417935] from Santo Antonio de Leverger) in the same clade, grouping with sequences of *A. phagocytophilum* (KU588997; KU588996) from *Ixodes pacificus* ticks from the USA (Figure 5).

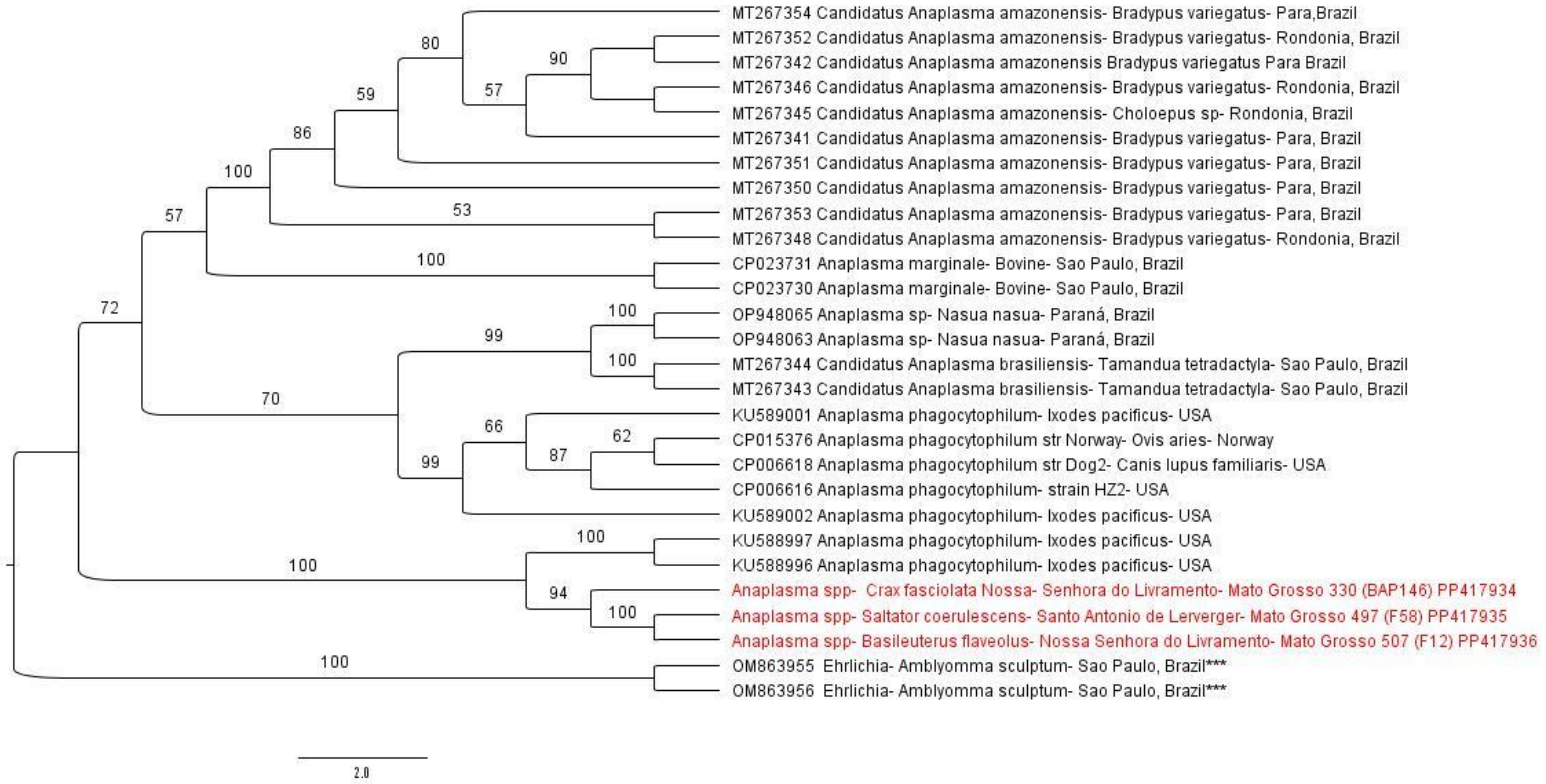

Figure 5. Phylogenetic analysis generated by the Maximum Likelihood method and evolutionary model TVM+G4+I+F based on an alignment of 430 bp of sequences from the 23S-5S intergenic region of *Anaplasma* sp., containing 26 homologous sequences from the 23S -5S intergenic region from *Anaplasma* sp. Two sequences from *Ehrlichia* spp. were used as outgroups (OM863955 and OM863956) indicated with (***). The sequences obtained in this project are highlighted in red. Bootstraps lower than 50 were not shown.

4. Discussion

Although Anaplasmataceae agents have been described in wild mammals worldwide [14], studies focusing on the occurrence and genetic diversity of these tick-borne α-proteobacteria in avian hosts are scarce. Up to now, these agents have been molecularly detected in avian hosts from South Africa [23], Chile [24], South Korea [20], Hungary [25], Romania [22], Cyprus [66], and Brazil [26–29].

In the present study, the molecular occurrence of *Ehrlichia*/*Anaplasma* in birds inferred by both conventional (16S rRNA) and real-time (*gro*EL) PCR assays used as screening tests was 9.2% (46/500), falling within previously reported prevalence in birds, which ranged from 0.73% ([25] up to 43.5% [28]. Such difference in molecular prevalence for *Ehrlichia*/*Anaplasma* in birds among different studies might be due to the variety of avian species tested, sensitivity/specificity of PCR protocols used, geographic region, level of infestation by tick vectors, susceptibility of the birds sampled with regard to the development of detectable bacteremia, among others.

In the present study, the Ehrlichia 16S rRNA sequences detected in specimens of *A*. *leucocephala* [#345 BAP267] [PP373702], *R*. *carbo* (#444 F68 [PP346773] and #264 BAP123 [PP346669]), and *E*. *albiceps* (#292 BAP72 [PP326052]) were positioned closely to *Ehrlichia* sp. Magellanica detected in a Magellanic penguin (*Spheniscus magellanicus*) from Chile [24]. In the phylogeny based on the dsb gene, *Ehrlichia* sequences detected in specimens of *A*. *cyanopus* [#389] and *B*. *flaveolus* [#418] grouped with *Ehrlichia minasensis*, suggesting that *Ehrlichia minasensis* or a closely related genotype might be transmitted by ticks to a variety of hosts besides cattle, such as sloths [67] and birds.

Herein, 3.7% of sampled birds were positive in the qPCR for *Anaplasma* spp. based on the groEL gene. Unfortunately, all samples positive in the qPCR were negative in conventional PCR assays, similarly to the results reported by [29] among carnivorous birds from Brazil.

The phylogeny based on the 23S-5S rRNA intergenic region presented well-established clades similar to those reported by Calchi [67] and Perles [68,69]. The ITS sequences obtained in the present work in blood samples from specimens of *C*.

fasciolata [#330 BAP146 (PP326053)], *B. flaveolus* [#507 F12 (PP417936)] and *S. coerulescens* [#497 F58 (PP417934)] were positioned in a clade closely related to *A. phagocytophilum*. Previously in Brazil, 16S rRNA genotypes of *Anaplasma* sp. detected in runner geese [28], vultures, caracaras [26], and curassows [27] were positioned closely to *A. phagocytophilum* and *Anaplasma* sequences previously detected in wild [70] and domestic felids [71]. In South Korea, *Anaplasma* 16S rRNA sequences detected in thrushes showed to be closely related to *A. phagocytophilum* [20]. In South Africa, the 16S rRNA genotype of *Anaplasma* sp. detected in African penguins was phylogenetically associated with *Anaplasma capra*, *A. marginale*, *A. ovis* and *A. centrale* [23]. Such phylogenetic positioning based on short fragments of the 16S rRNA should be interpreted with caution.

In the phylogenetic analysis based on the 16S rRNA gene, the genotypes detected in specimens of *C. fasciolata* [#330 BAP146 (PP326053)] and F. leucopus [#341 BAP428 (PP373692)] were positioned within the clade of *'Candidatus* Allocryptoplasma sp.'. Previous studies have shown that '*Candidatus* Cryptoplasma sp.', recently named as '*Candidatus* Allocryptoplasma sp.' [4], presents itself as a monophyletic clade [4,72]. When comparing the phylogenetic tree of the present study with that one described by Ouass [4], it is possible to observe the proximity of '*Candidatus* Allocryptoplasma' and *Anaplasma* spp. While in the phylogeny of the present study it was possible to observe a polyphyletic clade of '*Candidatus* Allocryptoplasma', a monophyletic profile was observed for the available sequences of '*Candidatus* Allocryptoplasma sp.' in the studies developed by Ouass [4] and Mendoza-Roldan [71]. This discrepancy can be explained by the inclusion of distinct and novel genotypes detected in the present study, which indicates that the phylogenetic positioning of such agents is far from being resolved.

'Candidatus Allocryptoplasma' has already been reported in *Ixodes ricinus* from Morocco and Tunisia [73], *Ixodes pacificus* from the United States of America [3], *Ixodes ricinus* from Serbia [74], *Ixodes ricinus* from France [75], *Amblyomma tholloni* and *Haemaphysalis parmata* from Uganda and *Amblyomma coelebs* from French Guiana [4], *Lacerta viridis* lizards and *Apodemus agrarius* rodents from Slovakia [76], Podarcis lizards from Italy [72] as well as in an *Amblyomma dissimile* tick from Brazil [77]. '*Candidatus* Allocryptoplasma spp.' present apparent worldwide distribution,

whose implications for animal and human health are still unknown. Since the majority of *'Candidatus* Allocryptoplasma' sequences were obtained from ticks (Ixodes ricinus, *Ixodes pacificus*, *Amblyoma tholloni*, *Amblyomma dissimile* and *Haemaphysalis longicornis*) so far, it's been suggested that ticks might play an important role in the transmission of such agents to hosts. When it comes to vertebrate hosts, *'Candidatus* Allocryptoplasma spp.' or closely related agents have only been detected in reptiles [72,78] and rodents [76]. The present work showed, for the first time, the occurrence of *'Candidatus* Allocryptoplasma spp.' or a closely related agent in birds. Altogether, these findings emphasize the need to unravel the diversity of Anaplasmataceae agents in non-classical vertebrate hosts, such as birds.

Taking into account that birds analyzed in the present work originated from a previous study that investigated tick infestation [34], only 2/37(5.55%) birds positive for *Anaplasma* spp. were parasitized by ticks at the sampling time: R. carbo #417 [F073] was infested by an *Amblyomma longirostris* nymph and *Eucometis penicillata* #51 [SL025] was found parasitized by an *Amblyomma nudosum* nymph. Future studies should be performed in order to unravel the tick species involved in the transmission of these novel Anaplasmataceae genotypes among birds.

5. Conclusions

Novel genotypes of *Ehrlichia* spp. (closely related to *Ehrlichia* Magellanica and *E*. *minasensis*), *Anaplasma* spp. (closely related to *A. phagocytophilum*) and '*Candidatus* Allocryptoplasma spp.' were molecularly detected in blood samples from tropical bird species sampled in the Pantanal wetland. More studies are needed to molecularly characterize and unravel the transmission dynamics of these Anaplasmataceae agents in tropical birds from Pantanal wetland, in order to investigate the role of wild birds in the dispersion of such agents. Whole Genome Sequencing approaches should be performed aiming at shed light on the real identity of these novel Anaplasmataceae agents infecting neotropical wild birds. This is the first molecular evidence of '*Candidatus* Allocryptoplasma spp.' in birds in the world.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, ***Table S1***. Number of avian species collected in Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul, central-western Brazil. ***Table S2.*** Conventional, nested and quantitative real-time PCR assays used in screening and molecular characterization for Anaplasmataceae agents targeting the 16S RNA, *groEL, dsb, gltA, sodB, omp-1, rpoB, ftsZ,* and *sucA* genes and intergenic region 23S-5S (ITS)**.** ***Table S3.*** Avian DNA samples positive in the multiplex quantitative (q) real-time qPCR for *Anaplasma* spp. and *Ehrlichia* spp. based on the *groEL* gene. ***Table S4.*** BLAST analyses results obtained for 16S rRNA, *dsb*, and ITS sequences obtained in PCR protocols for Anaplasmataceae agents from avian blood samples from the Brazilian Pantanal.

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Data Availability Statement: The sequences generated during the study were submitted in the NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

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Chapter 4. Molecular survey of vector-borne agents in wild birds from the Brazilian Pantanal

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Abstract:

Despite several records of Borrelia spp., Hepatozoon spp., piroplasmids, and onchocercid filariids parasitizing a wide variety of birds around the world, there are few reports of these vector-borne agents in avian hosts in Brazil. The present work aimed to investigate, using molecular assays, the occurrence of *Borrelia* spp., *Hepatozoon* spp., piroplasmids, and onchocercid filariids in tropical birds from the Brazilian Pantanal wetland, in the states of Mato Grosso and Mato Grosso do Sul. For this purpose, blood sampling and DNA extraction were performed on 500 birds from 93 different species and 13 avian orders. DNA samples positive to endogenous gene (avian β-actin) were submitted to PCR assays targeting the 18S rRNA gene of *Hepatozoon* spp. and piroplasmids, qPCR (16S rRNA) and PCR (16S rRNA and flaB genes) for *Borrelia* spp.; and PCR assays for filariids (cox1, 28S rRNA and 18S rRNA genes). As a result, one sample (0.2%; *Cyanocorax cyanomelas*) was positive in the qPCR for *Borrelia* spp., and two (0.4%; *Ramphocelus carbo* and *Taraba major*) for filariids. None was positive in the PCR assays for *Hepatozoon* spp. or piroplasmids. While the cox-1 sequence detected in R. carbo grouped with *Cardiofilaria* sp., the onchocercid cox-1 sequence obtained from T. major grouped with *Splendidofilaria* spp. This is the first molecular report of Borrelia spp. and onchocercid filariids closely related to *Cardiofilaria* spp. and *Splendidofilaria* spp. in birds from the Brazilian Pantanal.

Key-Words: Onchocercidae; piroplasmids*; Hepatozoon*; Pantanal; birds.

Resumo:

A diversidade de *Hepatozoon* spp., piroplasmídeos e filarídeos oncocercídeos que parasitam aves em todo o mundo tem sido subestimada, especialmente no Brasil. O presente trabalho teve como objetivo investigar, por meio de ensaios moleculares, a ocorrência de *Hepatozoon* spp., piroplasmídeos e filarídeos oncocercídeos em aves tropicais do Pantanal brasileiro, nos estados de Mato Grosso e Mato Grosso do Sul. Para isso, foram realizadas coletas de sangue e extração de DNA em 500 aves de 93 espécies diferentes e 13 ordens de aves. Amostras de DNA positivas para gene endógeno (β-actina aviária) foram submetidas a ensaios de PCR direcionados ao gene 18S rRNA de *Hepatozoon* spp. e piroplasmídeos e ensaios de PCR para filariídeos (genes cox1, 28S rRNA e 18S rRNA). Como resultado, dois (0,4%; *Ramphocelus carbo* e *Taraba major*) para filariídeos. Nenhum foi positivo nos ensaios de PCR para Hepatozoon spp. ou piroplasmídeos. Enquanto a sequência de cox-1 detectada em R. carbo se agrupou com *Cardiofilaria* sp., a sequência de cox-1 oncocercida obtida de T. major se agrupou com *Splendidofilaria* spp. Este é o *primeiro* relato molecular de filarídeos oncocercidas intimamente relacionados a Cardiofilaria spp. e *Splendidofilaria* spp. em aves do Pantanal brasileiro.

Palavras-chave: Onchocercidae, piroplasmideos, *Hepatozoon*, Pantanal, aves

1 Introduction

Birds represent the most diverse group comprehending a variety of lineages, morphologic characteristics, ecological and behavioral features [1,2]. Birds' capability to adapt to several ecological niches aids the spread of vector-borne agents, viruses, ticks and zoonotic pathogens through avian migration [3–9]. These characteristics make birds of interest to Public Health [5].

The genus *Borrelia* (Spirochetales: Borreliaceae) includes vector-borne spirochetes measuring 0.2-0.5mm wide and 3-30 mm length [10]. Borrelia genus is divided into three phylogenetic major groups: i) Lyme Borreliosis (LB) group, transmitted by ixodid ticks; ii) Relapsing fever (RF) group, transmitted by argasid ticks, and iii) Borrelia related to echidna, reptiles, and more recently, birds (from French Guyana), mainly transmitted by ticks of the genera Amblyomma, Bothriocroton, and Hyalomma [11,12]. So far, only two *Borrelia* species have been reported in bird-associated ticks from Brazil, namely *Borrelia anserina* [13,14] and *Borrelia* spp. closely related to *Borrelia turcica* [15].

Hepatozoon spp. (Adeleorina: Hepatozoidae) comprise protozoa whose life cycle includes arthropods (ticks, fleas, flies, mosquitoes or lice) as definitive hosts and vertebrates (amphibians, reptiles, mammals, and birds) [16–18] as intermediate hosts. The transmission to the intermediate hosts is mediated by the ingestion of the definitive hosts containing mature oocysts [19], vertical transmission [20], and predation [21]. *Ornithodorus peringueyi* ticks and *Xenopsylla trispinis* fleas are the only arthropods described harboring avian hemogregarinids (*Hepatozoon atticorae*), suggesting that birds become infected when they ingest these arthropods [22]. Hepatozoon spp description in birds has relied, mainly on morphometric and morphological features, as it was performed by Bennett and Peirce [23] for the description of *Hepatozoon parus*. Despite several descriptions of avian-related *Hepatozoon* species [23–29], *Hepatozoon* infections in birds are usually misclassified as *Haemogregarina* or *Atoxoplasma*, contributing

to overstimate the occurrence of these protozoa among avian hosts [25]. Merino [28] detected *Hepatozoon peircei* in a storm petrel (*Oceanodroma melania*) sampled in Baja California, Mexico, when targeting the 18S rRNA. Other attempts to detect *Hepatozoon* in birds reported the presence of sequences of other apicomplexan protozoa related to *Lankesterella* species, *Caryospora* and *Eimeria* in avian blood samples when targeting the 18S rRNA gene [29,30].

The Order Piroplasmida comprises tick-borne apicompleaxan protozoa belonging to four genera: *Babesia, Theileria, Cytauxzoon*, and *Rangelia* [31-33]. To date, 16 *Babesia* species have been reported in birds [34-40], along with several other unindentified *Babesia* spp. [41-49]. Most of the described species were identified using morphological methods based on blood smears or histopathology and were related to vertebrate hosts or vectors and geographical location. Out of the described species, only seven have been detected using molecular methods, mainly through nearly complete fragment of the 18S rRNA gene. Phylogenetic analysis has shown that these agents form a polyphyletic group divided into three paraphyletic clades: the first consists of *B. percei, B. poelea, B. ardeae, B. uriae*, and *B. ugwidiensis*, which form the Percei group, a sister clade to the Western group. The second clade is formed by *B. kiwiensis*, which is phylogenetically related to the clade of *Babesia* spp. sensu stricto. The third clade consists of *B. bennetti*, which is closely related to *B. bovis* and *B. ovis*, although there are suspicions that the provided sequence may be a chimera and that the species does not actually exist [39,50,51]. To date, *B. shortii* and *B.* uriae are considered pathogenic to birds [52]. The former, a species described in falcons, has been reported to cause clinical signs, such as somnolence, weight loss, anorexia, lethargy, vomiting, blood in feces, poor coordination, seizures, diarrhea, lowered head, dyspnea, and continuous vocalization. These clinical signs were resolved after treatment with imidocarb dipropionate [53]. *Babesia uriae*, on the other hand, has been associated with tissue lesions, primarily in the lungs of common murres (*Uria aalge*) [37].

Onchocercidae filariids of the subfamilies Dirofilariinae, Onchocercinae, Splendidofilariinae and Lemdaniinae are mostly hosted by birds and present a cosmopolitan distribution [54,55]. The morphological features of microfilariae must be complemented with morphological analysis of adult nematodes for a correct taxonomic identification [56].

The diversity of filariid parasites of birds is in fact unknown, with the most recent descriptions performed by Bartlett [57] and Binkienė [58]. Avian filariids are mainly transmitted by Nematocera dipterans from the Simuliidae, Culicidae and Ceratopogonidae families or lice (order Phthiraptera) [54]. Although avian-associated filariids may have implications in Public Health (e.g., *Pelecitus* spp.) [59,60], the knowledge on this subject is limited. For instance, filariids have been detected in birds from Canada, Iceland (Eulimdana spp.) [57], Colombia [56], Costa Rica [61], the USA (Eulimdana spp., Chandlerella spp., Splendidofilaria spp.) [57,62,63], Mexico (Filaroidea) [64], and Check Republic (*Eufilaria* delicata, *Ornithofilaria mavis*) [65]. Likewise, previous reports of unidentified filariids in birds from Brazil, were described by Silveira and Sebaio [66,67], *Pelecitus* spp. [68] in a specimen of *Athene cunicularia*, *Eufilaria* spp. microfilariae were detected in an *Antophilia galeata*'s blood smear [69] and *Aproctella alessandroi* was first collected and identified from *Saltator similis* in São Paulo state, southeastern Brazil [70] and deposited in the National Museom of Natural Nistory (deposit number: 117YU), Paris France. Later on, this specimen was characterized molecullarly targeting the cox-1, 12S rRNA, 18S rRNA, 28S rRNA, *myo*HC, *rbp*1 and *hsp*70 genes [71].

Considering the location in the basin of the Paraguai river comprising 140,000 km2 [72], a rainy season between November and March, with some variations between the northern and southern regions, varies from 1200 to 1300 mm, exceptionally reaching 2000 mm [73]. the wide diversity of resident and migratory avian population in the Brazilian Pantanal [4,74], the variety of ecosystems that aid in the preservation of the biodiversity [75], and the feasibility of the transmission of vector-borne pathogens among avian populations, the present study aimed to investigate, using molecular

techniques, the ocurrence of *Borrelia* spp., *Hepatozoon* spp., and onchocercid filariids in wild birds sampled in the Pantanal wetland in the Mato Grosso and Mato Grosso do Sul states, Central-western Brazil.

2 Material and methods

2.1 Study area and sampling

Twenty mist nets (36 mm mesh, 12 m long and 2.5 m height) were settled alongside the trails in four localities in the Pantanal region: Nossa Senhora do Livramento (99 samples) (16◦21′46.8″ S 56◦17′24.0″ W), Poconé (100 samples) (16◦29′56.4″ S 56◦24′46.8″ W), and Santo Antonio de Leverger (200 samples) (16◦44′34.8″ S 55◦33′10.8″ W) from the state of Mato Grosso and Corumbá (101 samples) (19◦34′37.2″ S 57◦01′08.4″ W) from the state of Mato Grosso do Sul. The sampling was performed over five days per each locality as previously described by Alabí Córdova [76,77] (Figure 1).

Bird identification was performed by an experienced ornithologist using field guides. The Birdtree project was used for taxonomical identification (see https://birdtree.org/taxonomy) (accessed on 7 October 2024) [78]. Avian blood sampling was performed with the proper permits according to Brazilian guidelines, approved by IBAMA (72548 e 72790), the “Ethics Committee on the Use of Animals” of the Faculty of Agricultural and Veterinary Sciences (FCAV/UNESP) (CEUA 268/21), and SISGEN (AF30FD1). In total, 517 birds belonging to 72 species and

13 orders were sampled [76,77].

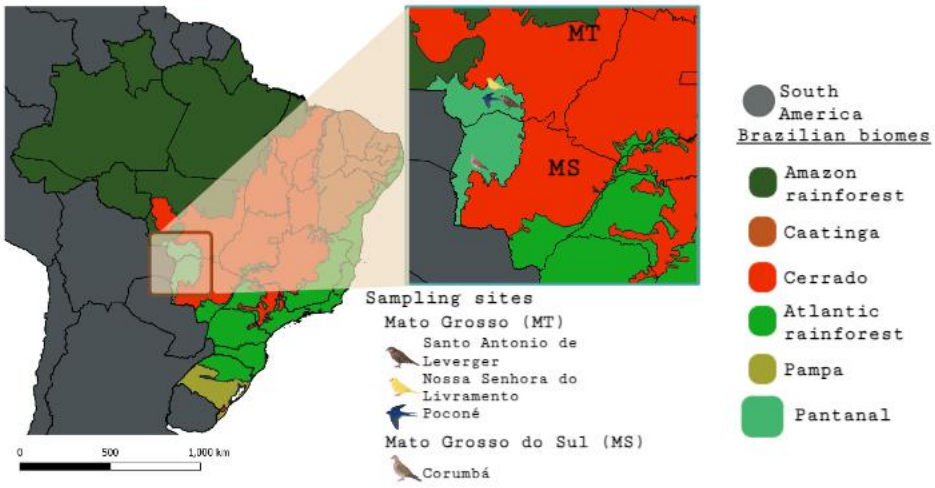

Figure 1. Sampling sites in the Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul, according to brazilian biomes.

2.2 DNA extraction and PCR for endogenous gene

DNA extraction was performed according to the FTA extraction protocol as previously described by de Sena Oliveira [79] followed by recommendations provided by Biopur Mini Spin Plus kit (Mobius Life Science, Paraná, Brazil). A conventional PCR assay targeting the avian β-actin gene was performed in previous studies [76,77] as described elsewhere [80]. The mean concentrations and 260/280 ratios of avian blood samples DNA extractions were detailed in previous studies [76]. Samples with concentrations higher than 100 ng//µL were diluted to a final concentration of 50 ng/µL with sterilized ultra-pure water (Invitrogen®, Carlsbad, CA, USA). Samples negative to endogenous gene were detailed in previous works and were excluded from the molecular screening for the selected vector-borne agents studied herein [76,77].

2.3 Molecular screening and molecular characterization for *Borrelia* spp., *Hepatozoon* spp., Piroplasmida and filariids

The molecular screening for Borrelia spp. was performed on avian β-actin-positive samples using a quantitative (q)PCR assay targeting the 16S rRNA gene [81] (**Supplementary Table 1**). The reactions consisted of 1 µL of template DNA, 0.2 µM of each primer and hydrolysis probe, Master Mix 2x (GoTaq™ Probe qPCR Master Mix, Promega Corporation, Madison, WI, USA), and sterilized ultra- pure water (Nuclease-FreeWater, Promega, Madison, WI, USA) to complete a final volume of 10 µL. Amplification reactions were carried out in a CFX96™ Thermal Cycler (BioRad, Hercules, CA, USA). Samples were tested in duplicates. Dilutions (2.0 × 107 copies/µL to 2.0 × 100 copies/µL) of a G-block containing a conserved fragment of the 16S rRNA gene from *Borrelia* sp. (148 bp) (Integrated DNA Technologies, Coralville, IA, USA) were used to obtain the efficiency and correlation coefficient of reactions. The number of target gene copies was determined according to the formula (Xg/µL DNA/ (plasmid size (bp) × 660)) × 6.022 ×1023 × G-block copies/µL. All analyses were carried out following the MIQE (“Minimum Information for Publication of Quantitative real-time PCR Experiments”) guidelines [82]. Positive samples with Cq values with a difference higher than 0.5 between duplicates were re-tested in triplicates. When the difference among the triplicates were greater than 0.5, the sample was considered positive but not quantifiable due to Monte Carlo effect [82]. Samples positive in the qPCR for Borrelia spp. were subjected to nested PCR assays aiming to amplify the 16S rRNA [83] and *fla*B genes [84]. *Borrelia theileri* DNA obtained from naturally-infected tapirs (*Tapirus terrestris*) [85] was used as a positive control. Ultra-pure sterilized water was used as a negative control.

Avian DNA blood samples positive to avian β-actin gene were also subjected to screening PCR assays targeting the 18S rRNA gene for *Hepatozoon* spp. [86,87] and piroplasmids [88], and *cox*-1 gene for filariids [89] (Supplementary Table 1). Each reaction had a total volume of 25 µL, containing 1.25 U Go Taq Hot Start Polymerase (Promega®, Madison, WI, USA), PCR buffer 10× (Promega®, Madison, WI, USA), sterilized ultra-pure water (Invitrogen®, Carlsbad, CA, USA), 0.2 mM of each deoxynucleotide, 0.4 µM of each oligonucleotide, 3.0 mM of MgCl2, and 3 µL DNA

template. In nPCR assays, 1 µL of the amplified product from the first PCR reaction was used as the target DNA in the second reaction. *Hepatozoon* sp. DNA from *Cerdocyon thous* blood DNA sample [90], *Babesia vogeli* DNA (Jaboticabal strains) [91], and *Dirofilaria immitis* DNA obtained from female adult worms (kindly provided by Dr. Norma Labarthe, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro) were used as positive controls. Ultra-pure sterilized water was used as a negative control. Avian DNA samples positive in the molecular screening for filariids were subjected to conventional PCR assays targeting the 18S rRNA and 28S rRNA genes [92] (**Supplementary Table 1**)

2.4 Sequencing and BLASTn analysis

Amplicons purification was performed using the Wizard SV Gel and PCR cleanup System kit (Promega, Wisconsin, USA), in accordance with manufacturers' recommendations.

The purified PCR products were submitted to sequencing by dideoxy-nucleotide chain termination method [93] in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Waltham, MA, USA) at "Centro de Recursos Biológicos e Biologia Genômica" (CREBIO -FCAV -UNESP, Jaboticabal, SP, Brazil). Consensus sequences were assembled using BioEdit v7.2.5 software [94] and compared with homologous sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) (accessed on 12 October 2024) [95] using BLASTn [96].

2.5 Phylogenetic analyses

Sequences were aligned with similar sequences downloaded from Genbank using the Clustal/W software. 18.1 [97] through Bioedit v. 7.0.5.3 [98]. Alignments saved in "FASTA" mode were used to infer evolutionary model and maximum likelihood analyses via IQ tree online version (http://iqtree.cibiv.univie.ac.at/) (accessed on 08 October 2024) [99]. The bootstrap values for the Maximum likelihood (ML) were analyzed with 100 repetitions [100]. The

phylogenetic tree was edited using Figtree V1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/) (accessed on 10 October 2024) [101].

3 Results

From the 517 samples submitted to the PCR targeting the avian β actin gene, 500 were positive as described in Alabi Cordova [76]. Out of the 500 DNA avian blood samples, one sample (0.2%) was positive in the qPCR assay for Borrelia spp. based on the 16S rRNA gene: 359 (BAP145) *Cyanocorax cyanomelas* from Nossa Senhora do Livramento, Mato Grosso State; Cq values for the positive samples ranged from 32.27 to 33.19, Y-int= 32.47. The single qPCR-positive sample was negative in the nested PCR assays for Borrelia spp. based on both the 16S rRNA and *fla*B genes. The singe individual positive to *Borrelia* spp. was not parasitized by ticks at the time of sampling (data not shown).

In the PCR assay for filariids based on the cox-1 gene, two samples (0.4%) were positive: 179 (BEP415) Ramphocelus carbo from Corumbá, Mato Grosso do Sul State; and 229 (BAP114) *Taraba major* from Poconé, Mato Grosso State. The sequence obtained from *Taraba major* 229 (BAP114) showed 91% identity (query cover = 99%; E-value = 0.0) with Onchocercidae (OR148297) obtained from *Neodrepanis coruscans* from Madagascar. The sequence obtained from *Ramphocelus carbo* 179 (BEP415) showed 92.3% identity (query cover = 99%; E-value = 0.0) with *Cardiofilaria pavlolvskyi* (KP760174) obtained from a European golden oriole (*Oriolus oriolus*). The phylogenetic analysis inferred by Maximum Likelihood method and TIM3+F+I+G4 evolutionary model and based on an alignment of 673 bp of the cox1 gene positioned the obtained sequences in two different clades along with other avian-associated filariids. The sequence detected in *Ramphocelus carbo* (PQ452779) grouped with *Cardiofilaria* spp. whereas the sequence obtained from a *Taraba major* (PQ699371) was closely related to *Splendidofilaria* spp. (Figure 2). All samples positive to cox-1 gene for onchocercid filariids were

negative in the additional molecular assays based on the 18S rRNA and 28S rRNA genes.

No sample was positive in the PCR assays for *Hepatozoon* spp. or piroplasmids based on the 18S rRNA gene.

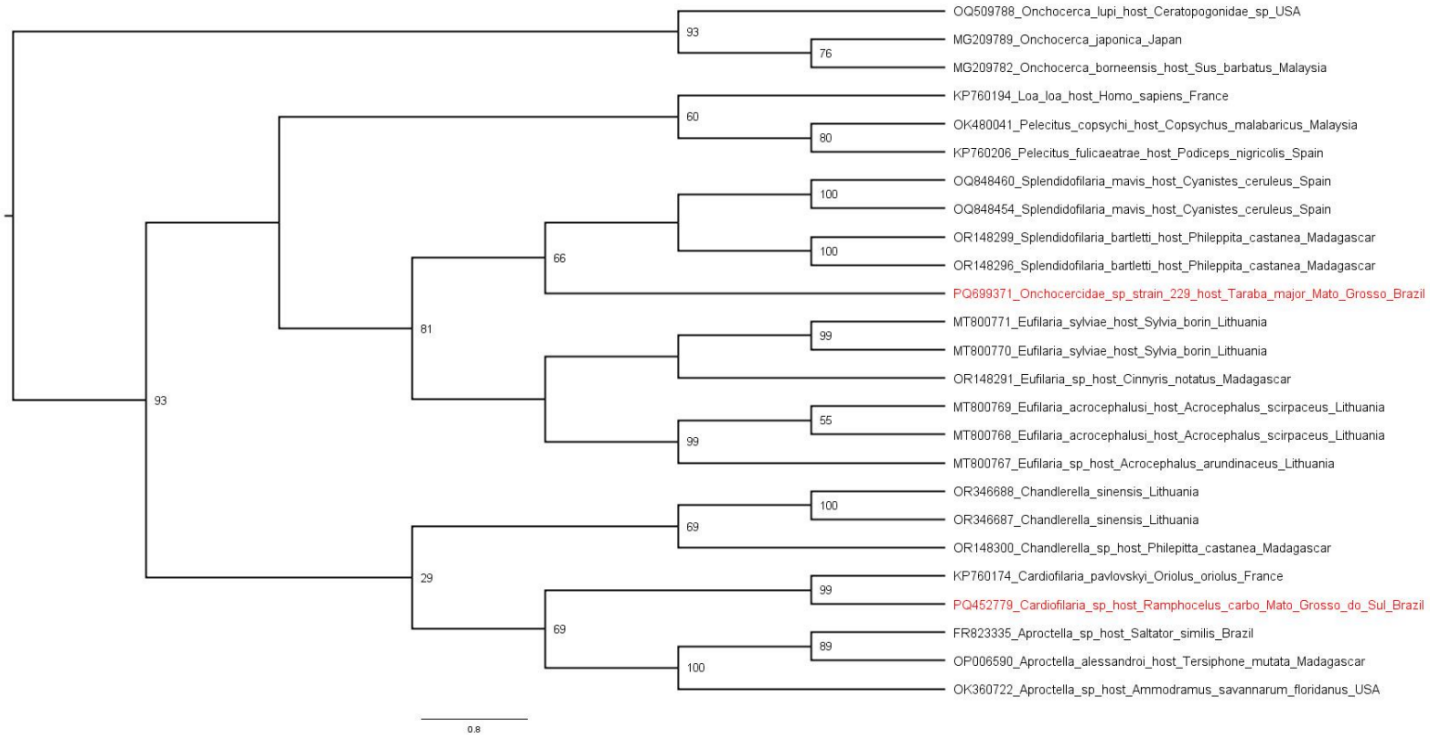

Figure 2. Phylogenetic analysis inferred by maximum likelihood method (TIM3+F+I+G4 evolutionary model) based on a 672 bp alignment of the cox1 gene for onchocercid filariids, containing 22 onchocercid filariids homologous sequences (*Aproctella*, *Eufilaria*, *Chandlerella*, *Pelecitus*, *Cardiofilaria*, *Splendidofilaria* and *Loa loa*). *Onchocerca* species (MG209782, MG209789 and OQ509788) were used as outgroups. Sequences obtained in present study are highlighted in red and with (***). Only the bootstraps with values of 50 or more are shown.

4 Discussion

The present study showed, for the first time, the presence of *Borrelia* spp. and Onchocercidae filariids in birds sampled in the Brazilian Pantanal.

Borrelia spp. have been detected in birds or avian-associated arthropods in Asia [102], Europe [103–107], North America [8,108,109], and South America [12,13,15,110]. While European birds can carry several *Borrelia* species (*Borrelia burgdorferi* s.l., *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia turdi*) [103-107], studies

reporting *Borrelia* infections in birds and/or associated ectoparasites in Brazil are scarce. While *B. anserina* [13,14] was detected in *Argas* (*Persicargas*) *miniatus* collected from a chicken coop in the state of Minas Gerais, Borrelia sp. closely related to *Borrelia turcica* was detected in a specimen of *Amblyomma longirostre* collected from a *Tangara seledon* in the state of Rio de Janeiro [15]. Unfortunately, the qPCR-positive sample detected in *C. cyanomelas* was negative in PCR assays targeting both 16S rRNA and *fla*B genes, precluding additional phylogenetic inferences.

Herein, onchocercid filariids DNA were detected only in passerine birds belonging to the Thraupidae and Thamnophilidae families. Previous study carried out in Brazil, based on morphological analysis, reported the occurrence of filariids in birds belonging to the following families: Parulidae, Picidae, Thamnophilidae and Pipridae in the Cerrado biome [66,69], Strigidae in Cerrado – Atlantic Forest interphase [68], and in Thamnophilidae, Tyrannidae, Fringillidae birds in Atlantic Forest biome [67,70,71]. In the present study, a molecular occurrence of 0.4% was detected among birds sampled in the Pantanal wetlands, in the states of Mato Grosso and Mato Grosso do Sul. Based on microscopy and/or molecular assays, previous studies reported an overall occurrence of onchocercid filariids in birds ranging from 0.4 to 13.8% [56,57,61–69].

The phylogenetic analyses based of the cox1 gene for avian-associated onchocercid filariids showed herein showed a similar topology as those previously presented by Hayashi [92] and Binkienė [58] based on the same molecular marker. Additionally, the topology presented in our study corroborate with phylogenetic analyses based on a concatenated dataset of several genes (12S rDNA, cox1, rbp1, hsp70, myoHC, 18S rDNA, and 28S rDNA) [71]. The *cox*-1 sequence detected in R. carbo grouped with *Cardiofilaria* sp.. Altogether, *Chanderella* spp., *Cardiofilaria* spp., and Aproctella spp. formed a large clade, as previously described [58,72]. The Onchocercidae cox-1 sequence detected in T. major was closely related to *Splendidofilaria* spp. The phylogenetic relatedness of *Splendidofilaria* spp. and *Eufilaria* spp. showed in our phylogenetic analysis also corroborated previous works [58,92]. Future studies aiming at adding more avian-associated *cox*-1 onchocercid sequences in GenBank database are needed in order to shed some light on the phylogenetic diversity of filariids among Neotropical birds. The search and

morphological characterization of adult filariids will contribute to unveling the diversity of Onchocercidae in wild birds from Brazil.

Differing from a previous study in which *Hepatozoon peircei* was detected [28] in an storm petrel (*Oceanodroma melanaria*), none of the samples of the present study were positive for Hepatozoon spp. The lack of infection by this parasite genus in Pantanal birds also differ from other studies that unexpectedly detected *Lankesterella* when aiming to detect *Hepatozoon* DNA [29,30]. The lack of positivity in the PCR assays for hemogregarines might be associated with low *Hepatozoon* parasitemia in birds. Additionally, the very few avian-associated-*Hepatozoon* spp. sequences available in Genbank hamper the design of specific primers for *Hepatozoon* infecting birds.

Piroplasmida DNA was not detected in the bird blood samples analyzed. It is well known that the occurrence of these agents in birds is low [44,52,111]. Indee, among the few studies conducted in Brazil, the reported positivity was low, ranging from 0.7% to 14.28%. The positive birds were *Sula leucogaster*, *Sula dactylatra*, *Neochen jubata*, and *Thalassarche chlororhynchos* [49,111,112]. The lack of detection or low positivity in birds may be related to low parasitemia. Although this study used the nPCR technique, which is more sensitive than conventional PCR, Piroplasmida DNA was not detected in the samples. It is likely that the use of more sensitive techniques, such as qPCR or digital PCR, could be more effective. Furthermore, primers commonly used to detect piroplasmids, which are very effective in mammals, are less efficient in birds, often due to their annealing to the host's DNA, which further complicates obtaining a reliable detection.

5 Conclusion

This is the first report of Borrelia sp. and onchocercid filariids in birds sampled in the Brazilian Pantanal wetland. Filariids DNA closely related to Cardiofilaria spp. and Splendidofilaria spp. are reported for the first time in birds from Brazil.

Ethical statement

This work was approved by IBAMA (72548 AND 72790), THE “Comissão de ética no uso de animais (CEUA)” of the School of Agricultural and Veterinarian Sciences, São Paulo State University “Júlio de Mesquita Filho” (FCAV/UNESP), (CEUA 268/21) and SISGEN (AF30FD1).

Declaration of interests.

Nothing to declare

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Chapter 5. Validation and standardization of a High-Throughput Microfluidic Real-Time PCR for the detection of vector-borne agents in wild birds from the Brazilian Pantanal

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Abstract

Despite numerous studies on haemosporidians in wild birds from Brazil, the presence of other vector-borne agents (VBA) such as *Anaplasma* spp., *Bartonella* spp., and Onchocercidae filariids in avian hosts remains largely unknown. The low occurrence of these VBAs might be due to the low sensitivity of traditional molecular techniques. The microfluidic real-time PCR assay, known for its high sensitivity, has emerged as a promising method to detect and study the occurrence and diversity of VBAs in both arthropod vectors and vertebrate hosts. To validate previously and standardize newly designed microfluidic real-time PCR protocols, selected positive avian blood DNA samples for *Anaplasma* spp., *Bartonella* spp., haemosporidians, and filariids were used. The molecular occurrence rates for the selected VBAs were 18.2% for *Anaplasma* spp., 0.36% for *Bartonella* spp., 6.2% for *Plasmodium* spp., 4.7% for *Haemoproteus* spp., and 6.5% for Onchocercidae filariids. The *Plasmodium* spp. *cyt*B sequence detected in a *Volatinia jacarina* clustered with *Plasmodium tejerai*, whereas the *Haemoproteus* spp. *cytB* sequence detected in a *Columbina squamata* clustered with *Haemoproteus columbae*. While Onchocercidae filariid *cox-1* sequences detected in specimens of *Ramphocelus carbo, Turdus amaurocalinus* and *Synallaxis albilora* grouped with *Aproctella* spp., one sequence detected in *R. carbo* was ancestral to the clade comprising *Splendidofilaria* spp. and *Eufilaria* spp. High-Throughput microfluidic real-time PCR assay can be used for screening VBAs in avian hosts from South America, but new primers/probe sets should be designed for VBA genotypes present in Brazil.

Keywords: Anaplasmataceae, Bartonellaceae, haemosporidians, Onchocercidae, microfluidic real-time PCR

Resumo. Apesar de numerosos estudos sobre hemosporídeos em aves silvestres do Brasil, a presença de outros agentes transmitidos por vetores (VBA), como *Anaplasma* spp., *Bartonella* spp. e Onchocercidae filariídeos em hospedeiros aviários, permanece amplamente desconhecida. A baixa ocorrência desses VBAs pode ser devido à baixa sensibilidade das técnicas moleculares tradicionais. O ensaio de PCR microfluídico em tempo real, conhecido por sua alta sensibilidade, surgiu como um método promissor para detectar e estudar a ocorrência e diversidade de VBAs em vetores artrópodes e hospedeiros vertebrados. Para validar anteriormente e padronizar protocolos de PCR microfluídicos em tempo real recém-projetados, foram utilizadas amostras selecionadas de DNA de sangue aviário positivo para Anaplasma spp., Bartonella spp., hemosporídeos e filariídeos. As taxas de ocorrência molecular para os VBAs selecionados foram de 18,2% para *Anaplasma* spp., 0,36% para *Bartonella* spp., 6,2% para *Plasmodium* spp., 4,7% para *Haemoproteus* spp. e 6,5% para Onchocercidae filariidae. A sequência de *Plasmodium* spp. cytB detectada em uma *Volatinia jacarina* agrupou-se com *Plasmodium tejerai*, enquanto a sequência de *Haemoproteus* spp. *cyt*B detectada em uma *Columbina squamata* agrupou-se com *Haemoproteus columbae*. Enquanto as sequências de Onchocercidae filariid cox-1 detectadas em espécimes de *Ramphocelus carbo*, *Turdus amaurocalinus* e *Synallaxis albilora* se agruparam com *Aproctella* spp., uma sequência detectada em *R. carbo* foi ancestral do clado que compreende *Splendidofilaria* spp. e *Eufilaria* spp. O ensaio de PCR microfluídico em tempo real de alto rendimento pode ser usado para triagem de VBAs em hospedeiros aviários da América do Sul, mas novos conjuntos de primers/sondas devem ser projetados para genótipos de VBA presentes no Brasil.

Palavras-chave: Anaplasmataceae, Bartonellaceae, haemosporidians, Onchocercidae, microfluidic real-time PCR

1 Introduction

Although the occurrence and diversity of haemosporidians in wild birds has been extensively investigated in Brazil [1–8], studies on other vector-borne agents (VBA) in avian hosts are limited when it comes to Anaplasmataceae agents, Bartonella spp., and filariids.

Anaplasmataceae agents (Order Rickettsiales) stands out among VBA of interest in both animal and human health [9]. Among tick-borne Anaplasmataceae agents, species from three genera are known to infect humans, namely Anaplasma, Ehrlichia, and '*Candidatus* Neoehrlichia' [10]. Despite that, few studies have investigated the occurrence of Anaplasmataceae in birds [11–16]. In Brazil, studies are scarce and reported the occurrence of *Anaplasma* and *Ehrlichia* 16S rRNA genotypes closely related to *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* [17–20]. Recently, '*Candidatus* Allocryptoplasma spp.', and *Ehrlichia* spp. closely related to Ehrlichia sp. (strain Magellanica) and *Ehrlichia minasensis* were detected in birds from the Brazilian Pantanal [21]

The genus *Bartonella* (order Hyphomicrobiales) comprises fastidious, facultative intracellular alpha-proteobacteria that infect primarily erythrocytes and endothelial cells [22,23]. Its transmission is mainly mediated by arthropod vectors [24–33], scratches, bodily fluids and bites [23,34]. While *Bartonella henselae*, *Bartonella koehlerae* and *Bartonella tamiae* were detected in wild bird blood samples in the USA [35,36], *B. henselae* and *B*. *machadoae* were detected in wild birds in the Brazilian Pantanal [37]. [21] [37]

Avian haemosporidians, the most studied blood parasites of birds, are represented by three main protozoa genera, namely Haemoproteus, *Leucocytozoon* and *Plasmodium* [38], which are transmitted by blood sucking dipterans [38,39]. Haemosporidians can be found infecting birds worldwide, except in the Antarctica [40]. Although the occurrence and diversity of *Plasmodium* spp. and *Haemoproteus* spp. has been extensively studied in Brazil, the studies were mostly based on conventional PCR assays [6,20], which might have hampered the detection in birds presenting low parasitemia.

Filariids from the Onchocercidae family (subfamilies Dirofilariinae, Onchocercinae, Splendidofilariinae and Lemdaniinae), with a worldwide distribution [39,41], are mainly transmitted by Simuliidae, Culicidae and Ceratopogonidae dipterans. Although microfilariae from *Eulimdana* spp. were detected in blood samples from Charadriiform birds the United States, Canada and Iceland [42], and *Eufilaria delicata* and *Ornithofilaria mavis* microfilariae were detected in Passeriformes from Czech Republic [43], the majority of the studies reported unidentified filariids in avian blood samples [44–47].

Microfluidic technology offers a variety of advantages in biology, chemistry and medicine [48]. The microfluidic real-time PCR system has demonstrated to be more efficient on surveillance of neglected diseases-causing pathogens. To date, the microfluidic real time PCR has been used to detect a wide variety of tick-borne pathogens [49–51], such as *Anaplasma* spp., *Borrelia* spp., *Ehrlichia spp*., *Rickettsia* spp., *Babesia* spp., *Haemoproteus* spp., *Plasmodium* spp., Leucocytozoon spp., *Trypanosoma* spp., and *Leptospira* spp. in ticks associated to birds and/or avian populations in France [51,52]. Considering the advantages of microfluidic real-time PCR as its versatility to detect several microorganisms in different samples, such as feces [53], cheese samples [54] and soil [55], simultaneous pathogen detection in multiple independent assays, high sensitivity, low probability of contamination, and time saving when compared to conventional or real-time PCR assays [56], the present study aimed to detect different vector-borne agents (VBA) in avian blood samples from the Pantanal wetland, central-western Brazil.

2 Material and methods

2.1 Sampling

Between 07/04/2022 and 07/08/2022, 20 mist nets with a 36 mm mesh, 12 m long and 2.5 m of height were placed sideways the trails in the Pantanal region, in the municipalities of Mimoso (-16°12'41"S -55°48'31"W) and São Lourenço (-16° 44'33"S -55° 33'12"). The mist nets were opened instantly after dawn and checked every 30 minutes for bird removal. The nets were closed at 11h00 A.M. every day. Over 5 days, 300 wild birds were sampled. All the specimens were photographed, identified, and weighed. Each specimen was marked with non-toxic nail-polish and released afterwards. The sampling was approved by the Ethical Commission for the Use of Animal (CEUA 268-21) of the São Paulo State University (FCAV/UNESP). The blood samples were collected from the brachial vein using needles and heparinized capillary tubes. A droplet of blood was used to prepare blood smears for microscopic examination and the remaining blood samples were stored up on FTA cards for molecular analysis. Blood smears were stained with Rosenfeld staining and analyzed with an Olympus BX43 microscope and photographed with an Olympus camera model DP73 and software cellSens Standard v 1.14.

2.2 Molecular assays

2.2.1 DNA extraction and PCR assay for endogenous gene

DNA extraction from avian blood samples was performed using the Biopur Mini Spin Plus DNA extraction kit (Mobius Life Science®, Paraná, Brazil), following the manufacturer instructions. Blood samples on Whatman® FTA cards were pre-treated as indicated by de Sena Oliveira et al. (2018). DNA concentration, 260/280 and 260/230 ratios from the DNA blood samples were verified in a spectrophotometer (Nanodrop, Thermo Scientific®). DNA samples were stored at -20°C until their use in the PCR for endogenous avian β actin gene [58], qPCR and the High-throughput microfluidic real-time PCR assays.

2.2.2 Test of primer and probes sets with DNA from Brazilian strains of vector-borne agents (*Ehrlichia* spp., *Anaplasma* spp., *Candidatus* Allocryptoplasma spp., *Bartonella* spp., *Dirofilaria immitis*, and avian-associated onchocercid filariids).

Among the set of primers and probes used to detect vector-borne agents (VBA) selected for the present study, two primer/probe sets previously designed for *Anaplasma* spp. (16S rRNA) and *Bartonella* spp. (*ssr*A) [59] were used. In addition, three primer/probe sets were designed for the following VBA: Onchocercid filariids (LSU rRNA), *Plasmodium* spp (*cyt*B), and *Haemoproteus* spp. (*cyt*B). Primers and probes were designed by targeting conserved regions from the selected genes and aligning different sequences from the specific gene for each vector-borne agent.

The sets of primers and probes obtained from [49,51,59] and those designed in the present study were tested by qPCR assays with positive controls obtained from previous studies from our research group that detected *Bartonella* spp. and Anaplasmataceae agents in Brazilian tropical bird blood samples [21, 37]. *Dirofilaria immitis* DNA obtained from naturally infected dogs from Brazil (kindly provided by Dr. Norma Labarthe, Oswaldo Cruz Foundation – FIOCRUZ, Rio de Janeiro, RJ, Brazil) and blood DNA sample from a *Ramphocelus carbo* (MIM222) showing microfilariae in the blood smears were used as positive controls in the qPCR assays for filariids. *Haemoproteus* sp. DNA obtained from a naturally infected *Columba livia* from Brazil (data not shown) showing *Haemoproteus* spp. trophozoites in blood smears was used as a positive control in the qPCR assays for haemosporidians.

Real-time PCR assays were performed with the kit LightCycler®480 Probe Master Mix 1× (Roche Applied Science, Penzberg, Germany), using 200 nM of primers and probes, and 2 µL of DNA in a final volume of 12 µL. The thermal condition was as follows: 95 ◦C for 5 min, 45 cycles at 95 ◦C for 10 s and 60 ◦C for 15 s, and one final cooling cycle at 40 ◦C for 10 s.

2.2.3 DNA Pre-amplification for Microfluidic Real-Time PCR

Total DNA was pre-amplified to improve detection of the selected pathogens DNA. The Preamp Master Mix (Standard Biotools, CA, United States) was used according to manufacturer's instructions. The reaction was performed in a final volume of 5 µL containing 1 µL Preamp Master Mix, 1.25 µL pooled primer mix (200nM for each primer (**Supplementary Table 1**), 1.5 µL distilled water and 1.25 µL DNA. The thermocycling program consisted of one cycle at 95° C for 2 minutes, 14 cycles at 95° C for 15 s and 4 minutes at 60°C. At the end of the thermal cycles, the reactions were diluted 1:10 in Milli-Q ultrapure water. All the pre-amplified DNA samples were stored at -20° C until usage.

2.2.4 High-Throughput Microfluidic Real-Time PCR

To detect DNA from the selected vector-borne agents, the BioMarkTM real-time PCR system (Standard Biotools, USA) was used for High-Throughput microfluidic real-time PCR, using 48.48 dynamic arrays. The chips distributed 48 PCR assay (primers and probe) and 48 samples into single chambers. The on-chip microfluidic assembled PCR reactions in specific chambers previously to thermal cycling. Primers and probes used in the microfluidic real-time PCR assays are listed in **Supplementary Table 1.**

Amplifications were performed using 6-carboxyfluorescein (FAM) and black hole quencher (BHQ1) labeled Taqman® probes with Taqman® Gene expression master mix according to the manufacturer's instructions (Applied biosystems, France). The thermal cycling conditions comprised 2 minutes of 50°C, 10 minutes at 95°C, followed by 40 cycles of 2-step amplification of 15 seconds at 95°C and 1 minute at 60°C. Data was collected on the BioMarkTM Real-Time PCR System and analyzed using the Fluidigm Real-Time PCR Analysis software to obtain crossing point (CP) values. Samples were run; one negative water control was included per chip, and one control for PCR inhibition using *E. coli* DNA.

2.2.5 Validation by PCR and Sanger sequencing

Conventional PCR assays using primers targeting molecular markers distinct from those of the BioMarkTM system were used to confirm the presence of the selected VBA DNA in bird blood samples **(Table 1).** For validation of the microfluidic real-time PCR assays, only samples showing low Cq values (< 30) were subjected to conventional PCR assays. Amplicons were sequenced by Eurofins MWG Operon (Germany) and then edited with BioEdit v7.2.5 software [62]. BLASTn [63] the obtained sequences with those previously deposited in GenBank database.

Agents	**Sequences (5'-3')**	**Size (bp)**	**Molecular marker**	**Reference**
Anaplasma spp./ *Ehrlichia* spp.	EHR1 GAACGAACGCTGGCGGCAAGC EHR2 AGTA(T/C) CG(A/G) ACCAGATAGCCGC EHR3 TGCATAGGAATCTACCTAGTAG EHR2 AGTA(T/C) CG(A/G) ACCAGATAGCCGC	693 592	16S rRNA	[62]
Anaplasma spp.	GE2F2 GTTAGTGGCAGACGGGTGAGT AE4-Fw GTACCYAYAGAAGAAGTCCCGGCA AE-Rv RCACCAGCTTCGAGTTAAGCCAAT	800	16S rRNA	[63–65]
Anaplasma spp.	ITSiF ATACCTCTGGTGTACCAGTTG	300	ITS 23S-5S	[66]

	ITSiR TTAACTTCCGGGTTCGGAATG			
Bartonella spp.	P-bhenfa TCTTCGTTTCTCTTTCTTCA P-benr1 CAAGCGCGCGCTCTAACC N-bhenf1a GATGATCCCAAGCCTTCTGGC N-bhenr AACCAACTGAGCTACAAGCC	Depends on the species	16S-23S rRNA (ITS)	[67]
	BhCS.781p F GGGGACCAGCTCATGGTGG BhCS.1137n R AATGCAAAAAGAACAGTAAACA	380-400	*glt*A	[68]
	CS443f GCTATGTCTGCATTCTATCA CS1210r GATCYTCAATCATTTCTTTCCA	767	*glt*A	[69]
Bartonella spp.	ftsZ F CATATGGTTTTCATTACTGCYGGTATGG ftsZ R TTCTTCGCGAATACGATTAGCAGCTTC	515	*fts*Z	[70]
	groEL F GGAAAAAGTGGGCAATGAAG groEL R TCCTTTAACGGTCAACGCATT	752	*gro*EL	[70]
	nuoG F GGCGTGATTGTTCTCGTTA	346	*nuo*G	[71]

	nuoG R CACGACCACGGCTATCAAT			
	165s GACTTCTGTTATCGCTTTGATTT 688as CACCACCAGCAAMATAAGGCAT	564	*pap*31	[72]
	1400F CGCATTGGCTTACTTCGTATG 2300R GTAGACTGATTAGAACGCTG	825	*rpoB*	[73]
	Barton-1 TAACCGATATTGGTTGTGTTGAAG Barton-2 TAAAGCTAGAAAGTCTGGCAACATAACG	585-588	*ribC*	[74]
	321s AGATGATGATCCCAAGCCTTCTGGCG 938as TGTTCTYACAACAATGATGATG	453-717	16S-23S rRNA (ITS)	[75]
Haemosporida (*Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp.)	HaemNFI-cytB-F CATATATTAAGAGAAITATGGAG HaemNR3-cytB-R ATAGAAAGATAAGAAATACCATTC HaemF-cytB ATGGTGCTTTCGATATATGCATG HaemR2-CYTb GCATTATCTGGATGTGATAATGGT	660 480 478	*cytB*	[76]

	HaemFL-cytB ATGGTGTTTTAGATACTTACATT HaemR2L-cytB CATTATCTGGATGAGATAATGGIGC			
Filariids	NTF-coxF TGATTGGTGGTTTTGGTAA NTR-coxR ATAAGTACGAGTATCAATATC	650	*cox-1*	[77]
Onchocercidae	988F CTCAAAGATTAAGCCATGC 1912R TTTACGGTCAGAACTAGGG	998	12S rRNA	[78]
Onchocercidae	Nematode 1 GCGGAGGAAAAGAAACTAA Nematode 2 ATCCGTGTTTCAAGACGGG	855	28S rRNA	[78]

2.2.6 Phylogenetic analsis

The obtained sequences were aligned via MAFFT [81] with homologous sequences retrieved from GenBank database. The alignment saved as “FASTA” was used to infer the evolutionary model. Phylogenetic inferences were performed using IQtree online version (http://iqtree.cibiv.univie.ac.at/) [82]. Clade supports for Maximum likelihood analyzes (ML) were evaluated using bootstraps analyses (Felsenstein, 1985) of 100 repetitions. The phylogenetic tree was edited using Figtree V1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) [83].

3 Results

3.1 DNA extraction and PCR assay for avian *β actin* endogenous gene

The extracted DNA samples presented an average concentration of 7.78 ng/uL and ratios 260/280 of 3.10 and 260/230 of -0.96. In the PCR assay, 275/282 (97.5%) avian DNA blood samples showed positive result for avian β-actin gene. Negative samples in the β-actin gene-based PCR assay were excluded for the subsequent molecular analyses.

3.2. Testing of primer and probe sets designed for microfluidic real-time PCR by qPCR assay

The designed primers/probe sets previously designed for Anaplasma spp. (16S rRNA) and Bartonella spp. (ssrA) [59] were capable to detect genotypes of Anaplasma spp./'Candidatus Allocryptoplasma spp.', and Bartonella spp. previously detected in avian blood DNA samples from Brazil [21, 37]. Then they were conserved to screen Brazilian samples through high-throughput microfluidic real-time PCR system.

When testing the positive samples previously obtained from bird blood samples from Brazil, five (100%) out of five samples showed positive results in the qPCR targeting the 16S rRNA for Anaplasma spp./'Candidatus Allocryptoplasma spp'. When targeting the ssrA gene for Bartonella spp., three (60%) out of five samples showed positive results.

3.3. Primer and probe set testing in High-Throughput Microfluidic Real-Time PCR assay

The following primers and probes were used in High-Throughput Microfluidic Real-Time PCR assay:

i.) designed in previous studies for: *Anaplasma* spp. (16S rRNA), *Bartonella* spp. (ssrA), Apicomplexa (18S rRNA), *Aegyptianella pullorum* (groEL), *Rickettsia* spp.

(gltA), *Rickettsia africae* (sca1), *B. vinsonii berkhoffii* (16S-23S rRNA ITS) and *Hepatozoon* spp. (18S rRNA) [59] *Borrelia* spp. (23S rRNA), *Rickettsia rickettsii* (23S-5S rRNA ITS), *Borrelia burgdorferi* s.s. (rpoB), *Borrelia garinii* (rpoB), *Borrelia valaisiana* (ospA), *B. henselae* (pap-31), *A. phagocytophilum* (msp2), *E*. *chaffeensis* (dsb), *Rickettsia massiliae* (23S-5S rRNA ITS), Spotted Fever Group Rickettsia spp. (gltA), *Babesia vogeli* (hsp70) [49] *Trypanosoma* spp. (18S rRNA) [51].

ii.) designed in the present study for: *Ehrlichia* spp. (groEL), *Borrelia* spp. (flaB), *Trypanosoma* spp. (18S rRNA), *Plasmodium* spp. (cytB), *Haemoproteus* spp. (cytB), *Leucocytozoon* spp (ssRNA), *Aproctella* spp. (LSU rRNA), *Chandlerella* spp. (cox1), *Eufilaria* spp (LSU rRNA).

When testing the 36 positive samples from bird blood samples from Brazil obtained in previous studies [21, 37],11 (30.5%) out of 36 showed positive results to different VBA in High-Throughput Microfluidic Real-Time PCR assay **(Supplementary Material Table SM2)**. Out of the 13 positive controls for Anaplasma spp./'Candidatus Allocryptoplasma spp.' (obtained by our research group in previous studies [21], five (38.5%) showed positive results in the microfluidic real-time PCR using primers previously described by Gondard [59]. Out of the nine positive controls for Bartonella spp. (obtained by our research group in previous studies – [37], four (44.4%) showed positive results in the microfluidic real-time PCR using primers previously described by Gondard [59]. The primers/probe sets designed in previous studies ([59] were able to amplify *Anaplasma* spp. '*Candidatus* Allocryptoplasma spp.', *Bartonella* spp. and Bartonella machadoae.

All samples were negative in microfluidic real-time PCR assays for the remaining vector-borne agents tested.

3.4 High-Throughput Microfluidic Real-Time PCR assay

The molecular occurrence for the selected VBA was as following: 18.2% (50/275) for *Anaplasma* spp., 0.36% (1/275) for *Bartonella* spp., 6.2 % (17/275) for *Plasmodium* spp., 5.09 % (14/275) for *Haemoproteus* spp., and 6.5% (18/275) for Onchocercid filariids.

The following single infections were observed (**Supplementary Material Table SM2**) in the microfluidic real-time PCR assays: *Anaplasma* spp. (n=35), *Bartonella* spp. (n=1), *Plasmodium* spp. (n=10), *Haemoproteus* spp. (n=8), and Onchocercidae filariids (n=8). The following co-infections were observed in the microfluidic real-time PCR assays: *Anaplasma* spp. + *filariids* = 9; *Anaplasma* spp. + *Plasmodium* spp. = 3; *Anaplasma* spp. + *Haemoproteus* spp. = 3; *Plasmodium* spp. + *Haemoproteus* spp. = 3; *Plasmodium* spp. + Onchocercidae filariids =1 **(Supplementary Material Table SM3**).

3.5 Validation of microfluidic real-time PCR results by conventional PCR assays and Sanger sequencing

Out of 50 positive samples for Anaplasma spp. in the microfluidic real-time PCR, 13 (26%) (SALOU4, SALOU15, SALOU61, SALOU82, SALOU83, SALOU93, SALOU94, SALOU103, SALOU115, SALOU125, SALOU126, MIM176, MIM243) samples were subjected to PCR assays targeting the 16S rRNA gene and 23S-5S intergenic region (ITS). One sample (*Eucometis pennicillata*) was positive (SALOU82 Genbank accession number PQ450485) showing 100% identity and a query cover of 90% with *Anaplasma* spp. (PP417936) previously detected in *Basileuterus flaveolus* from Brazil.

Out of 30 samples positive for hemosporidians (*Haemoproteus* spp. and *Plasmodium* spp.), 12 (38.7%) (Saolou48, MIM154, MIM218, MIM230, MIM236, MIM242, MIM243, MIM250, MIM289, MIM292, MIM296, MIM298) were subjected to a PCR targeting the cytB gene for *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp. As a result, five samples were positive, and two readable sequences were obtained: while sample MIM250 (*Columbina squammata*) (PQ450488) showed a 100% identity (100% query cover) with *Haemoproteus* spp. (KP686107), sample MIM292 (*Volatinia jacarina*) (PQ450487) showed 100% identity (97% query cover) with *Plasmodium* spp. (KY304999)

Out of 23 samples positive for Onchocercidae filariids, 15 (65.2%) (Saolou30, Saolou32, Saolou41, Saolou55, Saolou67, Saolou81, Saolou85, Saolou94, Saolou108, Saolou114, Saolou126, 179, 420, 441m and 497) were subjected to PCR

assays targeting the cox-1, 12S RNA and 28S rRNA genes. As a result, 10 samples were positive in the cox-1-based PCR, and nine sequences were obtained. Samples Saolou30 (*Ramphocelus carbo*) (PQ452771), Saolou32 (*Ramphocelus carbo*) (PQ452776), Saolou41 (*Ramphocelus carbo*) (PQ452777), Saolou55 (*Ramphocelus carbo*) (PQ452778), Saolou67 (*Turdus amaurochalinus*) (PQ452774), Saolou114 (*Synallaxis albilora*) (PQ452775) and Saolou126 (*Ramphocelus carbo*) (PQ452772) showed 97.8 - 100% identity (100% query cover) with *Aproctella* spp. (FR823331). The sample Saolou94 (*Ramphocelus carbo*) (PQ452773) showed 92.2% identity (99% of query cover) with *Eufilaria sylviae* (MT800771).

Although one blood sample from *Fomicivora rufa* was positive in the microfluidic PCR, no readable sequences were obtained in the PCR assays based on the *Bartonella* 16-23S rRNA (ITS), *glt*A, *gro*EL, *fts*Z, *nuo*G, *rib*C*, pap*31 and *rpo*B.

4 Phylogenetic analyses

The phylogenetic analysis based on the cytB gene (alignment of 328 bp) of haemosporidians inferred by Maximum likelihood method and GTR+F+I+G4 evolutionary model showed two major clades: (i) the avian related *Plasmodium* species clade, in which sequence of *Plasmodium* spp. detected in the present study (Genbank accession number PQ450487) was positioned within a clade containing sequences of

Plasmodium tejerai from Colombia (OP087638) and Brazil (KJ57552); and (ii) the *Haemoproteus* species clade, in which the sequence from the present study (Genbank accession number PQ450488) was positioned with *Haemoproteus columbae* sequences from Indonesia (LC606013, LC606008), Botswana (AF495554), Japan (LC647343) and Brazil (KU131583) (**Figure 1**).

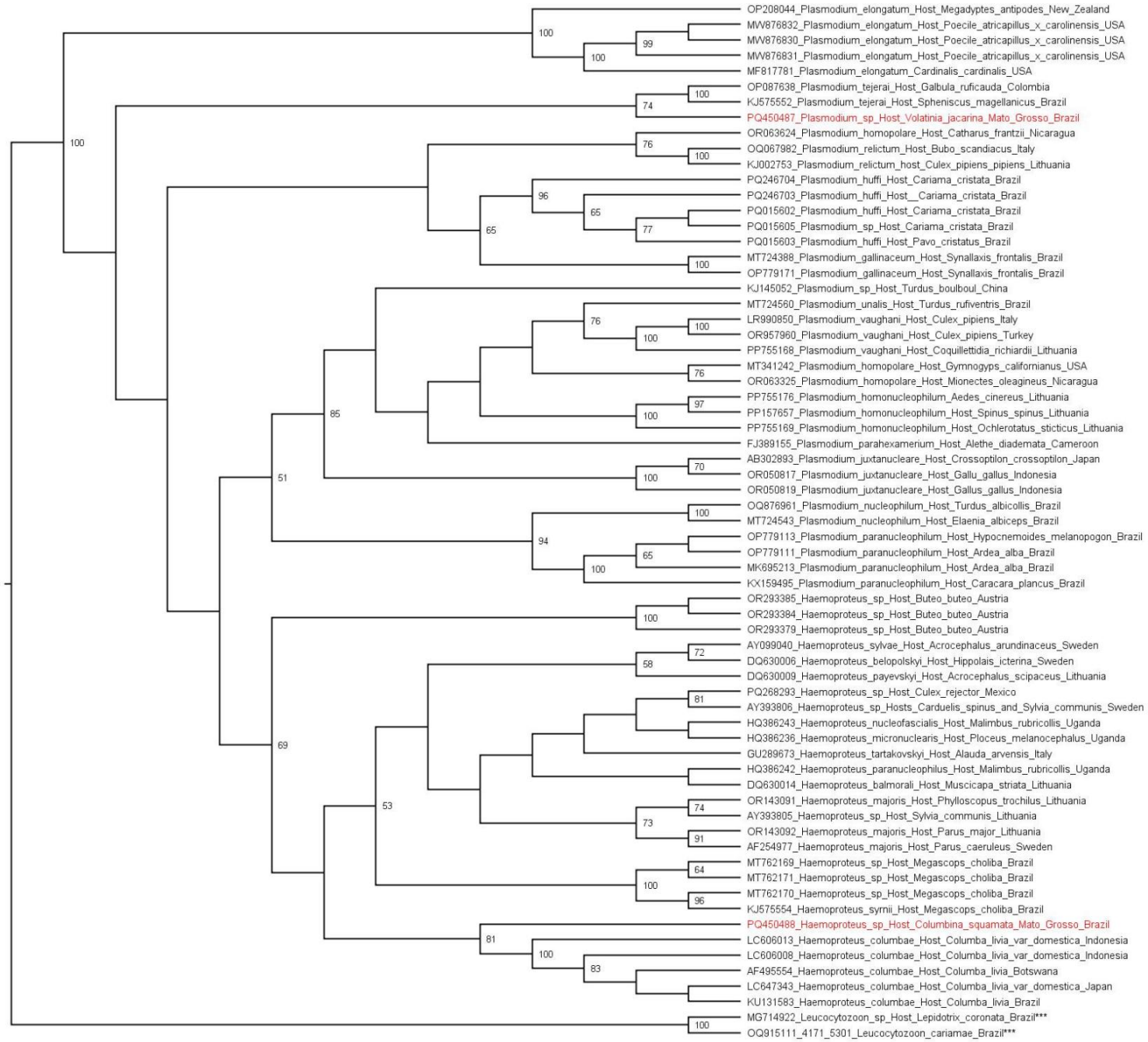


Figure 1. Phylogenetic analysis inferred by Maximum Likelihood method (GTR+F+I+G4 evolutionary model) based on a 328 bp alignment of the cytB gene containing 67 haemosporidian homologous sequences (*Plasmodium* species and *Haemoproteus* species). *Leucocytozoon* species (MG714922, MG209789 and OQ915111) were used as outgroups. Sequences obtained in present study are highlighted in red and the outgroups are indicated with "***". Only the bootstraps with values of 50 or more were shown.

The phylogenetic analyses based on the cox-1 gene of onchocercid filariids (alignment of 673 bp) inferred by Maximum likelihood method and TIM3+F+I+G4 evolutionary model showed seven different clades: (i) the first clade positioned all Dirofilarinae sequences; (ii) the second clade grouped *Splendidofilaria* species sequences; (iii) the third clade contained *Eufilaria* species sequences; (iv) the fourth clade contained a sequence of the present study obtained from *Ramphocelus carbo* (PQ452773) from Sao Lourenço, Mato Grosso, as an ancestral branch to *Eufilaria* and *Splendidofilaria*, (v) the fifth clade composed by *Aproctella* species from Madagascar (OP006590), USA (OK360722) and Brazil (FR823335). The majority of the sequences obtained in the present study were positioned within *Aproctella* clade, namely PQ452778 [*Ramphocelus carbo*], PQ452774 [*Turdus amaurocalinus*], PQ452777 [*Ramphocelus carbo*], PQ452776 [*Ramphocelus carbo*], PQ452771 [*Ramphocelus carbo*], PQ4552772 [*Ramphocelus carbo*] and PQ452775 [*Synallaxis albilora*]), which were closely related to a sequence previously detected in *Saltator similis* from Brazil (FR823335) (**Figure 2**).

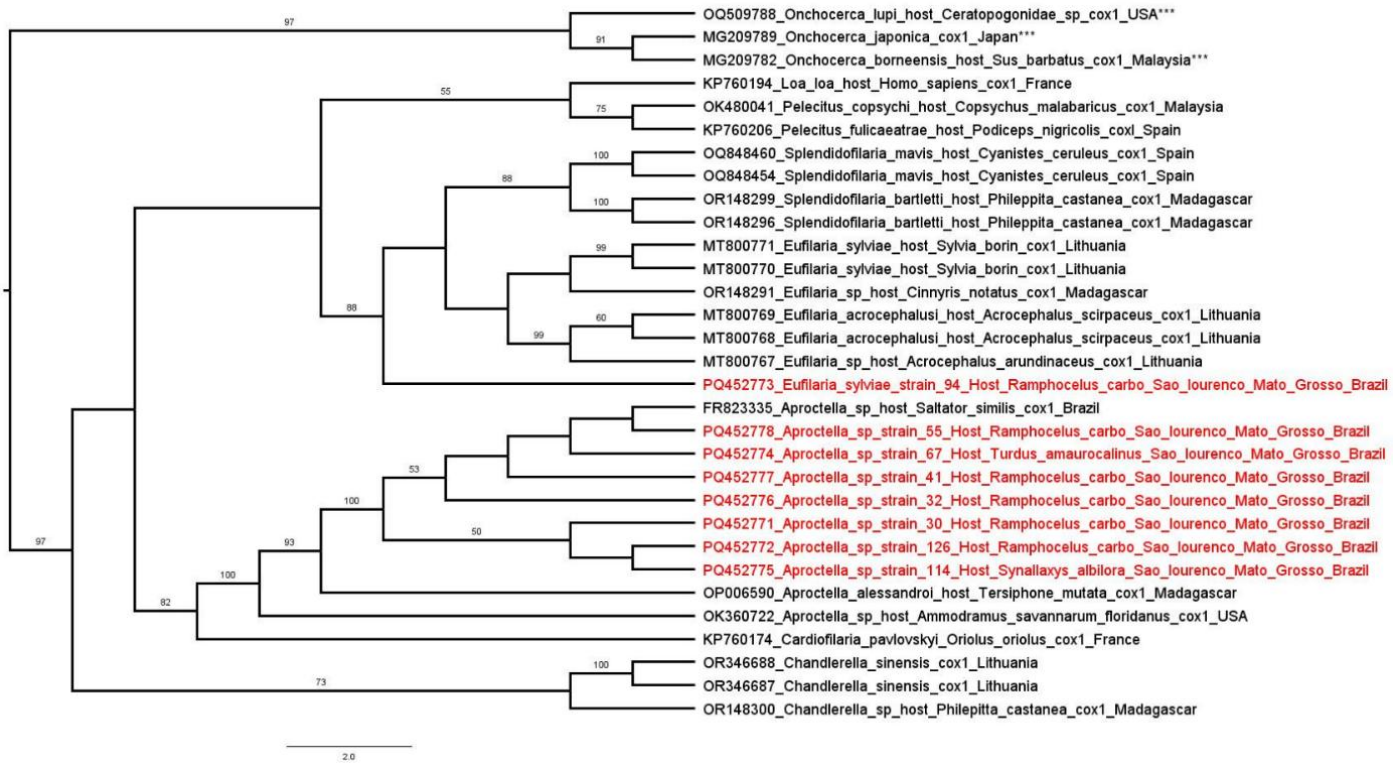
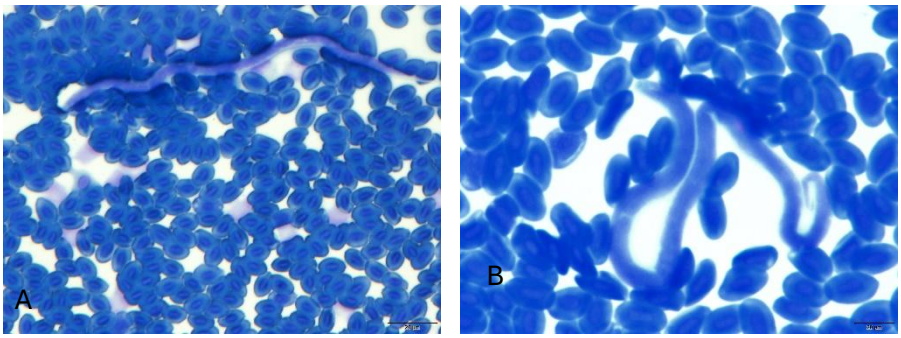


figure 2 Phylogenetic analysis inferred by maximum likelihood method (TIM3+F+I+G4) evolutionary model) based on a 673 bp alignment of the cox1 gene of 31 onchocercid filariid homologous sequences (*Aproctella*, *Eufilaria*, *Chandlerella*, *Pelecitus, Cardiofilaria, Splendidofilaria, Pelecitus* and *Loa loa*). Onchocerca species (MG209782, MG209789 and OQ509788) were used as outgroups. Sequences obtained in present study are highlighted in red and the outgroups with "***". Only the bootstraps with values of 50 or more were shown.

When examining the blood smears of the positive samples for onchfilariids and haemosporidians, microfilariae were found in blood smears from two specimens of *R. carbo* (SaoLou 32 and SaoLou 41) that showed to be positive for *Aproctella* spp. Microfilariae were also found in the blood smear of one specimen (SaoLou85) of *Saltator coerulescens* that was positive in the microfluidic real-time PCR but negative in the conventional PCR targeting the *cox*-1 gene (**Supplementary Material Figure SM3**).



Supplementary material Figure 3. Microfilaremia detected in avian sample blood smears. A) SaoLou32 and B) SaoLou41 were identified as *Aproctella* spp. due to sequencing and phylogenetic inference of the cox-1 gene.

5 Discussion

To the best of authors' knowledge, this is the first time that High-Throughput Microfluidic Real-Time PCR assay was applied to detect vector-borne pathogens DNA in avian blood samples collected in the Americas. In the present study, multiple primers and probes sets were designed to be implemented for the detection of vector-borne agents (*Plasmodium* spp., *Haemoproteus* spp., Onchocercid filariids) in DNA blood samples from birds sampled in the Pantanal wetland, Mato Grosso state, central-western Brazil. In previous studies, High-Throughput Microfluidic Real-Time was used to detect DNA from selected pathogens in ticks associated to birds and avian blood samples from France [49–51]. The use of this technique ensures multiple independent assays [56] and efficiency in the surveillance of neglected pathogens [49–51].

Primers and probes designed in previous studies [59]and targeting the 16S rRNA gene of *Anaplasma* agents and *ssr*A genes of *Bartonella* spp. agents were able to detect DNA of Anaplasmataceae/Bartonellaceae agents in bird blood DNA samples from Brazil that showed to be previously positive for *Anaplasma* spp./'*Candidatus* Allocryptoplasma spp.', *Bartonella* spp. [21, 37]. Indeed, although the previously described primers were designed to catch the common genotypes circulating in Europe, which are genetically distinct from those found

in birds and wildlife from Brazil, they were able to catch some novel *Bartonella* and *Anaplasma*/Allocrytoplasma spp. recently reported in Brazil.

While microfluidic real-time PCR revealed a positivity rate of 18.2% for Anaplasma/Allocryptoplasma spp. among birds sampled in the Pantanal of Mato Grosso state, our previous study performed in the states of Mato Grosso and Mato Grosso do Sul reported an occurrence of 7.9% using both real-time quantitative (q)PCR and conventional PCR assays [60,61]. One Anaplasma spp. sp. 23S-5S rRNA (ITS) sequence showed 100% identity and a query cover of 90% with *Anaplasma* spp. previously detected in *B. flaveolus* from Brazil. This finding validates the specificity of the primers/probe used for *Anaplasma* spp. previously designed by Gondard [59], showing that they can catch *Anaplasma* species that so far have only been detected in Brazil [21, 37].

The topology of phylogenetic analysis based on the haemosporidians cytB gene performed herein corroborated with previously reported [6,83,84], displaying the separation between *Plasmodium* and *Haemoproteus* species. While the *Plasmodium* spp. *cyt*B sequence detected in a specimen of *V. jacarina* clustered with *Plasmodium tejerai*, the *Haemoproteus* spp. *cyt*B sequence detected in a specimen of *C. squamata* clustered with *Haemoproteus columbae* obtained from other Columbiformes. These findings demonstrated that the primers/probes designed herein are able to catch *Haemoproteus*/*Plasmodium* lineages from Brazil [83, 84]

The topology of phylogenetic analysis based on the cox-1 gene of onchocercid filariids presented herein showed similar results to those presented by Binkienė and Hayashi [79, 86]. The Onchocercidae filariid cox-1 sequence detected in a specimen of *Ramphocelus carbo* showed to be ancestral to the clade comprising *Splendidofilaria* spp. and *Eufilaria* spp. On the other hand, Onchocercidae filariid cox-1 sequences detected in specimens of *R. carbo*, *T. amaurocalinus* and *S. albilora* grouped with *Aproctella* spp. Therefore, the primers and probe designed in the present study were able to detect at least two distinct avian-associated Onchocercidae filariid species. Unfortunately, no

readable sequence was obtained from positive samples for Bartonella spp., precluding assessing the *Bartonella* species detected in birds sampled in this study. This finding could be related to the expected low *Bartonella* bacteraemia in birds. Instead, a single sequence of *Bartonella machadoae* was obtained from a *F. rufa* blood sample used as a positive control [21] (data not shown) (accession number PV083549).

6 Conclusion

High-Throughput microfluidic real-time PCR assay based on previous designed primers for Europe and the Caribbean regions was able to detect several positive samples to vector-borne agents from present study samples, suggesting the field samples from different regions should be tested first with the primers designed for the European and Caribbean regions and in case of variability due to different genotypes new primers and probes should be designed. High-Throughput microfluidic real-time PCR assay can be used to screen *Anaplasma* spp., *Bartonella* spp., *Plasmodium* spp., *Haemoproteus* spp., and Onchocercidae filariids associated with avian hosts from South America.

Ethics Statement

The sampling was accredited by the Ethical commission for the use of animal (CEUA 3241/122) of the São Paulo State University (UNESP) and The "*Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBIO)" for the birds collection and sampling (SISBIO# 83622-1).

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Data linking

The sequences generated and analysed during the present study were submitted in the NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences can be accessed by the following accession numbers: PQ450485, PQ450488, PQ450487, PQ452771, PQ452776, PQ452777, PQ452778, PQ452774, PQ452775, PQ452772, FR823331, PQ452773 and PV083549

Authors contributions

ASAC: Methodology, Validation, Investigation, Data curation, Writing-Original Draft, Visualization, Writing- original draft, Writing -review and editing; **JBP:** Methodology, Resources, Writing -review and editing; **AGP:** Methodology; Writing – review & editing; **CG:** Methodology, Writing – review & editing; **TVF:** Methodology; Writing – review & editing; **LFN:** Methodology, Writing – review & editing; **RZM:** Resources, Writing – review & editing; **SM:** Conceptualization, Funding acquisition, Resourcers, Supervision, Writing - review and editing; **MRA:** Conceptualization, Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interests

The authors declare there is no conflict of interest that could have influenced the results obtained and reported in this research.

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Chapter 6. Final considerations

The present work showed that tropical birds from Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul can be parasitized by a variety of vector-borne pathogens.

When referring to Anaplasmataceae agents, *Anaplasma* spp. and '*Candidatus* Allocryptoplasma spp.' showed a higher occurrence when compared to *Ehrlichia* spp. The *Ehrlichia* genotypes detected herein were closely related to *Ehrlichia minasensis* and *Ehrlichia* sp. Magellanica. This finding of new genotypes of Anaplasmataceae agents in birds from Pantanal wetland suggests that the genetic diversity is richer than previously thought and that vectors parasitizing birds may play an important role in the transmission of such agents. The isolation of avian-associated Anaplasmataceae agents from wild bird blood samples and involved tick vectors should be considered, aiming at fully characterizing these novel agents. Additionally, primers targeting distinct molecular markers should be designed in order to improve the detection and characterization of avian-associated Anaplasmataceae agents. Several primers previously designed and used in the literature to detect and characterize these agents in mammal biological samples generated nonspecific amplification of the avian hosts DNA in the present study (data not shown).

When referring to *Bartonella*, sequences closely related to *B. henselae* and *B. machadoae* were detected in the sampled birds. Previously, *Bartonella vinsonii berkhoffii,* which is known to infect canids, was detected in bird blood samples and associated fleas. This also could be happening in the Pantanal wetland, where a close relationship among arthropod vectors, birds and mammals may favor an enzootic cycle for *Bartonella* species detected in present study. This finding emphasizes the need for additional studies to unveil the role of birds and associated arthropod vectors in the *Bartonella* spp. epidemiology. Future studies should be performed using more sensitive approaches, such as digital PCR, in both the avian hosts and the arthropod parasitizing these birds, as well as in arthropods present in avian nests. Although not yet performed in the literature, *Bartonella* culturing attempts from avian blood samples are encouraged.

Unfortunately, *Borrelia* spp. was detected only in qPCR assay, making it impossible to characterize through conventional PCR assays and sequencing, possibly due to a very low bacteremia. Nonetheless, this finding shows, for the first time, that *Borrelia* spp. is circulating in wild birds from Pantanal. Isolation of *Borrelia* spp. from avian blood and associated ticks is much needed to fully characterize the strains circulating in Neotropical birds.

Distinct onchocercid filariids were found infecting birds in the present study. It is imperative the deposit of more representative sequences of several molecular makers in GenBank, due to the scarcity of avian-associated onchocercid sequences. Alternatively, future studies should collect avian blood samples at night as previously recommended by a previous study carried out in the Unites States. DNA extraction from buffy coat is also encouraged aiming at gaining higher sensitivity. To fully characterized the avian-associated filariids, blood smears and necropsy to collect adult filariid stages are needed to effectively identify the genus and species involved.

The High-throughput microfluidic real time PCR assay was shown to be very useful to detect a variety of vector-borne agents in a sole reaction. If the assay works as a screening method, the results should be further confirmed by conventional PCR and sequencing. It turns out to be highly efficient when the primers and probe sets are well designed for the agents of interest or able to detect unknown genotypes in the samples. In the present work, previous designed sets could detect some genotypes previously detected in wild birds from Brazil. However, in order to allow an improved detection of *Bartonella*, *Anaplasma* and *Ehrlichia* in blood samples from Neotropical birds, primers and probe sets should be designed specifically for genotypes of these vector-borne pathogens circulating in Brazil. Additionally, new sets of primers and probes were designed herein to detect onchocercid filariids and haemosporidians (*Haemoproteus* and *Plasmodium*). The design of primers/probes could be limited by the availability of representative sequences of distinct molecular markers deposited in the GenBank database.

Appendices

Appendices Chapter 3. Novel tick-borne Anaplasmataceae genotypes in tropical birds from the Brazilian Pantanal wetland.

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Table S1. Number of avian species collected in Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul, central-western Brazil.

Scientific name	Common name	Number of sampled birds
Agelaioides badius	Grayish baywing	5
Agelasticus cyanopus	Unicolored blackbird	3
Antilophia galeata	Helmeted manakin	1
Arremon flavirostris	Saffron-billed sparrow	7
Arundinicola leucocephala	White-headed marsh tyrant	4
Basileuterus flaveolus	Flavescent warbler	23
Basileuterus hypoleucus	White-bellied warbler	4
Cacicus cela	Yellow-rumped cacique	10
Cacicus solitarius	Solitary cacique	2
Campylorhynchus turdinus	Thrush-like wren	2
Cantorchilus leucotis	Buff-breasted wren	6
Casiornis rufus	Rufous casiornis	1
Cercomacra melanaria	Mato grosso antbird	9

Certhiaxis cinnamomeus	Yellow-chinned spine tail	18
Cnemotriccus fuscatus	Fuscous flycatcher	5
Coereba flaveola	Bananaquit	4
Conirostrum speciosum	Chesnut-vented conebill	1
Coryphospingus cucullatus	Red-crested finch	1
Cranioleuca vulpine	Rusty-backed spinetail	3
Cyanocorax chrysops	Plush-crested jay	1
Cyanocorax cyanomelas	Purplish jay	3
Cyclarhis gujanensis	Rufous-browed peppershrike	1
Dendroplex picus	Straight-billed woodcreeper	6
Donacobius atricapilla	Black-capped donacobius	5
Elaenia albiceps	White-crested elaenia	2
Elaenia spectabilis	Large elaenia	1
Eucometis penicillata	Gray-headed tanager	5
Euscarthmus meloryphus	Fulvous-crowned scrub tyrant	3
Fluvicola albiventer	Black-backed water tyrant	8
Furnarius leucopus	Pale-legged hornero	13
Furnarius rufus	Rufous hornero	20
Hemitriccus margaritaceiventer	Pearly-vented Tody-tyrant	1
Hemitriccus striaticollis	Stripe-necked Tody-tyrant	5
Herpsilochmus longirostris	Large-billed antwren	1
Hylophilus pectoralis	Ashy-headed greenlet	3

Hypocnemoides maculicauda	Band-tailed antbird	7
Icterus cayanensis	Epaulet oriole	2
Icterus croconotus	Orange-backed troupial	1
Legatus leucophaius	Piratic flycatcher	4
Lepidocolaptes angustirostris	Narrow-billed woodcreeper	1
Machetornis rixosa	Cattle tyrant	5
Molothrus oryzivorus	Giant cowbird	1
Myiarchus ferox	Short-crested flycatcher	6
Myiophobus fasciatus	Bran-colored flycatcher	3
Myiozetetes cayanensis	Rusty-margined flycatchter	5
Paroaria capitata	Yellow-billed cardinal	36
Pipra fasciicauda	Band-tailed manakin	7
Pitangus sulphuratus	Great kiskadee	25
Poecilotriccus latirostris	Rusty-fronted Tody-flycatcher	8
Progne tapera	Brown-chested martin	1
Pseudoseisura unirufa	Grey-crested cacholote	8
Ramphocelus carbo	Silver-beaked tanager	101
Saltator coerulescens	Bluish-gray saltator	18
Sicalis flaveola	Saffron finch	2
Sporophila angolensis	Chesnut-belliend finch	9
Sporophila coerulescens	Double-collared seedeater	1
Sporophila collaris	Rusty-collared seedeater	11
Sporophila lineola	Lined seedeater	1

Stelgidopteryx ruficollis	Southern roughed-wing swallow	3
Synallaxis albilora	White-lored spinetail	10
Taraba major	Great antshrike	4
Thraupis palmarum	Palm tanager	1
Thraupis sayaca	Sayaca tanager	3
Thryothorus genibarbis	Moustached wren	1
Todirostrum cinereum	Common-Tody flycatcher	2
Turdus amaurochalinus	Creamy-bellied thrush	3
Turdus hauxwelli	Hauxwell's thrush	1
Turdus leucomelas	Pale-breasted thrush	8
Turdus rufiventris	Rufous-bellied thrush	7
Tyrannus melancholicus	Tropical kingbird	3
Vireo olivaceus	red-eyed vireo	1
Volatinia jacarina	Blue-black grassquit	3
Total		500

Table S2. Conventional, nested and quantitative real-time PCR assays used in screening and molecular characterization for Anaplasmataceae agents targeting the 16S RNA, *gro*EL, *dsb*, *glt*A, *sod*B, *omp*-1, *rpo*B, *fts*Z, and *suc*A genes and intergenic region 23S-5S (ITS).

Agent	Target gene	Primer sequences (5'-3')	PCR product size (pb)	Thermal protocol	Reference
***Ehrlichia* spp./ *Anaplasma* spp.**	16S rRNA	EHR16SD GGTCCYACAGAAGTCC EHRSR TAGCACTCATCGTTTACAGC	345	95°C for 5 minutes; 40 cycles: 94°C for 1 minute, 54°C for 30 seconds and 72°C for 30 seconds and final extension 72°C for 5 minutes	(Parola et al., 2000)
***Ehrlichia* spp.**	*dsb*	dsb-330 GATGATGTCTGAAGATATGAAA CAAAT dsb-728 CTGCTCGTCTATTTTACTTCTTA AAGT	409	95°C for 2 minutes; 50 cycles: 95°C for 15 seconds, 58°C for 30 and 72°C for 30 seconds and final extension 72°C for 5 minutes	(Doyle et al., 2005)
***Anaplasma* spp.**	16S rRNA	AnaplsppF, AGAAGAAGTCCCGGCAAACT AnaplR 3GAGACGACTTTTACGGATTAG CTC	800	94°C for 3 minutes; 30 cycles: 94°C for 30 seconds, 50°C for 30 and 72°C for 1 minute and final extension 72°C for 10 minutes	[50]
***Anaplasma* spp./*Ehrlichia* spp.**	16S rRNA	AE1-F AAGCTTAACACATGCAAGTCGA A AE1-R AGTCACTGACCCAACCTTAAAT G	1406	94°C for 3 minutes; 35 cycles: 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 1 minute and final extension 72°C for 5 minutes	[20]
***Ehrlichia* spp.**	*omp-1*	conP28-F1 AT(C/T)AGTG(G/C)AAA(AG)TA(T/C)(A/G)T (G/A)CCAA conP28-R1 TTA(G/A)AA(A/G)G(C/T)AAA(C/T)CT(T/G)CCTCC conP28-F2 CAATGG(A/G)(T/A)GG(T/C)CC(A/C)AGA (A/G)TAG conP28-R2 TTCC(T/C)TG (A/G)TA(A/G)G(A/C)AA(T/G)TTTAGG	700	94°C for 3 minutes; 30 cycles: 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute and final extension 72°C for 5 minutes	[49]
***Ehrlichia* spp.**	*sodB*	sodbEhr1600-F ATGTTTACTTTACCTGAACTTCC ATATC sodbEhrl600-R ATCTTTGAGCTGCAAAATCCCAA TT	600	94°C for 3 minutes; 55 cycles: 94°C for 10 seconds, 58°C for 10 seconds and 72°C for 15 seconds, extension 72°C for 30 seconds and final extension 72°C for 5 minutes	[48]

***Anaplasma* spp./*Ehrlichia* spp.**	*gltA*	F4b CCGGGTTTTATGTCTACTGC Rb1 CGATGACCAAAACCCAT EHR-CS136F TTYATGTCYACTGCTGCKTG EHR-778R GCNCCMCCATGMGCTGG	800 650	95°C for 5 minutes; 40 cycles: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minutes and final extension 72°C for 10 minutes 95°C for 5 minutes; 34 cycles: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minutes and final extension 72°C for 10 minutes	[47]
***Ehrlichia* spp.**	16S rRNA	Eh_16S21F1 GGCTCAGAACGAACGCTGG Eh-16S 1494 R1 AGCCGCAGGTTCACCTACA EH 16S 31F2 GAACGCTGGCGGCAAGCC EH 16S 1467 R2 GTTACGACTTCACCMTAGTCA	1474 1437	94°C for 3 minutes; 45 cycles: 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute and final extension 72°C for 10 minutes. * Same thermal conditions for the second reaction.	[46]
***Ehrlichia* spp.**	*gltA*	Eh_gltA112 F1 GGRRTRTTAACTTATGATCCAGG Eh_gltA686 R1 GCATTYTGYTCATGATCAGCATG Eh_gltA137 F2 TTATGTCTACTGCTGCTTGTGA Eh_gltA614 R2 TARGAAGAAAYRTCAAACATCATATG	575 478		[46]
***Ehrlichia* spp.**	*rpoB*	Eh_rpoB241 F1 AGTTATAGTATTGGTGARCCRCA Eh_rpoB821 R1 ARYCTAACWCCYCTRAAYCTATC Eh_rpoB305 F2 CTGTWCCTATACGTATAGTKYTG CG Eh_rpoB623 R2 TCTARCCAKGAWCCYCTRTARG G	581 319	94°C for 3 minutes; 45 cycles: 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute and final extension 72°C for 10 minutes. * Same thermal conditions for the second reaction.	[46]
***Ehrlichia* spp.**	*ftsz*	Eh_ftsZ242 F1 GTARAGGWGCWGCWGAAGART CAA Eh_ftsZ703 R1 CWGCTTCTCCTGTRCCCATCAT Eh_ftsZ313 F2 ACTGCYGGAATGGGTGGWGGA Eh_ftsZ679 R2 TTTRCCCATYTCRCTCATTATTGC	462 367	94°C for 3 minutes; 45 cycles: 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute and final extension 72°C for 10 minutes. * Same thermal conditions for the second reaction.	[46]
***Ehrlichia* spp.**	*groEL*	Eh_groEL64 F1 TTRGAAGAYGCWGTAGGATGYAC Eh_groEL593 R1 CCWCKRTCAAAYTGCATRCCATC Eh_groEL107 F2 CYGTAGCWATTRGYAARYCYTATGG Eh_groEL341 R2 CWNAYAATATCTGCHCCAGCAGC	530 235	94°C for 3 minutes; 45 cycles: 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute and final	[46]

				extension 72°C for 10 minutes. * Same thermal conditions for the second reaction.	
***Ehrlichia* spp.**	*groEL*	Ehrli-gro67F GAAGATGCWGTWGGWTGTACKGC Ehrli-gro776R AGMGCTTCWCCTTCWACRTCYTC Ehrli-gro217F ATTACTCAGAGTGCTTCTCARTG Ehrli-gro581R TGCATACCRTCAGTYTTTTCAAC	710 365	94°C for 3 minutes; 45 cycles: 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute final extension 72°C for 10 minutes * Same thermal conditions for the second reaction.	(Su et al., 2021; Tabara et al., 2007)
***Anaplasma* spp.**	16S rRNA	16SF GCGATTTTAGAGTGYGGAGATTG 16SR TACAATACCGGAGTAAAAGTCAA	1133	94°C for 3 minutes; 40 cycles: 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute. final extension 72°C for 10 minutes	[52, 53]
***Anaplasma* spp.**	16 S rRNA	AE4-Fw GTACCYAYAGAAGAAGTCCCGG CA AE-Rv RCACCAGCTTCGAGTTAAGCCAA T GE2F2 GTTAGTGGCAGACGGGTGAGT	800	98°C for 3 minutes; 40 cycles: 98°C for 55 seconds, 57.5°C for 20 seconds and 72°C for 40 seconds final extension 72°C for 5 minutes * Same thermal conditions for the second reaction.	(Parola et al., 2000)
***Anaplasma* spp.**	*groEL*	GROESL1F TATAGCTAGCATAATTACCCAGAGC GROESL1R GGTTAGTTCTGCTTTCGATGC GROESL2F TTATGTCTATGCGCCGTG GROESL2R CGGACCTTGCCACATTTT	842 339	94°C for 3 minutes; 30 cycles: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1.5 minutes final extension 72°C for 10 minutes 2ª reação 94°C for 3 minutes; 30 cycles: 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute final extension 72°C for 10 minutes	[57]
***Anaplasma* spp.**	ITS 23S-5S	ITSiF ATACCTCTGGTGTACCAGTTG ITSiR TTAACTT- CCGGGTTCGGAATG	300	94°C for 2 minutes; 35 cycles: 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute final extension 72°C for 5 minutes	[56]

***'Candidatus* Allocryptoplasma spp.'**	*sucA*	Crypto_sucA_F1 GTTATGGGNTTTGAGTAYGG Crypto_sucA_R2 GGGCTCTTCYTGRCACCA	636	93°C for 3 minutes; 35 cycles: 93°C for 30 seconds, 52°C For 1 minute and 72°C for 1 minute final extension 72°C for 5 minutes	(Ouass et al., 2023)
***'Candidatus* Allocryptoplasma spp.'**	*groEL*	Crypto_GroEL_F1 CCTTCYTCAACAGCAGCYCTAG Crypto_GroEL_R2 ACNGTTGAAGARAGTAARGG	713		

Table S3. Avian DNA samples positive in the multiplex quantitative (q) real-time qPCR for *Anaplasma* spp. and *Ehrlichia* spp. based on the *gro*EL gene.

Sample ID	Avian species	Sampling site	State	Agent detected	Cq
4 BAP413	*Certhiaxis cinnamomeus*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	38.32
51SL 025	*Eucometis penicillata*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	38.30
70 BEP393	*Thraupis sayaca*	Corumbá	Mato Grosso do Sul	*Anaplasma* spp.	37.78
78 F002	*Leptotila verreauxi*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	37.61
81 BAP435	*Cantorchilus leucotis*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	36.89
167 BEP304	*Ramphocelus carbo*	Corumbá	Mato Grosso do Sul	*Anaplasma* spp.	38.68
191 BEP340	*Tigrisoma lineatum*	Corumbá	Mato Grosso do Sul	*Anaplasma* spp.	39.22
208 BAP04	*Ramphocelus carbo*	Poconé	Mato Grosso	*Anaplasma* spp.	37.79
232 BAP87	*Legatus leucophaius*	Poconé	Mato Grosso	*Anaplasma* spp.	37.95
244 BAP112	*Cacicus cela*	Poconé	Mato Grosso	*Anaplasma* spp.	28.94
264 BAP123	*Ramphocelus carbo*	Poconé	Mato Grosso	*Anaplasma* spp.	39.24
266 BAP65	*Sporophila angolensis*	Poconé	Mato Grosso	*Anaplasma* spp.	39.24
281 BAP118	*Saltator coerulescens*	Poconé	Mato Grosso	*Anaplasma* spp.	38.50
286 BAP42	*Ramphocelus carbo*	Poconé	Mato Grosso	*Anaplasma* spp.	35.73
288 BAP128	*Ramphocelus carbo*	Poconé	Mato Grosso	*Anaplasma* spp.	38.22
299 BAP15	*Certhiaxis cinnamomeus*	Poconé	Mato Grosso	*Ehrlichia* spp	37.31

318 BAP254	*Furnarius rufus*	Nossa Senhora do Livramento	Mato Grosso	*Ehrlichia* spp	37.52
332 BAP335	*Pitangus sulphuratus*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	39.43
333 BAP257	*Sporophila collaris*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	37.40
337 BAP330	*Agelasticus cyanopus*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	37.14
350 BAP143	*Busarellus nigricollis*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	36.53
356 BAP262	*Chloroceryle americana*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	36.86
361 BAP332	*Agelasticus cyanopus*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	35.50
365 BAP220	*Tyrannus melancholicus*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	34.57
384 BAP139	*Guira guira*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	35.96
388 BAP263	*Paroaria capitata*	Nossa Senhora do Livramento	Mato Grosso	*Anaplasma* spp.	37.21
400 SL80	*Icterus cayanensis*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	36.68
403 F14	*Leptotila verreauxi*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	36.82
417 F73	*Ramphocelus carbo*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	38.20
423 F40	*Leptotila verreauxi*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	38.12
436 F86	*Ramphocelus carbo*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	36.89
461 F56	*Pitangus sulphuratus*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	36
463 F67	*Pipra fasciicauda*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	36.53
480 SL32	*Cranioleuca vulpina*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	34.66
487 SL42	*Phaethornis nattereri*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	35.82
489 F27	*Basileuterus flaveolus*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	39.05
496 F42	*Ramphocelus carbo*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	33.46
498 SL57	*Dendroplex picus*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	33.47
				Ehrlichia spp.	33.24

516 F50	*Ramphocelus carbo*	Santo Antonio de Leverger	Mato Grosso	*Anaplasma* spp.	33.40
				Ehrlichia spp.	37.89

Table S4. BLAST analyses results obtained for 16S rRNA, *dsb*, and ITS sequences obtained in PCR protocols for Anaplasmataceae agents from avian blood samples from the Brazilian Pantanal.

Sample ID	Bird species/ Locality	Molecular marker	Sequence size (bp)	Query cover (%)	E-value	Identity (%)	Best Match	Host	Accession number	Country	Reference of PCR protocol used
264	*Ramphocelus carbo* (BAP123), Poconé Mato Grosso.	16S rRNA	209	100	1.00 E^{-96}	98.58	*Ehrlichia* spp.	*Amblyomma sculptum*	MT514732	Brazil	[44]
292	*Elaenia albiceps (BAP72)* Poconé, Mato Grosso	16S rRNA	249	100	2.00 E^{-139}	99.65	*Ehrlichia* spp.	*Erinaceus amurensis*	MH879869	China	[44]
441	*Pitangus sulphuratus* (F10) Santo Antonio de Leverger, Mato Grosso	16S rRNA	205	100	1.00 E^{-96}	99.51	*Anaplasma* spp.	*Amblyomma hebraeum*	MG351089	Eswatini	[44]
444	*Ramphocelus carbo* (F68) Santo Antonio de Leverger, Mato Grosso	16S rRNA	281	100	2.00 E^{-139}	100	*Ehrlichia* spp.	*Haemaphysalis elliptica*	MZ351092	Eswatini	[44]
330	*Crax fasciolata* (BAP146) Nossa Senhora do Livramento, Mato Grosso	16S rRNA	269	100	2.00 E^{-128}	98.88	*Anaplasma* spp.	*Amblyomma dissimile*	MG437272	Brazil	[44]
				100	3.00 E^{-127}	98.51	*Candidatus* Allocryptoplasma spp.	*Lacerta viridis*	MG924904	Slovakia	
330	*Crax fasciolata* (BAP146) Nossa Senhora do Livramento, Mato Grosso	16S rRNA	884	100	0	98.6	'*Candidatus* Alloryptoplasma sp.'	*Lacerta viridis*	MG924904	Slovakia	[54]
				99	0	98.98	*Anaplasma* sp.	*Haemaphysalis parmata*	OQ092428	Uganda	

330	*Crax fasciolata* (BAP146) Nossa Senhora do Livramento, Mato Grosso	16S rRNA	908	100 99	0 0	99.56 99.56	'*Candidatus* Alloryptoplasma sp.' '*Candidatus* Alloryptoplasma sp.'	*Amblyomma coelebs* *Amblyomma coelebs*	OQ724839 OR854270	French Guyana French Guyana	Consensus sequence
341	*Furnarius leucopus* (BAP428) Nossa Senhora do Livramento, Mato Grosso	16S rRNA	832	100 100	0 0	98.9 99.3	*Anaplasma* sp. *Anaplasma* sp.	*Haemaphysalis parmata* *Amblyoma tholloni*	OQ092428 OQ092427	Uganda Uganda	[54]
345	*Aurindinicola leucocephala* (BAP267) Nossa Senhora do Livramento, Mato Grosso	16S rRNA	832	100 100	0 0	97.4 97.3	*Ehrlichia* sp. *Ehrlichia* sp.	*Dasyurus geoffroii* *Ixodes auritulus*	MW633161 MW628650	Australia Uruguay	[54]
389	*Ageslastycus cyanopus* (BAP289) Nossa Senhora do Livramento, Mato Grosso	*dsb*	401	99	0	99	*Ehrlichia minasensis*	*Bradypus variegatus*	MT212414	Rondônia, Brazil	[54]
418	*Basileuterus flaveolus* (F54) Poconé, Mato Grosso	*dsb*	402	100	0	99.25	*E. minasensis*	*B. variegatus*	MT212414	Rondônia, Brazil	[54]
330	*Crax fasciolata* (BAP146) Nossa Senhora do Livramento, Mato Grosso	23S-5S rRNA (ITS)	261	100 73	$6E^{-84}$ $1E^{-61}$	89.53 90.10	*Anaplasma phagocytophilum* *Anaplasma marginale*	*Scelosporus occidentalis* *Bos taurus*	JF487930 CP023731	USA Brazil	[56]
497	*Saltator coerulescens* (F58) Santo Antonio de Leverger, Mato Grosso	23S-5S rRNA (ITS)	270	77 88	$5E^{-55}$ $5E^{-55}$	86.19 86.19	*A. phagocytophilum* *A. phagocytophilum*	*Ovis aries* *Homo sapiens*	CP015376 CP035303	Norway South Korea	[56]

507	*Basileuterus flaveolus* (F12) Nossa Senhora do Livramento, Mato Grosso	23S-5S rRNA (ITS)	267	89 100	$4E^{-101}$ $33E^{-77}$	91 83.84	*A. phagocytophilum* *A. marginale*	*S. occidentalis* *B. taurus*	JF487930 CP023731	USA Brazil	[56]

Appendices Chapter 4. Molecular survey of vector-borne agents in wild birds from the Brazilian Pantanal

*This chapter corresponds to a future publication in the scientific journal Veterinary Research Communications.

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Supplementary material Supplementary material Conventional, nested, and quantitative real-time PCR assays used in screening for endogenous gene, *Hepatozoon*, *Borrelia* and onchocercid filariids and molecular characterization for onchocercid filariids and *Borrelia* spp.

Target gene and amplicon size	Agent/Molecular assay/ Purpose	Primer	Thermal conditions	References
β-actin (700 bp)	Birds /PCR/ Endogenous control	β-actin F ATCTCGTCTTGTTTTATGCG β-actin R TATCCGTAAGGATCTGTATG	95°C for 5 minutes for initial amplification and 35 cycles of 95°C for 30s, 54°C for 30s, 72°C for 1 min and a final extension 72ºC for 5 min	(Hatai et al., 2008)
18S rRNA (900 pb)	*Hepatozoon* spp. /PCR/ Screening	HEMO1 TATTGGTTTTAAGAACTAATT TTATGATTG HEMO2 CTTCTCCTTCCTTTAAGTGATAAG GTTCAC	94°C for 3 minutes for initial amplification and 35 cycles of 94°C for 45s, 48°C for 1min, 72°C for 1 min and a final extension 72ºC for 7 min	(Perkins and Keller, 2001)
18S rRNA (600 bp)	*Hepatozoon* spp. /PCR/	HepF300	94°C for 3 minutes for initial amplification and 35 cycles of 94°C for 30s, 60°C for 30s,	(Ujvari et al., 2004)

	Screening	GTTTCTGACCTATCAGCTTTCGAC G Hep900 CAAATCTAAGAATTTCACCTCTGA C	72°C for 1 min and a final extension 72ºC for 2 min	
cox-1 (650 bp)	Filariids /PCR/ Screening	COIintF TGATTGGTGGTTTTGGTAA COIintR ATAAGTACGAGTATCAATATC	94°C for 3 minutes for initial amplification and 40 cycles of 94°C for 45s, 52°C for 45s, 72°C for 90s and a final extension 72ºC for 7 min	(Casiraghi et al., 2001)
18S rRNA (998 pb)	Onchocercid filariids /PCR/ Characterization	988F CTCAAAGATTAAGCCATGC 1912R TTTACGGTCAGAACTAGGG	94°C for 1 minute for initial amplification and 40 cycles of 98°C for 10s, 55°C for 15s, 68°C for 30s and a final extension 72ºC for 7 min	(Hayashi et al., 2024)
28S rRNA (855 pb)	Onchocercid filariids /PCR/ Characterization	Nematode1 GCGGAGGAAAAGAAACTAA Nematode2 ATCCGTGTTTCAAGACGGG	94°C for 1 minute for initial amplification and 40 cycles of 98°C for 10s, 55°C for 15s, 68°C for 30s and a final extension 72ºC for 7 min	(Hayashi et al., 2024)
16S rRNA (148 pb)	*Borrelia* spp. /qPCR/ Screening	Bor16S3F AGCCTTTAAAGCTTCGCTTGTAG Bor16S3R GCCTCCCGT AGGAGTCTGG Bor16S3P FAM-CCGGCCTGAGAGGGTGAA CGG	50°C for 2 minutes for pre-PCR cycle, 95°C for 15 minutes, and 39 cycles of 95°C for 30s and 60°C for 1 min	(Parola et al., 2011)
16S rRNA (1489 bp)	*Borrelia* spp. /PCR/ Characterization	1st Round FD3 F AGAGTTTGATCCTGGCTTAG T50 R	First reaction 94C for 3 minutes for initial amplification and 35 cycles of 93°C for 1min, 55°C for 1min,	(Marti Ras et al., 1996)

		GTTACGACTTCACCCTCCT 2nd Round FD3 F AGAGTTTGATCCTGGCTTAG 16s-1 R TAGAAGTTCGCCTTCGCCCTG 3rd Round 16s-2 F TACAGGTGCTGCATGGTTGTCG T50 R GTTACGACTTCACCCTCCT 4th Round Rec4 F ATGCTAGAAACTGCATGA Rec9 R TCGTCTGAGTCCCCATCT	72°C for 1min and a final extension 72ºC for 7 min. Second and third reaction 94°C for 3 minutes for initial amplification and 35 cycles of 94°C for 1min, 56°C for 1min, 75°C for 1min and a final extension 72ºC for 7 min. Fourth reaction 94°C for 3 minutes for initial amplification and 35 cycles of 93°C for 1min, 54°C for 30 sec, 72°C for 30sec and a final extension 72ºC for 7 min.	
flab (665 pb)	*Borrelia* spp. /PCR/ Characterization	FlaRL F GCAATCATAGCCATTGCAGATTGT FlaLL R 5 ACATATTCAGATGCAGACAG AGGT	95°C for 3 minutes for initial amplification and 40 cycles of 95°C for 1min, 55°C for 1min, 75°C for 1min and a final extension 72ºC for 5 min.	(Stromdahl et al., 2003)

Appendices Chapter 5. Validation and standardization of a High-Throughput Microfluidic Real-Time PCR for the detection of vector-borne agents in wild birds from the Brazilian Pantanal

*This chapter corresponds to a future publication in the scientific journal Comparative Immunology, Microbiology and Infectious Diseases.

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Supplementary Table 2. Description of primers (F= forward; R=reverse), probes (P) and target genes used in the microfluidic real-time PCR assays for the selected vector-borne agents in avian blood samples from the Brazilian Pantanal.

Agents	Sequences (5'-3')	Length (bp)	Target Gene	Reference
Anaplasma spp.	F: CTTAGGGTTGTAAAACTCTTTCAG R: CTTTAACTTACCAAACCGCCTAC P: ATGCCCTTTACGCCCAATAATTCCGAACA	160	16S rRNA	[59]
Anaplasma phagocytophilum	F: GCTATGGAAGGCAGTGTTGG R: GTCTTGAAGCGCTCGTAACC P: AATCTCAAGCTCAACCCTGGCACCAC	77	*msp*2	[49]
Ehrlichia spp.	F: GCAACGCGAAAAACCTTACCA R: AGCCATGCAGCACCTGTGT P: AAGGTCCAGCCAAACTGACTCTTCCG	98	16S rRNA	[49]
Ehrlichia chaffeensis	F: TATTGCTAATTACCCTCAAAAAGTC R: GAGCTATCCTCAAGTTCAGATTT P: ATTGACCTCCTAACTAGAGGGCAAGCA	117	*dsb*	[59]
Aegyptianella pullorum	F: AGCCAGTATTATCGCTCAAGG R: GCCTCACGTGCCTTCATAAC P: TGCTTCTCAGTGTAACGACAGGGTTGG	165	*groEL*	[59]
Ehrlichia spp. [AA]	F: GTGGTGGATTACATGTAGCAG R: CTTAGCAGTACCCAATTCAGC P: CAGTGAAGGCTCCAGGATTTGGTGATAGAA	164	*groEL*	Present study
Bartonella spp.	F: CGTTATCGGGCTAAATGAGTAG R: ACCCCGCTTAAACCTGCGA P: TTGCAAATGACAACTATGCGGAAGCACGTC	118	*ssrA*	[59]
Bartonella hensellae	F: CCGCTGATCGCATTATGCCT R: AGCGATTTCTGCATCATCTGCT P: ATGTTGCTGGTGGTGTTTCCTATGCAC	107	*pap*31	[49]
Bartonella vinsonii berkhoffi	F: GGAATTGCTTAACCCACTGTTG R: CCTTATTGATTTAGATCTGATGGG P: AGAAACTCCCGCCTTTATGAGAGAAATCTCT	141	16-23S rRNA (ITS)	[59]
Hepatozoon spp.	F: ATTGGCTTACCGTGGCAGTG R: AAAGCATTTTAACTGCCTTGTATTG P: ACGGTTAACGGGGGATTAGGGTTCGAT	175	18S rRNA	[49]
Apicomplexa protozoa	F: TGAACGAGGAATGCCTAGTATG R: CACCGGATCACTCGATCGG P: TAGGAGCGACGGGCGGTGTGTAC	104	18S rRNA	Present study
Trypanosoma spp.	F: GTAATTCCAGCTCCAAAAGCG R: TCAGGAAGGAACCACTCCC P: ACCTCAAGGGCATGGGTCACCAATCC	178	18S rRNA	[59]
Babesia vogeli	F: TCACTGTGCCTGCGTACTTC	87	*hsp*70	[51]

	R: TGATACGCATGACGTTGAGAC P: AACGACTCCCAGCGCCAGGCCAC			
Borrelia garinii	F: TGGCCGAACTTACCCACAAAA R: ACATCTCTTACTTCAAATCCTGC P: TCTATCTCTTGAAAGTCCCCCTGGTCC	88	*rpo*B	[49]
Borrelia valaisiana	F: ACTCACAAATGACAGATGCTGAA R: GCTTGCTTAAAGTAACAGTACCT P: TCCGCCTACAAGATTTCCTGGAAGCTT	135	*osp*A	[49]
Borrelia sp.	F: GAGTCTTAAAAGGGCGATTTAGT R: CTTCAGCCTGGCCATAAATAG P: AGATGTGGTAGACCCGAAGCCGAGT	73	23S rRNA	[59]
Borrelia burgdorferi sensu stricto	F: GCTTACTCACAAAAGGCGTCTT R: GCACATCTCTTACTTCAAATCCT P: AATGCTCTTGGACCAGGAGGACTTTCA	83	*rpo*B	[49]
Rickettsia spp.	F: GTCGCAAATGTTCACGGTACTT R: TCTTCGTGCATTTCTTTCCATTG P: TGCAATAGCAAGAACCGTAGGCTGGATG	78	*gltA*	[49]
Rickettsia rickettsii	F: TCTACTCACAAAGTTATCAGGTTAA R: CCTACGATACTCAGCAAAATAATTT P: TCGCTGGATATCGTTGCAGGACTACAG	124	23S-5S rRNA ITS	[49]
Rickettsia massiliae	F: GTTATTGCATCACTAATGTTATACTG R: GTTAATGTTGTTGCACGACTCAA P: AGCCCCGCCACGATATCTAGCAAAAA	128	23S-5S rRNA ITS	[59]
Rickettsia africae	F: GATACGACAAGTACCTCGCAG R: GGATTATATACTTTAGGTTCGTTAG P: CAGATAGGAACAGTAATTGTAACGGAACCAG	122	Sca1	[59]
Spotted fever group *Rickettsia*	F: CCTTTTGTAGCTCTTCTCATCC R: GCGATGGTAGGTATCTTAGCAA P: TGGCTATTATGCTTGCGGCTGTCGGT	145	*gltA*	[51]
Borrelia spp. [AA]	F: GCTGAAGAGCTTGGAATGCAG R: GCAATTGCTTCATCCTGATTTG P: CCTGCAAAAATTAACACACCAGCATCTCTATC	116	*fla*B	Present study
Haemoproteus spp. [AA]	F: CTTGGGGTCAAATGAGTTTCTG R: GGTAGGGTCACTAACAATATATC P: CACCACAAATCCATGAGACTAATCCAGGTATA	240	*cyt*B	Present study
Plasmodium spp. [AA]	F: TACCTGGTCTTGTCTCATGG R: CCCTAAAGGATTTGTGCTACC P: TTTGTGGTGGATATCTTGTAAGCGACCCAAC	339	*cytB*	Present study
Trypanosoma spp. [AA]	F: TGAACCAAAGGGACGCTCTC R: CTTCTGGGTGTTACTGCCG P: CTGTTCCGGCGATGGGGCAACTC	112	18S rRNA	Present study
Leucocytozoon spp. [AA]	F: GTAATGTAGAAACTGCGAACGG R: CTTATACGTGTCGCTTCTTTGT P: TTAGGACTCCCCACTTGTCTTTTTCTTGAAA	177	*ssRNA*	Present study
Aproctella spp [AA]	F: GCATATTTTTACACCCAAAGTCC R: CTAAAGTGTAACCGTACCGTAAC P: CTTGAGCGGGGCTACAGTCCATAGAAG	98	LSU rRNA	Present study
Eufilaria spp. [AA]	F: ACCCAAAGTCCCCCTTGAG R: CGTAACGTTTTTCACCCGTAC P: CGAGGCCATTATCCATAGAAGGTGCTAG	72	LSU rRNA	Present study
Chandlerella spp. [AA]	F: TATTGGGATCTCCTGAAATGGC R: AGGCTGACCTTCAACACTTAATG_ P: TGGATAAAAAGTTCAACTTCTACCAGGGCCA	158	*cox-1*	Present study

[AA]: Stands for primers and probes designed in the present study**.**

Supplementary Table 2. Results of High-Throughput Microfluidic Real-Time PCR assay with positive controls obtained from neotropical birds sampled in Pantanal wetland, Mato Grosso state, central-western Brazil (Alabí-Córdova et al., 2024 a,b).

Positive samples on microfluidic PCR	Scientific name	*Anaplasma* spp* 16S rRNA	*Bartonella* spp. * *ssr*A	*Plasmodium* spp.[AA] *cyt*B	*Haemoproteus* spp.[AA] *cyt*B	Onchocercidae filariids [AA] LSU rRNA
4[AN]	*Certhiaxis cinnamomeus*					
12[BRT]	*Formicivora rufa*					
81[AN]	*Cantorchilus leucotis*					
33[BRT]	*Tyrannus melancholicus*					
102[BH]	*Furnarius rufus*					
103[BH]	*Paroaria capitata*	X				
108[BRT]	*Chloroceryle americana*	X			X	
110[BRT]	*Cantorchilus leucotis*					
111[BRT]	*Paroaria capitata*					
117[BRT]	*Furnarius leucopus*					
167[AN]	*Ramphocelus carbo*					
244[AN]	*Cacicus cela*					
333[AN]	*Sporophila collaris*	X				
337[AN]	*Agelasticus cyanopus*				X	
341[AN]	*Furnarius leucopus*	X				
350[AN]	*Busarellus nigricollis*			X		
361[AN]	*Agelasticus cyanopus*					
420[BRT]	*Ramphocelus carbo*		X			X
441[AN]	*Pitangus sulphuratus*				X	X
497[AN/Cal]	*Saltator coerulescens*		X			X
507 [AN/Cal]	*Basileuterus flaveolus*		X			
516[ANN/EH]	*Ramphocelus carbo*	X				
555[HAEM]	*Leptotila verreauxi*					
Occurrence for positive controls tested		5 (21.73%)	3 (13.04%)	1 (4.34%)	3 (13.04%)	3 (13.04%)

AA: Stands for primers and probes designed in the present study. The occurrence of each VBA was calculated based on the 40 samples used as positive controls. The following superscripts stand for the next agents *Anaplasma* spp. AN, *Bartonella* spp. BRT, *Bartonella henselae* BH, Haemosporidians HAEM, '*Candidatus* Allocryptoplasma spp.'CAl. Samples indicated with the superscripts were positive in qPCR and PCR obtained from (Alabí Córdova, Fecchio, Calchi, Dias, Machado et al., 2024a; Alabí Córdova, Fecchio, Calchi, Dias, Mongruel et al., 2024a)(Alabí Córdova, Fecchio, Calchi, Dias, Machado et al., 2024a; Alabí Córdova, Fecchio, Calchi, Dias, Mongruel et al., 2024a).. Asterisks indicate the reference from where the primers were obtained. *:[59]

Table SM3. Results of High-Throughput Microfluidic real-time PCR showing co-positivity for selected vector-borne agents in neotropical birds sampled in Pantanal wetland, Mato Grosso state, central-western Brazil.

Positive samples on microfluidic PCR	Scientific name	*Anaplasma* spp. 16S rRNA*	*Bartonella* spp. *ssrA**	*Plasmodium* spp.[AA] *cytB*	*Haemoproteus* spp.[AA] *cytB*	Onchocercidae filariids. [AA] LSU rRNAe
Salou4	*Furnarius rufus*	X				
Salou9	*Ramphocelus carbo*					X
Salou12	*Synallaxis albilora*					X
Salou13	*Crotophaga ani*	X				
Salou15	*Pseudoseisura unirufa*	X				
Salou19	*Crotophaga ani*	X				
Salou23	*Ramphocelus carbo*	X				
Salou30	*Ramphocelus carbo*					X
Salou32	*Ramphocelus carbo*	X				X
Salou34	*Eucometis pennicillata*	X				
Salou36	*Ramphocelus carbo*					X
Salou40	*Ramphocelus carbo*					X
Salou41	*Ramphocelus carbo*	X				X
Salou42	*Ramphocelus carbo*					X
Salou48	*Myiothlypis flaveola*			X		
Salou49	*Ramphocelus carbo*	X				
Salou51	*Eucometis pennicillata*	X				
Salou54	*Ramphocelus carbo*	X				X
Salou55	*Ramphocelus carbo*	X				X
Salou57	*Leptotila verreauxi*	X				
Salou59	*Synallaxis albilora*	X				

Salou61	*Myiothlypis flaveola*	X					X		
Salou65	*Ramphocelus carbo*				X		X		
Salou67	*Turdus amaurochalinus*	X							X
Salou70	*Eucometis pennicillata*	X							
Salou71	*Myiothlypis flaveola*	X							
Salou72	*Eucometis pennicillata*	X							
Salou74	*Hypocnemoides maculicauda*	X							
Salou77	*Turdus amaurochalinus*			X					
Salou78	*Eucometis pennicillata*	X							
Salou79	*Eucometis pennicillata*	X							
Salou80	*Synallaxis albescens*	X							
Salou81	*Ramphocelus carbo*								X
Salou82	*Eucometis pennicillata*	X							
Salou83	*Turdus amaurochalinus*	X							
Salou85	*Saltator coerulescens*	X							X
Salou86	*Cranioleuca vulpine*	X							
Salou88	*Dendroplex picus*	X							
Salou90	*Icterus pyrrhopterus*	X							
Salou93	*Rupornis magnirostris*	X							
Salou94	*Ramphocelus carbo*	X							X
Salou95	*Fluvicola albiventer*	X			X				
Salou97	*Myiothlypis flaveola*	X							
Salou99	*Ramphocelus carbo*	X							
Salou103	*Ramphocelus carbo*	X							
Salou105	*Ramphocelus carbo*	X							
Salou106	*Myiothlypis flaveola*	X							
Salou107	*Myiothlypis flaveola*	X							
Salou108	*Leptotila verreauxi*								X

Salou114	*Synallaxis albilora*	X							X
Salou115	*Myiophobus fasciatus*	X							
Salou117	*Pheugopedius genibarbis*			X					
Salou121	*Arremon flavirostris*			X					
Salou122	*Taraba major*	X							
Salou124	*Synallaxis albilora*								X
Salou125	*Ramphocelus carbo*	X		X					
Salou126	*Ramphocelus carbo*	X		X					X
Saolou136	*Myiophobus fasciatus*	X							
MIM149	*Volatinia jacarina*								
MIM150	*Myiarchus ferox*	X							
MIM152	*Machetornis rixosa*	X							
MIM153	*Volatinia jacarina*								
MIM154	*Volatinia jacarina*					X			
MIM166	*Myiarchus ferox*	X							
MIM175	*Ramphocelus carbo*			X					
MIM176	*Volatinia jacarina*	X							
MIM178	*Columbina talpacoti*					X			
MIM186	*Volatinia jacarina*								
MIM218	*Columbina talpacoti*					X			
MIM230	*Turdus leucomelas*			X					
MIM234	*Thraupis palmarum*								
MIM236	*Leptotila verreauxi*					X			
MIM241	*Volatinia jacarina*			X					
MIM242	*Columbina talpacoti*					X			
MIM243	*Columbina talpacoti*	X				X			
MIM250	*Columbina squammata*					X			
MIM251	*Volatinia jacarina*			X		X			

MIM272	*Volatinia jacarina*			X			
MIM278	*Columbina squammata*				X		
MIM289	*Columbina talpacoti*			X	X		
MIM290	*Volatinia jacarina*			X			
MIM292	*Volatinia jacarina*			X			
MIM296	*Volatinia jacarina*			X			
MIM298	*Columbina talpacoti*	X			X		
Totql		50	1	17	13	18	

[AA]: Stands for primers and probes designed in the present study. Asterisks indicate the reference from which the primers where obtained *:[84]