FREDERICO FONTANELLI VAZ

Survey and molecular characterization of pathogens in wild Amazon parrot nestlings and recently seized nestlings from illegal trade

São Paulo

2020

FREDERICO FONTANELLI VAZ

Survey and molecular characterization of pathogens in wild Amazon parrot nestlings and recently seized nestlings from illegal trade

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Departamento:
Patologia

Área de Concentração:
Patologia Experimental e Comparada

Orientadora:
Profa. Dra. Tânia de Freitas Raso

São Paulo

2020

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Título traduzido: Pesquisa e caracterização molecular de patógenos em filhotes de papagaios *Amazona sp.* de vida livre e recém apreendidos do tráfico.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Patologia, São Paulo, 2020.

Programa de Pós-Graduação: Patologia Experimental e Comparada.

Área de concentração: Patologia Experimental e Comparada.

Orientadora: Profa. Dra. Tânia de Freitas Raso.

1. *Chlamydia psittaci*. 2. Circovírus. 3. Herpesvírus. 4. Poxvírus. 5. Psitacídeos. I. Título.

Ficha catalográfica elaborada pela bibliotecária Maria Aparecida Laet, CRB 5673-8, da FMVZ/USP.

CERTIFICADO

Certificamos que o Projeto intitulado "OCORRÊNCIA DE AGENTES INFECCIOSOS EM PAPAGAIOS DO GÊNERO Amazona DE VIDA LIVRE E RECÉM-APREENDIDOS ORIUNDOS DO TRÁFICO: IMPLICAÇÕES PARA A CONSERVAÇÃO", protocolado sob o CEUA nº 9545290116, sob a responsabilidade de **Tânia de Freitas Raso** *e equipe; Frederico Fontanelli Vaz* - 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 - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMZV) na reunião de 06/04/2016.

We certify that the proposal "Occurrence of infectious agents in Amazon parrots from wildlife and birds caught from illegal trade: implications for conservation", utilizing 100 Birds (males or females), protocol number CEUA 9545290116, under the responsibility of **Tânia de Freitas Raso** *and team; Frederico Fontanelli Vaz* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of São Paulo (CEUA/FMZV) in the meeting of 04/06/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 02/2016 a 02/2020 Área: Patologia Experimental E Comparada

Procedência:	Não aplicável biotério						
Espécie:	Aves	sexo:	Machos ou Fêmeas	idade:	1 a 12 meses	N:	100
Linhagem:	Papagaio Amazona sp			Peso:	100 a 600 g		

Resumo: O Brasil é o país com a maior diversidade de psitacídeos. Das 375 espécies conhecidas, 85 são encontradas no território nacional (CBRO, 2013). Apesar disso, aves da Ordem Psittaciformes estão entre as mais ameaçadas de extinção no Brasil; com 22 espécies na Lista de Espécies Ameaçadas da União Internacional para a Conservação da Natureza (IUCN, 2015). Populações de papagaios-de-cara-roxa (Amazona brasiliensis), papagaios-charão (A. pretrei) e os papagaios-de-peito-roxo (A. vinacea) sofrem com ações antrópicas e são consideradas ameaçadas. O papagaio-verdadeiro (A. aestiva) está na categoria pouco preocupante, no entanto, há um especial interesse na espécie por ser o principal alvo do comércio ilegal para suprir o mercado de aves de estimação. Informações sobre a saúde destas aves são escassas na literatura. Sendo assim, pesquisas de patógenos devem ser encorajadas pois contribuem para o conhecimento do status sanitário de uma população bem como podem contribuir para as atividades de conservação das espécies. Entre os vários patógenos que afetam psitacídeos, três são importantes no contexto brasileiro: o circovírus, agente da Doença do Bico e das Penas dos Psitacídeos; o herpesvírus, agente da Doença de Pacheco e; a Chlamydia psittaci, agente da clamidiose. Deste modo, este estudo objetiva pesquisar estes agentes infecciosos em filhotes das espécies de papagaios A. aestiva, A. brasiliensis, A. vinacea e A. pretrei em condições de vida livre e em filhotes destas espécies recém-apreendidos do comércio ilegal; comparar a ocorrência e/ou prevalência dos agentes infecciosos entre os papagaios de vida livre e papagaios apreendidos amostrados e; avaliar o potencial impacto destes patógenos para a conservação das populações estudadas. Amostras de swabs de cloaca e orofaringe e amostras de sangue das aves serão coletadas para realização dos exames moleculares (PCR). Posteriormente, as amostras positivas serão caracterizadas por sequenciamento e análise de DNA. Os resultados deste estudo poderão preencher lacunas existentes no monitoramento da saúde dos papagaios em vida livre e oriundos do tráfico e possibilitarão determinar o possível impacto dos patógenos nas populações analisadas, traçando assim as possíveis implicações para a conservação de espécies de psitacídeos ameaçadas no Brasil.

Local do experimento: Laboratório de Ornitopatologia II, VPT, FMVZ-USP

São Paulo, 06 de abril de 2016

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 17 de dezembro de 2019
CEUA N 9545290116

IImo(a). Sr(a).
Responsável: Tânia De Freitas Raso
Área: Patologia Experimental E Comparada

Título da proposta: "PESQUISA E CARACTERIZAÇÃO MOLECULAR DE PATÓGENOS EM FILHOTES DE PAPAGAIOS AMAZONA sp. DE VIDA LIVRE E RECÉM APREENDIDOS DO TRÁFICO".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 005549)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 12/dezembro/2019) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Alteração do título: Solicitamos a alteração para o título "PESQUISA E CARACTERIZAÇÃO MOLECULAR DE PATÓGENOS EM FILHOTES DE PAPAGAIOS AMAZONA sp. DE VIDA LIVRE E RECÉM APREENDIDOS DO TRÁFICO" por estar mais adequado ao conteúdo final do trabalho, sendo retirado a parte final do título ficando mais claro e objetivo. A nova versão em ingles proposta é: SURVEY AND MOLECULAR CHARACTERIZATION OF PATHOGENS IN WILD AMAZON PARROT NESTLINGS AND NEWLY SEIZED FROM THE ILLEGAL TRADE. Agradecemos a compreensão da CEUA quanto a nossa solicitação. ".

Comentário da CEUA: "Alteração aprovada.".

Profa. Dra. Anneliese de Souza Traldi
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 43876-1	Data da Emissão: 25/07/2017 11:32	Data para Revalidação*: 24/08/2018

* 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: Gláucia Helena Fernandes Seixas	CPF: 497.323.150-91
Título do Projeto: Psitacídeos no Pantanal, Cerrado e Mata Atlântica de Mato Grosso do Sul: informações para a gestão das espécies e seus habitats	
Nome da Instituição : FUNDAÇÃO NEOTRÓPICA DO BRASIL	CNPJ: 73.684.789/0001-10

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Definição dos sítios de amostragem in loco	06/2017	05/2018
2	Verificação do uso de ambientes pelos psitacídeos	06/2017	05/2021
3	Coleta de material biológico e dados sobre sucesso reprodutivo dos psitacídeos (2o semestre)	06/2017	05/2021
4	Comunicação sobre o projeto em diferentes meios (eventos, midia, outros)	06/2017	05/2021
5	Submissão de artigos científicos e técnicos para revistas	06/2017	05/2021
6	Elaboração de relatórios do projeto	11/2017	05/2021
7	Análise laboratorial do material biológico (genética e sanidade)	05/2018	05/2021
8	Análise de dados e elaboração de mapas de uso de ambientes pelos psitacídeos e para ecoturismo	05/2018	05/2021

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	1. Esta autorização não exime seu titular da necessidade de atender ao disposto na Instrução Normativa Ibama nº 27/2002, que regulamenta o Sistema Nacional de Anilhamento de Aves Silvestres. É obrigatório ao pesquisador portar autorização de anilhamento durante as expedições de campo que envolvam essa atividade; 2. O volume máximo de sangue coletado não deve ultrapassar 1% da massa corporal da ave; 3. A marcação com o uso de rádio-transmissor deve ser adequada ao tamanho da ave, de modo que não prejudique sua mobilidade nem sua rotina; 4. Não está autorizada a coleta de ovos viáveis, somente a sua manipulação com posterior devolução ao ninho.

Equipe

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Código de autenticação: 96655827

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Autorização para atividades com finalidade científica

Número: 25694-6	Data da Emissão: 04/10/2016 20:43	Data para Revalidação*: 03/11/2017

* 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: NÊMORA PAULETTI PRESTES	CPF: 514.754.640-53
Título do Projeto: ESTUDO DE SIMPATRIA ENTRE O PAPAGAIO-CHARÃO E O PAPAGAIO-DE-PEITO-ROXO	
Nome da Instituição : FUNDAÇÃO UNIVERSIDADE DE PASSO FUNDO	CNPJ: 92.034.321/0001-25

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	instalação de rádio-colares para avaliar deslocamentos	09/2016	09/2018
2	monitorar a população das duas espécies nos dormitórios	09/2016	09/2018
3	coleta de material genético	09/2016	09/2018
4	instalação de caixas-ninho	09/2016	09/2018
5	identificar áreas de ocorrência das espécies em estudo	09/2016	09/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
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5	Este documento NÃO exime o pesquisador titular da necessidade de atender ao disposto na Instrução Normativa Ibama nº 27/2002, que regulamenta o Sistema Nacional de Anilhamento de Aves Silvestres.
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7	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
8	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
9	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.
10	As atividades contempladas nesta autorização abrangem espécies brasileiras constante de listas oficiais (de abrangência nacional, estadual ou municipal) de espécies ameaçadas de extinção, sobreexplotadas ou ameaçadas de sobreexplotação.

Outras ressalvas

1	Cadastrar projeto no SNA.net, de acordo com a IN 27/2002, visto que contém dentre os métodos de marcação o rádio colar.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Jaime Martinez	Coordenador do projeto	307.592.770-87	4031437901 SSP-RS	Brasileira

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Autorização para atividades com finalidade científica

Número: 35621-8	Data da Emissão: 06/12/2019 15:46:48	Data da Revalidação*: 01/10/2020

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: ELENISE ANGELOTTI BASTOS SIPISNKI	CPF: 722.516.209-82
Título do Projeto: Conservação do papagaio-de-cara-roxa	
Nome da Instituição: INSTITUTO DE PESQUISA EM VIDA SELVAGEM E EDUCACAO AMBIENTAL	CNPJ: 78.696.242/0001-59

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Realizar censo populacional do papagaio-de-cara-roxa	11/2015	03/2020
2	Monitorar os filhotes de papagaio-de-cara-roxa no PR	11/2015	03/2020
3	Estudo de fenologia de cinco espécies vegetais utilizadas como recurso alimentar pelo papagaio-de-ca	11/2015	03/2020
4	Monitorar os filhotes de papagaio-de-cara-roxa em SP	11/2015	03/2020
5	Realizar prospecção do papagaio-de-cara-roxa no litoral sul do Paraná e norte de Santa Catarina	11/2015	03/2020

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Rafael Meirelles Sezerban	pesquisador	010.231.069-65	Brasileira
2	Patricia Pereira Serafini	pesquisadora	027.472.819-22	Brasileira
3	Roberta Lúcia Boss	Pesquisadora	033.006.399-56	Brasileira

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FOLHA DE AVALIAÇÃO

Autor: VAZ, Frederico Fontanelli

Título: Survey and molecular characterization of pathogens in wild Amazon parrot nestlings and recently seized nestlings from illegal trade

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Data: ____/____/____

Banca examinadora

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Dedico este trabalho a todos os animais selvagens que conseguem resistir e persistir frente a tantas ameaças.

AGRADECIMENTOS

À minha mãe **Marizilda Fontanelli**, que sempre apoiou e incentivou a minha carreira e sem a qual eu não teria chegado a este momento, obrigado por todo amor que recebo todos os dias. À minha irmã **Lívia Fontanelli Vaz**, por ter sido alicerce à nossa família durante a minha ausência. Ao meu avô **Redeu Fontanelli** (*in memoriam*) e à minha avó **Iaiá Fontanelli** (*in memoriam*), que com certeza me guiaram nesta jornada.

À minha orientadora Profa. Dra. **Tânia de Freitas Raso**, por ter aberto as portas pra mim, ter me aceito como orientado e confiado no meu trabalho. Jamais esquecerei seus ensinamentos, seu exemplo de profissionalismo e sua amizade. Obrigado por contribuir tanto no meu crescimento profissional e em todas as etapas do projeto.

À **Maria Catalina Ospina Pinto**, pela amizade, conversas e discussões produtivas que tivemos no laboratório e por toda ajuda dedicada ao encerramento do projeto. Ao **Jorge Oyakawa** e **Herculano Pinho Pereira**, pela ajuda na esterilização de materiais e no preparo de soluções.

Aos biólogos do *Projeto de Conservação do Papagaio-de-cara-roxa*, **Elenise Angelotti Bastos Sipinski**, **Roberta Lucia Boss** e **Rafael Meireles Sezerban** por toda ajuda na coleta de amostras a campo.

À zootecnista **Gláucia Helena Fernandes Seixas** do *Projeto Papagaio-Verdadeiro*, obrigado pelo envio das amostras dos filhotes do Pantanal e por colaborar na coleta de amostras dos filhotes do *Centro de Reabilitação de Animais Silvestres* de Campo Grande.

Aos biólogos do *Projeto Papagaio-Charão* **Nêmora Pauletti Prestes** e **Jaime Martinez**, e ao **Roberto Tomasi Jr.** por toda ajuda na coleta de amostras a campo.

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RESUMO

VAZ, F. F. **Pesquisa e caracterização molecular de patógenos em filhotes de papagaios *Amazona* sp. de vida livre e recém apreendidos do tráfico.** 114 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

Aves da ordem Psittaciformes estão entre as mais ameaçadas do Brasil. Das 86 espécies existentes, 24 (27,9%) estão na Lista Vermelha da União Internacional para Conservação da Natureza. Os papagaios do gênero *Amazona* possuem um destaque por estarem entre os mais traficados, principalmente o papagaio-verdadeiro (*Amazona aestiva*). O papagaio-de-cara-roxa (*A. brasiliensis*) se encontra na categoria quase ameaçada e o papagaio-charão (*A. pretrei*) na vulnerável, ambos necessitando manejo em vida livre. Além da perda de habitat e do tráfico, a disseminação de patógenos é uma ameaça emergente a essas espécies, em decorrência da ampla movimentação, comércio e manipulação das mesmas. Considerando a falta de informações sobre a saúde desses animais, o objetivo deste estudo foi de investigar patógenos selecionados em filhotes de *A. aestiva, A. brasiliensis* e *A. pretrei* de vida livre e *A. aestiva* apreendidos do tráfico. Amostras de 235 *Amazona* sp. de vida livre foram coletadas de quatro estados brasileiros e amostras de 90 *A. aestiva* foram coletadas de filhotes apreendidos do tráfico e encaminhados a um Centro de Reabilitação de Animais Silvestres (CRAS). As amostras foram testadas por meio da PCR para *C. psittaci, Psittacid alphaherpesvirus 1*, poxvírus e *Beak and feather disease vírus* (BFDV). O DNA de *C. psittaci* foi detectado em amostras de cinco filhotes de vida livre. O DNA dos outros patógenos não foi detectado nas amostras das aves de vida livre ou do tráfico. O sequenciamento da *C. psittaci* na amostra de um *A. brasiliensis* revelou alta similaridade com isolados encontrados em psitacídeos no Brasil, pertencentes ao genótipo A. Os resultados do presente estudo demonstram que a prevalência de patógenos em aves de vida livre é bastante baixa e que patógenos exóticos, como o circovírus, parecem ainda não ter atingido essas populações, apesar de já estarem presentes em cativeiro no Brasil. Isso reforça a necessidade de proteger a nossa avifauna de ameaças iminentes de introdução e disseminação desses vírus na natureza. Novos protocolos de avaliação de saúde devem ser discutidos e seguidos rigorosamente para a reintrodução de psitacídeos na natureza. Com relação às aves

do CRAS, estas foram isoladas e amostradas logo que chegaram ao centro, não sendo acompanhadas para avaliar os efeitos do cativeiro em longo prazo na saúde das mesmas. Medidas preventivas nunca devem ser negligenciadas em psitacídeos introduzidos em um plantel, pois pesquisas revelam ocorrência de surtos e a detecção de patógenos relevantes para a conservação dessas aves. Novos estudos devem ser encorajados para um melhor conhecimento da epidemiologia de patógenos em psitacídeos de vida livre e para ampliar o conhecimento dos seus impactos sobre a conservação das espécies.

Palavras-chave: *Chlamydia psittaci.* Circovírus. Herpesvírus. Poxvírus. Psitacídeos.

ABSTRACT

VAZ, F. F. **Survey and molecular characterization of pathogens in wild Amazon parrot nestlings and recently seized nestlings from illegal trade.** 114 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

Birds of the order Psittaciformes are among the most threatened birds in Brazil. Of the 86 species recorded, 24 (27.9%) are in the International Union for Conservation of Nature Red List. Amazon parrots have a highlight for being among the most trafficked birds, especially the blue-fronted Amazon parrot (*Amazona aestiva*). The red-tailed Amazon parrot (*A. brasiliensis*) is in the near threatened category and the red-spectacled Amazon parrot (*A. pretrei*) is threatened in the vulnerable category, both of them needing management in the wild. In addition to habitat loss and illegal trade, the spread of pathogens is an emerging threat to these species due to their wide movement, trade and manipulation. Considering the lack of information on the health of wild parrots, the aims of this study were to investigate selected pathogens on wild *A. aestiva, A. brasiliensis* and *A. pretrei* nestlings and in *A. aestiva* seized from illegal trade. Samples from 235 wild Amazon parrots were collected in four Brazilian states, and samples from 90 *A. aestiva* were collected from nestlings seized from illegal trade and submitted to a Wildlife Rehabilitation Center (CRAS). Samples were tested by PCR for *Chlamydia psittaci, Psittacid alphaherpesvirus 1*, poxvirus and *Beak and feather disease virus* (BFDV). *Chlamydia psittaci* DNA was detected in swab samples from five wild nestlings. The DNA of the other pathogens was not detected in the wild and trafficked bird samples. Sequencing of *C. psittaci* in the sample of one *A. brasiliensis* revealed high similarity with isolates found in parrots in Brazil, belonging to genotype A. The results of the present study demonstrate that the prevalence of pathogens in wild parrots is very low, and exotic pathogens such as BFDV may not yet have reached these populations, although they are present in captivity in Brazil. This reinforces the need to protect our bird fauna from imminent threats of introducing and spreading these viruses into the wild. Novel health assessment protocols should be discussed and strictly followed for the reintroduction of parrots in the wild. Regarding the birds from CRAS, they were isolated and sampled soon after their arrival at the center, and were not monitored to evaluate long-term effects of captivity on their health. Preventive measures should never be

neglected in psittacine birds introduced in a flock, as studies reveal outbreaks and the detection of relevant pathogens to the conservation of these birds. Further studies should be encouraged to better understand the epidemiology of pathogens in wild parrots, to expand the knowledge of their impacts on species conservation.

Keywords: *Chlamydia psittaci*. Circovirus. Herpesvirus. Poxvirus. Psittacine birds.

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LIST OF ABREVIATIONS

CBRO	Brazilian Council of Ornithological Records
CEUA	Committee on Ethics in the Use of Animals
BFDV	Beak and feather disease virus
EDTA	Ethylenediaminetetraacetic acid
ICMBio	Chico Mendes Institute for Biodiversity Conservation
IUCN	International Union for Conservation of Nature
OIE	Food and Agriculture Organization
OMP	Outer membrane protein
PBFD	Psittacine beak and feather disease
PCR	Polymerase chain reaction
PD	Pacheco’s disease
PsHV	Psittacid herpesvirus
UL	Unique long
WHO	World Health Organization
WRC	Wildlife Rehabilitation Center

SUMMARY

1. GENERAL INTRODUCTION

Brazil is considered a megadiverse country, containing the third largest bird diversity in the world, counting on 1919 recorded species. The largest number of Psittaciformes is found in the country, as among the 411 registered species (IUCN, 2019), 86 (20.9%) occur in the Brazilian territory, being 24 endemic birds (PIACENTINI et al., 2015). Some national species occur in very restricted areas and are sensitive to environmental alterations (GALLETTI et al., 2006; NUNES, 2017), and can often serve as bioindicators for their habitat imbalances.

Parrots have long been considered plant antagonists, acting only as predators of them, as they consume, destroy and waste seeds. However, recent studies have proved that these birds play an important ecological role in seed dispersal, pollination and healing of the plants they consume, as they feed on their parasites (BLANCO; HIRALDO; TELLA, 2018). In addition, parrots are charismatic birds and have great potential to act as flagship species for the protection of critical ecosystems, for the development of ecotourism programs and environmental education (SNYDER et al. 2000).

Despite the importance of parrots to the environment and their rich diversity found in Brazil, psittacine species are among the most endangered national birds, with 17 species included in the Brazil Red Book of Threatened Species of Fauna (ICMBIO, 2018). Almost 30% (112/411) of Psittaciformes in the world are on the Red List of the International Union for Conservation of Nature (IUCN, 2019). Their colorful feathers and high sociability create a domestic demand and make them intensely sought after to serve as pet animals. The Amazon parrots are highlighted considering the native species, being first among the most trafficked psittacine birds in Brazil. The Amazon genus has 12 species occurring in the country and the populations face two major threats: wild nestlings being caught to supply the illegal trade; and loss, alteration and fragmentation of all the biomes where they inhabit. A quarter of native Amazon species are considered threatened (ICMBIO, 2018).

Another currently challenge for parrot conservation is the spread of pathogens, especially in a large and biodiverse country like Brazil. The emergence of pathogenic infectious diseases can be triggered by introduction of pathogens, infected animals or vectors into the environment or into captive aviaries; wildlife translocation; human

and domestic animals encroachment; spill-over events; and ecological manipulation (DASZAK; CUNNINGHAM; HYATT, 2000). Considering that South American parrots are susceptible to at least three of these practices (BERKUNSKY et al., 2017), the health survey on these animals is an important addition to the species conservation efforts.

Wild parrot nestlings caught from the wild to supply the pet trade are commonly seized by environmental authorities and sent to wildlife rehabilitation centers (IBAMA, 2016). Birds of different origins are usually mixed and placed in direct or indirect contact with the existing captive birds in the centers. Once rehabilitated, the parrots are often released into inappropriate habitats and without health criteria (MARINI; GARCIA, 2005). Therefore, pathogen infectious acquired throughout this course can be introduced and / or disseminated into the wild (FITZGERALD, 2007). In addition, exotic Psittaciformes importation without appropriate health criteria keeps a risk for the introduction of exotic diseases in Brazil.

In captivity, some of the outcome of these anthropogenic activities for the health of Amazon parrots has been investigated. An outbreak of *Chlamydia psittaci* have been reported in 58 *A. aestiva* nestlings from the illegal trade submitted to a wildlife rehabilitation center, showing a mortality rate of 96.5% (RASO et al., 2004). The prevalence of the bacteria in 26 seized *Anodorhynchus hyacinthinus* nestlings also from the illegal trade and maintained under poor husbandry conditions reached 65.4% (RASO et al., 2013). Another pathogen considered important for parrots, the Pacheco's disease (PD) herpesvirus (PsHV), has been also detected in 30 parrots from a wildlife center (LUPPI et al., 2011). The birds were recovered from illegal trade or from illegal domestic custody and showed unspecific clinical signs or sudden death. It is not known whether herpesvirus and *C. psittaci* reported at the wildlife centers were brought from the wild or acquired by the birds in the captivity, although both of them has already been investigated and observed in wild parrots in Brazil (RASO et al., 2006; ALLGAYER, 2009).

Psittacinepox is considered an important pathogen for parrot aviculturists as it can result in high losses in a short time (RITCHIE, 1995). The poxvirus has already been detected in a conservation breeding facility (ESTEVES et al., 2017) and an outbreak has been reported recently in captive exotic birds in Brazil (MURER et al., 2018). In addition, the Psittacine Beak and Feather Disease (PBFD) circovirus, an

exotic pathogen, was introduced into the country (WERTHER et al., 1999) and occasionally has been found infecting native species in wildlife rehabilitation centers (ARAUJO et al., 2015), in a zoo (HIDASI et al., 2018) and in exotic and native pet animals (AZEVEDO, 2014). However, they have never been studied in wild Brazilian parrots.

Considering the importance of psittacine birds, the threats that anthropic actions impose on the health of avian populations and the lack of information about the occurrence of diseases in wild parrots in Brazil, the aims of this thesis was to investigate the presence of selected pathogens in wild Amazon parrot nestlings in four Brazilian states and in recently seized *Amazona aestiva* nestlings from the illegal trade and submitted to a wildlife rehabilitation center.

1.1 CHAPTERS PRESENTATION

The organization of this thesis comprises six chapters, including this introductory chapter 1.

Chapter 2 briefly presents general aspects about the biology and conservation of Amazon parrots in the American continent, especially in Brazil, highlighting the species evaluated in this study. The purpose of this general introduction and chapter 2 is to make clear the interpretation of later chapters.

Chapters 3, 4 and 5 are written for publication in scientific journals. Chapter 3 is a literature review that summarizes the main characteristics of the viruses and *Chlamydia psittaci* studied in this thesis. Thus, the reader can reflect on the importance of each pathogen for Amazon parrots and Psittaciformes in general, and also an epidemiological and molecular comparison with the results obtained here. The article will be submitted to a veterinary science Journal co-authored by Tânia de Freitas Raso.

Chapter 4 presents an extensive pathogen research conducted on wild Amazon parrot nestlings, *Amazona pretrei, Amazona brasiliensis* and *Amazona aestiva* in four states of Brazil. The epidemiology of these pathogens poorly evaluated in populations and their consequences or not for the conservation of the species are explored. The article will be submitted to a multidisciplinary sciences Journal co-

authored by Elenise Angelotti Bastos Sipinski, Gláucia Helena Fernandes Seixas, Nêmora Pauletti Prestes, Jaime Martinez and Tânia de Freitas Raso.

Chapter 5 investigates the occurrence of the same pathogens in recently seized *Amazona aestiva* nestlings from illegal trade and submitted to a wildlife rehabilitation center. The article will be submitted to an infectious disease and veterinary sciences Journal co-authored by Gláucia Helena Fernandes Seixas and Tânia de Freitas Raso.

Chapter 6 concludes this thesis including final considerations, reflections and discussions about the consequences of the results found in the studies here developed. Actions that can be expected in the future and that can be added to the conservation efforts of Brazilian psittacine birds are also discussed.

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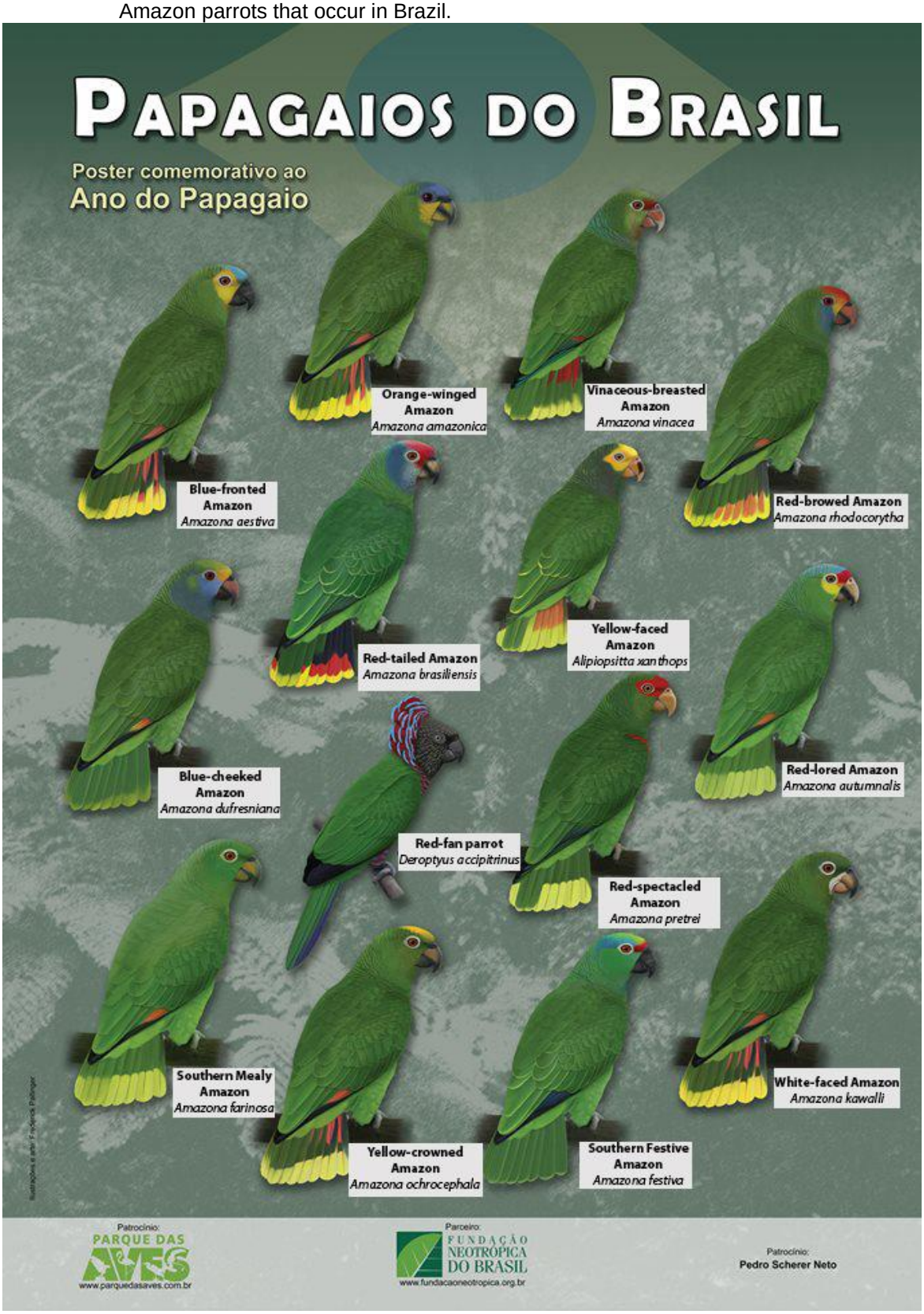
2. GENERAL ASPECTS OF AMAZON PARROTS BIOLOGY AND CONSERVATION

Psittacine birds are distributed throughout all the tropical zones around the world, extending their habitat to subtropical and slightly colder areas (IUCN, 2019). The order Psittaciformes is one of the few orders that have very specific anatomical characteristics, which allow its immediate recognition, especially for having a large and curved beak. The richness and abundance of parrots in Brazil, the country with its greatest diversity, was already described in the maps of the 1500s, which nominate the discovered area as the “Land of the Parrots” Since then, South American parrots have been included in animal trade, and records from the 1980s and 1990s reported translocations of 20,000 to 30,000 South American parrots, 3,000 to 6,000 Amazon parrots, and an estimated 7,000 trafficked *Amazona aestiva* (SICK, 1997).

Parrots of the *Amazona* genus occur only in Latin America, from Mexico to northern Argentina, although some species have been introduced in the southern United States (IUCN, 2019). The number of species currently recognized varies among the Brazilian Council of Ornithological Records (CBRO) and International Union for Conservation of Nature (IUCN) lists, according to the subspecies approach. The IUCN considers 34 existing species, 19 of which (55.9%) are included in its Red List (Table 2.1). In Brazil, the CBRO recognizes 12 Amazon species (PIACENTINI et al., 2015) (Figure 2.1), including *A. dufresniana* in the Brazilian territory, and the Chico Mendes Institute for Biodiversity Conservation (ICMBIO) recognizes three species (*A. pretrei, A. rhodocorytha, A. vinacea*) as threatened (ICMBIO, 2018). The IUCN list records one less Amazon species (n=11) in Brazil and one more endangered species (n=4), as it recognizes *A. diadema* as a separate species from *A. autumnalis*. It includes *Amazona diadema* in the “endangered” category due to its limited distribution in the Brazilian Amazon territory, unrecognizing *A. autumnalis* in Brazil (IUCN, 2019).

Many species of parrots, including the *Amazona* genus, are known to have gregarious social behavior, mainly for the formation of communal roots and foraging activities (MARTINEZ, 2004). Although they can live in groups, Amazon parrots form lifelong couples and the female lays an average of two eggs per reproductive period.

Figure 2.1 - Commemorative poster for the “Year of the Parrot”, showing the 12 species of Amazon parrots that occur in Brazil.



Source: Adapted from Sociedade de Zoológicos do Brasil. Illustration: Frederick Pallinger

Table 2.1 - Demographic, geographical and conservation characteristics of Amazon parrots.

Species*	Conservation status*	Estimate population	Population trend	Geographic distribution
Amazona aestiva	Least concern	Unknown	Decreasing	Brazil, Paraguay, Argentina, Bolivia
Amazona agilis	Vulnerable	6000-15000	Decreasing	Jamaica
Amazona albifrons	Least concern	Unknown	Increasing	Mexico, Guatemala, Nicaragua, Honduras, Costa Rica
Amazona amazonica	Least concern	Unknown	Decreasing	Brazil, Bolivia, Peru, Ecuador, Colombia, Venezuela, Guiana, French Guiana, Suriname
Amazona arausiaca	Vulnerable	850-1000	Increasing	Dominic Republic
Amazona auropalliata	Endangered	Unknown	Decreasing	Mexico, Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica
Amazona autumnalis	Least concern	Unknown	Decreasing	Mexico, Guatemala, Nicaragua, Honduras, Costa Rica, Panam, Colombia, Venezuela, Ecuador
Amazona barbadensis	Vulnerable	1700-5600	Unknown	Venezuela
Amazona bodini	Near threatened	Unknown	Decreasing	Venezuela, Guiana, Colombia
Amazona brasiliensis	Near threatened	6000-6700	Increasing	Brazil
Amazona collaria	Vulnerable	6000-15000	Decreasing	Jamaica
Amazona diadema	Endangered	Unknown	Decreasing	Brazil
Amazona dufresniana	Near threatened	Unknown	Decreasing	Brazil, Suriname, Guiana, French Guiana, Venezuela
Amazona farinosa	Near threatened	Unknown	Decreasing	Brazil, Bolivia, Peru, Ecuador, Colombia, Venezuela, Guiana, French Guiana, Suriname, Panama
Amazona festiva	Near threatened	Unknown	Decreasing	Brazil, Colombia, Ecuador, Peru, Guiana
Amazona finschi	Endangered	4700-6700	Decreasing	Mexico
Amazona guatemalae	Near threatened	Unknown	Decreasing	Mexico, Guatemala, Honduras, Nicarágua, Costa Rica, Panama
Amazona guildingii	Vulnerable	250-1000	Increasing	Saint Vincent and Grenadines

Amazona imperialis	Endangered	160-240	Increasing	Dominic Republic
Amazona kawalli	Near threatened	Unknown	Stable	Brazil
Amazona leucocephala	Near threatened	13600-23000	Decreasing	Cuba, Bahamas, Cayman islands
Amazona lilacina	Endangered	600-1700	Decreasing	Ecuador
Amazona mercenarius	Least concern	Unknown	Decreasing	Bolivia, Peru, Ecuador, Colombia, Venezuela
Amazona ochrocephala	Least concern	Unknown	Decreasing	Brazil, Suriname, Guiana, French Guiana, Venezuela, Colombia, Panama, Ecuador, Peru, Bolivia, introduced in Puerto Rico
Amazona oratrix	Endangered	4700	Decreasing	Mexico, Guatemala, Honduras, introduced in USA and Puerto Rico
Amazona pretrei	Vulnerable	Unknown	Decreasing	Brazil
Amazona rhodocorytha	Vulnerable	2500-10000	Decreasing	Brazil
Amazona tucumana	Vulnerable	6000-15000	Decreasing	Bolivia, Argentina
Amazona ventralis	Vulnerable	6000-15000	Decreasing	Dominic Republic, Haiti, introduced in Puerto Rico
Amazona versicolor	Vulnerable	230-330	Increasing	Saint Lucia
Amazona vinacea	Endangered	1000-2500	Decreasing	Brazil, Paraguay, Argentina
Amazona viridigenalis	Endangered	2000-4300	Decreasing	Mexico and introduced in USA and Puerto Rico
Amazona vittata	Critically Endangered	01-49	Unkown	Puerto Rico
Amazona xantholora	Least concern	Unknown	Stable	Guatemala

*According to the IUCN Red List of Threatened Species. Source: IUCN (2019)

Nesting occurs in natural hollows formed in tree trunks and branches present in the environment (SICK, 1997). Deforestation and habitat destruction reduce the supply of suitable nest cavities and food available to parrots, being the main factors of population decline of Amazon species. The second risk factor for the conservation of these birds is the catching of wild nestlings to supply the illegal trade (SNYDER, 2000). In addition, the annual hurricane season is also responsible for the decline of endemic island populations in Central America (WHITE et al., 2005).

In Brazil, conservation projects have been created for Amazon parrots, which are part of the National Action Plan for the Conservation of Atlantic Forest Parrots, an initiative of the ICMBio that includes actions to achieve conservation goals for these species (SCHUNCK et al., 2011).

The Red-tailed Amazon Parrot Conservation Project was created in 1997 by the Society for Wildlife Research and Environmental Education (SPVS) to protect the species. The main activities of SPVS include increase of scientific knowledge about the species; environment education; monitoring of breeding, feeding and communal roosts sites; annual censuses and installation of artificial nest boxes on the coast of Paraná (SCHUNK et al., 2011). The Red-tailed Amazon parrot (*A. brasiliensis*) occurs in a restricted coastal area from northern Santa Catarina to southern São Paulo (Figure 1.2). Its largest population (80%) is concentrated in the state of Paraná, counting on about 9,112 individuals (SPVS, 2018). The species was considered vulnerable until the release of the latest ICMBio list, in which its category was raised to near threatened. Based on data obtained from SPVS efforts, the population of *A. brasiliensis* has been stable over the years (ICMBIO, 2018; SIPINSKI et al., 2014). Nevertheless, the Red-tailed Amazon parrot is especially vulnerable to environmental disturbance because of to its occurrence in a narrow coastal strip, and in some places the population still needs management in the wild. In Paraná, the *A. brasiliensis* is mostly found in protected areas. However, they lack proper supervision, enabling nest poaching and logging of forest species (SCHERER-NETO, 1989; SIPINSKI, 2003). In São Paulo, urban growth, deforestation and nest poaching are still intense impacts affecting the parrot population (MARTUSCELLI, 1995; SCHUNCK et al. 2011). In

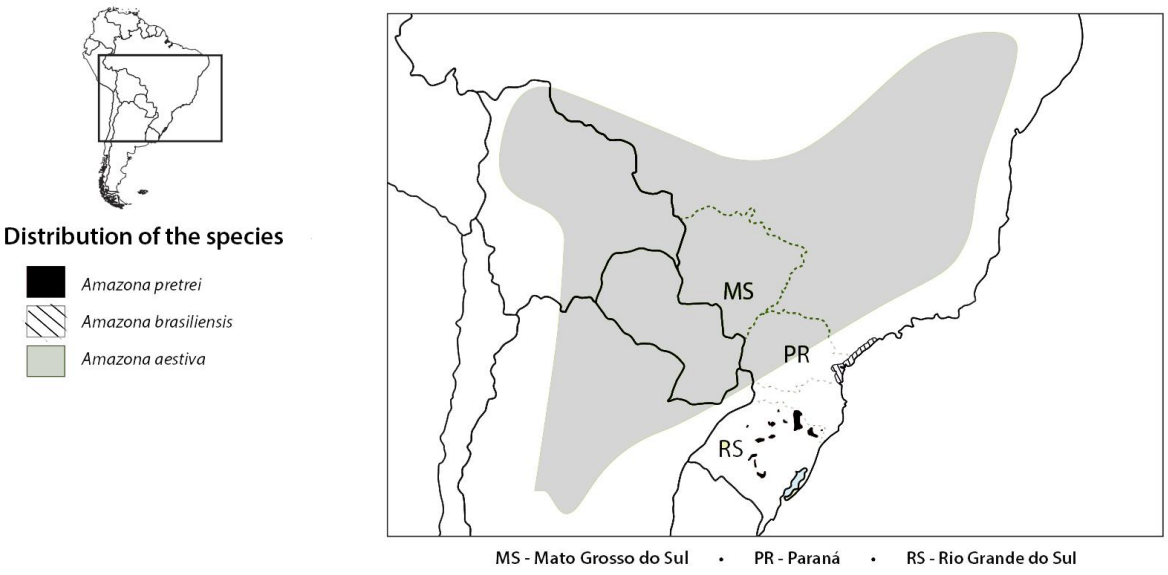
the state of Santa Catarina, the species has no current records and lacks information, which makes conservation actions difficult in the area (SPVS, 2018).

The Blue-fronted Amazon Parrot Project was created in 1997 by the Mato Grosso do Sul Environmental Institute (Imasul)/Wildlife Rehabilitation Center (CRAS), and from 2004 onwards it was implemented by the Neotropic Foundation of Brazil (SEIXAS, 2009). The project proposes actions to conserve the species and its environment and curb illegal exploitation, as well as expand knowledge about its biology, ecology and interaction with the habitat. The *A. aestiva* occurs in several biomes, such as the Cerrado, Caatinga, Pantanal and Chaco, occupying almost all regions of Brazil (Northeast, Southeast, Midwest and North), as well as eastern Bolivia, southern Paraguay and northern Argentina (FORSHAW; COOPER, 1989) (Figure 1.2). Despite categorized in the least concern category, the species is intensely pressured by deforestation activities and the collection of wild nestlings in all environments where it occurs, being the most commonly parrot species found in wildlife rehabilitation centers in Brazil (SEIXAS; MOURÃO, 2000; IBAMA, 2016). Over the past 30 years, more than 10,000 nestlings have been recovered from illegal trade and sent to a wildlife rehabilitation center in Campo Grande, state of Mato Grosso do Sul (TOMAS et al., 2019). Hundreds of nestlings are seized every year by environmental authorities, and it is likely that this is only a small part of the total parrot chicks taken from natural nests to supply the illegal domestic and international trade (TOMAS et al., 2019), but not registered for lack of supervision or die during capture, transportation or in confinement by the trapper (IÑIGO-ELIAS; RAMOS, 1991).

The Red-Spectacled Amazon Parrot Project was created in 1991 by the Associação Amigos do Meio Ambiente and the Institute of Biological Sciences of the Passo Fundo University. The project operates mainly in the preservation of the remaining native forests occupied by the red-spectacled Amazon parrot (*A. pretrei*), such as the Araucarias Forest, with the creation of conservation units. The project also encourages forest replacement programs, conducts a captive breeding program, environmental education programs, radiotelemetry and nesting boxes installation (MARTINEZ; PRESTES, 2002). The red-spectacled Amazon parrot occurs in the states of Santa Catarina and Rio Grande do Sul in Brazil, mainly in private properties and not in protected areas, being rarely seen in Argentina (IUCN, 2019) (Figure 1.2).

It is the only Amazon species to have sexual dimorphism, as the male presents greater extension of the red color on the head and wings (SCHUNCK et al., 2011). The *A. pretrei* is within the vulnerable category in the ICMBio Red List, having an estimate population of 20,000 wild individuals (ICMBIO, 2018). During the breeding period, which occurs from August to January, the populations occupy a vast region of the state of Rio Grande do Sul. After this period, from March to August, the parrots migrate to the southeast of Santa Catarina to feed on *Araucaria angustifolia* seeds, your main food item. The destruction of Araucaria forests is the main factor of the species populations decline, which also are pressured by the capture of wild nestlings to supply regional illegal trade of parrots (MARTINEZ; PRESTES, 2002; MARTINEZ, 2004).

Figure 2.2 – Distribution of *Amazona pretrei*, *Amazona brasiliensis* and *Amazona aestiva* in South America.



Source: Adapted from IUCN (2019)

The three species mentioned above, *A. aestiva, A. brasiliensis* and *A. pretrei*, are part of the National Action Plan for the Conservation of Parrots from the Atlantic Forest (SCHUNCK et al., 2011), included in the plan because they are vulnerable to anthropogenic actions and/or are endangered. An epidemiologic study of these three species populations could provide an overview of the health of parrots in areas with

intense anthropic activity and in more isolated and preserved areas, as the species occur in different habitats and regions of Brazil, with different dynamics of population and environmental impacts. In addition, sample collection from these populations is facilitated by the logistics already established by conservation projects that have been in existence for decades.

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3. *CHLAMYDIA PSITTACI* AND VIRAL DISEASES IN PSITTACINE BIRDS

3.1 ABSTRACT

Parrots are very popular pet animals because of their social and beauty characteristics, which created a global domestic demand and an international trade of these species. The movement of psittacine birds also allowed the spread of their diseases, which are currently a concern for captive birds and have increased conservation issues of wild populations. The present review was elaborated to compile and critically review the information available in the literature about relevant parrot pathogens, *Chlamydia psittaci, Psittacid alphaherpevirus 1*, poxvirus and *Beak and feather disease virus.* Epidemiology and treatment/control updates were reunited and summarized, to clarify characteristics of the diseases for their better understanding.

3.2 INTRODUCTION

One of the most studied groups of birds is the Psittaciformes. A demand for information about this group was created as parrots have been favored as human pets and need care in captivity. Parrots are very popular because of their beauty, sociability and ability to imitate the human speech (SNYDER et al., 2000). These characteristics led psittacine birds to be intensively traded around the world, which also allowed the spread of their diseases and have increased conservation issues (PHALEN, 2015).

Pathogens believed to be originated in South America parrots have been seen in wild Australian cacatuids, as demonstrate for *Chlamydia psittaci* (SUTHERLAND et al., 2019). Australia has been identified as the most likely origin of the *Beak and feather disease virus* (BFDV), which are now spread in wild populations in the African, European and Asian continents (FOGELL et al., 2018).

So, for a better understanding of the current psittacine disease epidemiology, control and treatment updates, this review was elaborated. The information available in the literature about *C. psittaci* and selected viral pathogens, *Psittacid*

alphaherpevirus 1, poxvirus and BFDV were examined and the main characteristics of the disease were summarized, trying to make it clear for veterinarians to read.

3.3 CHLAMYDIOSIS

The chlamydiosis caused by *Chlamydia psittaci* is a bacterial infectious disease of birds and mammals, including the human species, in which it is denominated psittacosis, being one of the main zoonoses transmitted by birds, mainly due the human contact with pet birds (CROSTA; MELILLO; SCHNITZER, 2016). It is a respiratory disease, usually systemic and is some cases fatal, and despite its importance for several species, it is often underestimated, having a worldwide distribution (RASO, 2014).

3.3.1 Etiology

Chlamydia psittaci is a gram-negative obligate intracelullar bacteria belonging to the order Chlamydiales, Family Chlamydiaceae, which also includes other species that have recently been discovered in birds, *C. avium* and *C. gallinacea* (SACHSE et al., 2014). To date, nine serotypes corresponding to nine genotypes are known (KNITTLER; SACHSE, 2015) (Table 3.1).

C. psittaci has a single chromosome containing 1.1 Mb, and a conserved plasmid containing approximately 8 kb (KNITTLER et al., 2014). The bacterium has two morphologically distinct forms. The elementary body (EB) is the inactive, infectious form that attaches to the host epithelial cells for bacterial entry (GERLACH, 1994a). In the cell, surrounded by an intracellular membrane, the EB transforms into larger, metabolically active reticular body (RB). Reticular bodies divide by binary fission and within 24-72h become mature, and transform back into infective new EBs, which are released from the cell to infect new neighboring cells and new hosts (KNITTLER; SACHSE, 2015).

Table 3.1 - *Chlamydia psittaci* genotypes most commonly associated with disease in their respectively hosts.

***Chlamydia psittaci* genotypes**	**Host predilection**
A	Psittaciformes
B	Pigeons and turkeys
C	Water fowl
D	Turkeys
E	Pigeons, ducks and other species
E/B	Ducks
F	Parakeets
WC	Cattle
M56	Rodents

Source: Adapted from Knittler and Sachse (2015).

3.3.2 Pathogenesis

Chlamydia psittaci shedding occurs mainly by feces and respiratory secretions in an infected bird, but also is transmitted by urine, tears, oropharyngeal mucus and crop milk secretions (in Columbiformes). Susceptible birds in contact with hosts may become infected by inhaling or ingesting the pathogen. *Chlamydia* first replicates within 24 hours in lungs and / or air sacs, epithelial cells and macrophages when inhaled (KNITTLER; SACHSE, 2015). After 48 hours, chlamydemia occurs probably within monocytes (BEECKMAN; VANROMPAY, 2010), and the bacteria reaches other organs such as the conjunctiva, gastrointestinal tract, spleen, kidneys, and liver (PAGE; BANKOWSKI, 1959; KNITTLER; SACHSE, 2015), being eliminated in the faeces after 72 hours, a cycle that provides rapid dissemination (CROSTA; MELILLO; SCHNITZER, 2016).

The incubation period varies from three days to a few weeks. Altricial nestlings can become infected mainly when fed by their parents through regurgitation, and precocial chicks can be infected through infectious exudates and contaminated faeces around their nests (VAROMPAY, 2013). Vertical transmission has been demonstrated in some species (LUBLIN et al., 1996), and invertebrates (mites and bloodsucking insects) can serve as bacteria carriers (EDDIE, 1962; ROSSI-PERAZZA; RASO, 2017), helping to spread them.

Chlamydia has several immune system escape mechanisms. The first of them is the inhibition of fusion of its membrane (acquired by the cell) with the lysosome, shortly after entry into the host cell, which prevents its digestion by the phagolysosome, especially when infecting macrophages (KNITTLER; SACHSE, 2015). The second mechanism involves the presence of genus-specific lipopolysaccharides in the RBs, which increase membrane viscosity and prevent the action of T lymphocytes. In the third mechanism, the host cell activates an enzyme system that destroys itself, but retains its replicative properties, remaining permanently infected and continuously releasing EBs without its lysis (CROSTA; MELILLO; SCHNITZER, 2016). However, at the end of their developmental stage, the bacteria produce proteases that lyse the cells for complete release of EBs. In addition, when macrophages undergo mitosis, chlamydiae survive within these cells, and can infect newly generations, avoiding the extracellular environment and circulating antibodies (GERLACH, 1994a). Finally, in the presence of growth inhibitors, such as IFN-γ, or stress situations in the host cell, such as the reduction of available nutrients, intracellular chlamydiae can develop into a non-replicating aberrant and persistent form until normal conditions are restored (VANROMPAY, 2013; KNITTLER; SACHSE, 2015). All of these mechanisms can cause the host to have a long time of infection, and often a lifelong infection.

The pathogenesis of chlamydia does not only rely on host cell lysis, which will cause the lesions and later associated clinical signs (VANROMPAY, 2013). It also depends on the virulence of the highly variable EBs outer membrane protein (MOMP). Strains of *C. psittaci* adapted to a particular host species are likely to be of low virulence, causing chronic infection and mild to moderate disease, but of high virulence to other species, causing lethal reactions, especially at high bacteria levels (ANDERSEN; FRANSON, 2007).

3.3.3 Epizootiology

In many countries where it has been studied, *C. psittaci* is considered endemic and has been detected in more than 467 wild or captive avian species within 30 orders, being the majority of positive birds within the order Psittaciformes (45% - 153/342) (KALETA; TADAY, 2003). All avian species can be considered susceptible

to the bacterium, but this high percentage observed in psittacine species may be a result of the amount of analyzes and studies performed on birds of this order, as they are very popular pet animals and possible sources of infection for humans (ANDERSEN; FRANSON, 2007). Wild animals have been recognized as important reservoirs of the agent (RASO et al., 2006), and in captivity the morbidity rate may be high, with a percentage of inapparent carrier parrots ranging from 10% to 40%, reaching 100% in some places (FUDGE, 1996; RASO; JUNIOR; PINTO, 2002), mainly in young birds (ANDERSEN; FRANSON, 2007).

The clinical manifestation and increased shedding of the bacteria may be triggered by stressful situations, which occur, for example, in birds seized from illegal trade, which experience extreme situations of overpopulation, inappropriate hygiene, handling, temperature and feeding (FUDGE, 1996). In addition, many birds are carriers of the bacterium and remain chronically infected without showing clinical signs until they go through such situations, and intermittently sheds EBs, representing a significant source of infection for humans and other birds (RASO, 2014). However, although studies have been performed, the prevalence of chlamydiosis in birds is considered underestimated, as its definitive diagnosis is difficult, the clinical signs are unspecific, and standardized commercial tests and reagents are lacking (RASO, 2014).

The presence of *C. psittaci* in Brazil was first reported in 1998 in captive Amazon parrots by direct immunofluorescence reaction, with a prevalence of 35.8% (34/95) (RASO; JUNIOR; PINTO, 2002). Since then, similar prevalence has been found in captive parrots (28.3% in 237 individuals of *Amazona, Ara, Anodorhynchus, Pionus* and *Pionites*) (RASO; CARRASCO; PINTO, 2009) and parrots from illegal trade (35% in 26 *Anodorhynchys hyacinthinus*) (RASO et al., 2013). The morbidity rate is usually high because of chlamydia cycle and its type of transmission, including inapparent carriers, but it depends on the bird species, handling, the establishment of persistent infections and the virulence of the strain (VANROMPAY, 2013). The mortality rate also depends on these factors and can reaches up 96.5%, as observed in an outbreak involving 58 *A. aestiva* nestlings from illegal trade (RASO et al., 2004).

In the wild, *C. psittaci* DNA has been detected in *A. aestiva* (2/32, 6.25%) and *A. hyacinthinus* (16/45, 35.5%) nestlings in Pantanal, Mato Grosso do Sul (RASO et al., 2006), and in an *A. brasiliensis* nestling in Rasa Island, Paraná (1/117, 1.2%)

(RIBAS et al., 2014). In another study performed in Rasa Island, *C. psittaci* DNA was not found in 58 *A. brasiliensis* (VAZ et al., 2017). Therefore, the bacterium is endemic in Brazilian parrots, but it is likely to have a low prevalence in Amazon species and medium prevalence in Hyacinth macaws and does not appear to cause worrying mortality (RASO et al., 2006; VAZ et al., 2017). In wild Australian Psittaciformes, the prevalence of *C. psittaci* (1.81% in 55 cockatoos) (SUTHERLAND et al., 2019) has been similar to the ones reported in Brazil.

3.3.4 Clinical signs

Chlamydiosis can be divided into four clinical categories: superacute, acute, chronic and inapparent. In the superacute form, which occurs mainly in young birds, the parrots do not show clinical signs and die within a few hours. In the acute form, clinical signs are nonspecific and include anorexia, lethargy, dehydration, regurgitation, vomiting, greenish diarrhea, ruffled feathers, ocular and nasal secretions, conjunctivitis, respiratory changes, greenish-yellow urates, and neurological signs such as tremors or torticollis (LONGBOTTOM; COULTER, 2003; RASO et al., 2004; ORNELAS-EUSEBIO et al., 2016). The chronic form includes progressive weight loss, mild respiratory signs and subtle conjunctivitis. In the inapparent form, the birds are carriers, do not show clinical signs, can spread the agent intermittently, and may manifest the disease when immunosuppressed (LONGBOTTOM; COULTER, 2003).

3.3.5 Diagnosis

Clinical signs of chlamydiosis and basic complementary exams provide a basis to help diagnose the disease, but confirmation of suspicion is only made by laboratory testing and direct agent detection (RASO, 2014). Hematological and biochemical tests help in the diagnosis and may reveal anemia, leukocytosis with heterophilia and monocytosis, increases in bile acids, AST and uric acid. Radiographic and endoscopic examinations reveal signs of pneumonia, aerosaculitis, splenomegaly and hepatomegaly (CROSTA; MELILLO; SCHNITZER, 2016).

Hepatomegaly and splenomegaly are common findings at necropsy associated with chlamydiosis, with or without necrotic foci on their surfaces. The liver may be friable and yellowish, the spleen darkened, soft and covered with whitish gray spots (VANROMPAY, 2013). Necrotizing lesions can also be found in the respiratory system, in the pericardium, associated by fibrinopurulent serositis (ANDERSEN; FRANSON, 2007). The air sacs may be thickened and contain fibrinous exudate (VANRONPAY, 2013). Microscopic lesions include multifocal necrosis and inflammatory reactions in the affected organs, especially in the liver and spleen, with the presence of basophilic inclusion corpuscles in the macrophage cytoplasm. Lymphocyte depletion can be observed in lymphoid tissues such as the spleen, which are replaced by macrophages (SUWA et al., 1990).

The diagnosis of the disease can be difficult, and a single testing method might not be adequate. A diagnosis can be conclusive when positive Gimenez or Macchiavello staining techniques are associated with positive PCR for *C. psittaci* in the same tissue. In addition, isolation (gold standard) of *C. psittaci* can be performed on cell culture or SPF embryonated chicken eggs, also proving the clinical suspicion, but requires a Biosafety Level 3 Laboratory. Confirmation of disease occurrence is also performed when serum samples collected at two-week intervals reveal a four-fold or greater rise in antibody titer. Finally, in situ hybridization can be performed followed by PCR in the same tissue for the diagnosis of bacteria (BALSAMO et al., 2017).

3.3.6 Treatment and control

The treatment of choice for chlamydiosis in birds is based on tetracyclines. In any of the therapeutic protocols used, treatment of *C. psittaci* can be very challenging and unsafe, adverse effects can occur, no complete elimination of infection is guaranteed, and it always should be supervised by an avian veterinarian (BALSAMO et al., 2017).

Doxycycline is the first choice antibiotic employed because of its characteristics of bioavailability, low hepatotoxicity and nephrotoxicity, and low influence on calcium intake relative to other tetracyclines (CROSTA; MELILLO; SCHNITZER, 2016). Studies have shown that administration of doxycycline in drinking water and/or food

may be effective in some cases, but in others studies it did not eliminate the infection in the aviary (RASO et al., 2002; PADILLA; FLAMMER; MILLER, 2005). These cases prove that drug administration without control of food/water intake, proper cleaning and disinfection of certain environments is not effective in controlling the disease. Oral or injectable administration of antibiotics, along with proper cleaning and disinfection of the environment, can ensure successful treatment (BALSAMO et al., 2017).

Elementary bodies may resist many weeks in organic matter outside the host, but when “free” in the environment they are unstable and inactivated within a few days. *Chlamydia psittaci* is sensitive to heat, and are inactivated by 1% formaldehyde, 1:1000 dilution of quaternary ammonium compounds, 3% hydrogen peroxide, 70% ethanol and chlorine solutions (GERLACH, 1994a; BALSAMO et al., 2017).

Birds being treated should be kept housed in a separated air space from other birds, in clean cages, free of stressful situations and within adequate husbandry and population density. These birds should be cared for after healthy birds housed in the same aviary. Caution is recommended when introducing new birds into aviaries or birds returning from exhibitions and other events, establishing quarantine periods of at least 30 days and testing before adding them to a group using serial samples (RASO, 2014). Individual screening of these birds can be performed using a PCR assay on cloacal, oropharyngeal and conjunctival swab samples (BALSAMO et al., 2017).

Avian veterinarians must act as educators, informing persons at risk, like bird caretakers and pet bird owners, about potential health risks. Avian chlamydiosis is a notifiable disease in Europe, but is not in Brazil, so the prevalence is underestimated (RASO, 2014).

3.4 PACHECO’S DISEASE

Pacheco’s disease (PD) is almost exclusively a Psittaciformes disease, first reported in Brazil as an acute, fatal, inflammatory liver syndrome in the 1930s (PACHECO; BIER, 1930). The disease agent, a herpesvirus, was only confirmed in 1975 in the United States by electron microscopy of bird hepatocytes that died in an

aviary, which had PD-like lesions (SIMPSON; HANLEY; GASKIN, 1975). Herpesvirus establishes long-term latent infection in animals that recover from the disease or even show no clinical signs, eliminating intermittently the pathogen and keeping the agent in the population (RITCHIE, 1995). In addition, depending on the genotype involved, the virus can cause neoplasia and papillomas in the mucosa of parrots that survive the PD (JOHNE et al., 2002; PHALEN, 2016).

3.4.1 Etiology

Pacheco's disease is caused by a herpesvirus belonging to the Order Herpesvirales, Family Herpesviridae, Subfamily Alphaherpesvirinae, genus *Iltovirus*, species *Psittacid alphaherpesvirus 1* (PsHV) (ICTV, 2018). There is only one other species within the genus *Iltovirus*, the *Gallid alphaherpesvirus 1*, which causes infectious laryngotracheitis in poultry. Herpesviruses are double-stranded, enveloped DNA ranging from 120 to 200 nm in diameter, being the PsHV genome composed of approximately 163,025 base pairs (THUREEN; KEELER, 2006).

Four genotypes and three serotypes have been identified for PsHV, which contains certain similarities (Table 3.2) but different biological characteristics (TOMASZEWSKI; KALETA; PHALEN, 2003). In addition, variants have been found within PsHV genotypes, evidencing the presence of viral polymorphism, with six (LUPPI et al., 2016), ten (TOMASZEWSKI et al., 2001) and up to 12 patterns (SCHRÖDER-GRAVENDYCK et al., 2001) identified by RFLP and PCR.

Table 3.2 - Serotypes and potential Psittaciformes diseases for the four *Psittacid Herpesvirus 1* genotypes

Genotype	Serotype	Pacheco's Disease	Mucosal papilloma	Bile duct carcinoma
1	1	Amazon parrots, Australian species	Uncommon	No
2	2	Amazon parrots, *Psittacus erithacus*	Uncommon	No
3	3	Amazon parrots	Very common	Yes
4	1	Most species	No	No

Source: Adapted from Phalen (2016).

3.4.2 Pathogenesis

Excretion of PsHV by infected parrots occurs through feces and pharyngeal secretions (GASKIN; ROBBINS; JACOBSON, 1978). Birds with clinical disease can shed a high viral load, reaching 10,000,000 virions/g of feces in budgerigars after 48 hours of exposure to the virus (RITCHIE, 1995). Viral excretion occurs on average three to seven days after exposure to PsHV in other avian species. Virions present in feces and respiratory tract secretions may be inhaled or ingested by other birds, which become infected by horizontal transmission, and vertical transmission is not yet proven for PsHV (RITCHIE, 1995; KALETA; DOCHERTY, 2007). The incubation period of PD can vary from five to seven days (PHALEN, 2016), and once in the bird's organism, PsHV has mainly liver tropism, in which it multiplies, causes cell lysis and can lead to a severe liver disease (RITCHIE, 1995).

Herpesviruses have some immune system escape mechanisms. Once in the host, the first dissemination occurs from cell to cell, without reaching the extracellular environment and possible circulating antibodies (RITCHIE, 1995). However, the main escape mechanism of herpesviruses is their strategy of latency in the host, which can remain lifelong infected. *Gallid alphaherpesvirus-1*, for example, evades to the trigeminal ganglion, and when the bird recovers or even in the subclinical form of the disease, it can eliminate the virus when exposed to stressful conditions (GARCÍA; SPATZ; GUY, 2013). There are no studies showing the location of PsHV latency in parrots, but intermittent shedding for at least five years has been observed in samples collected from virus-infected hosts (TOMASZEWSKI; WIGLE; PHALEN, 2006). In addition, the study showed the presence of PsHV in the oral and cloacal mucosa, in organs such as the duodenum, jejunum, colon, spleen, crop, ventricle, oviduct, sciatic nerve and adrenal gland.

3.4.3 Epizootiology

The course of the disease, its intensity and incubation period vary according to the avian species and its susceptibility, as well as the viral strain, viral load and route of exposure, and parrots of any age and gender may succumb to PD. Mortality is

varied and only some individuals can be affected (O-TOOLE et al., 1992), but it can reaches 30 to 50% of the aviary (BISTYÁK et al., 2007; BARBOSA et al., 2020 *in press*), and up to 100% of certain species from the collection (GASKIN; ROBBINS; JACOBSON, 1978). Amazon parrots are the most susceptible species (RITCHIE, 1995), mainly to genotypes 1, 2 and 3. Genotype 4 is the most common cause of PD in macaws and small and medium size parrots, such as *Aratinga* species (PHALEN, 2005). Nevertheless, genotype 1 has already been found in macaws, *Aratinga* sp., *Pionus* sp., *Pyrrhura* sp. and other species (LUPPI et al., 2016). Cockatoos, cockatiels and other Australian birds are relatively resistant to the disease. However, all species of Psittaciformes are potentially susceptible to infection (PHALEN, 2005). Rare cases have been observed in other avian orders, such as Passeriformes (TOMASZEWISCK et al., 2004) and Piciformes (BISTYÁK et al., 2007).

Pacheco's disease outbreaks have been reported in captive birds in the American (GASKIN; ROBBINS; JACOBSON, 1978; BARBOSA et al., 2020 *in press*), European (RANDAL et al., 1979), African (KALINER, 1975), Asian (TSAI et al., 1993) and Oceania (DURHAM; GUMBRELL; CLARK, 1977) continents, currently having worldwide distribution; a consequence of the international bird trade (RITCHIE, 1995). Most of the outbreaks have a history of birds with latent herpesvirus infection being introduced into naive aviaries or birds that have been transported to exhibitions with other individuals, become infected and brought the virus to their aviaries.

Shedding and spread of PsHV is also facilitate by the illegal trade route, where wild birds are caught, undergo intense stress, are placed in captivity, being in close contact with each other in high population density and with improper husbandry (LUPPI et al., 2011). An extremely significant outbreak occurred in the years of 1977/1978 in animals imported from Paraguay, which may have been caught in Brazil and Argentina, to the United States, in which nearly 7,000 birds of eleven species died, including 1,380 *A. aestiva*, with a loss of US$ 150,000 to US$ 250,000 (GASKIN; ROBBINS; JACOBSON, 1978).

The PD was first reported in Brazil and soon after in birds imported from South America to the United States. South America has been cited as the most likely origin of PsHV (RITCHIE, 1995). Serological studies in Bolivia (DEEM et al., 2005) and Peru (KARESH et al., 1997) have reported anti-PsHV antibodies in wild parrots. Another study detected PsHV DNA in *Anodorhynchus hyacinthinus* in the Pantanal

through nested-PCR, with a prevalence of 12.4% (11/112) (ALLGAYER, 2009). Nevertheless, the molecular characterization of the agent in wild birds is a gap in the literature, as PsHV gene sequences from these individuals are not available in GenBank.

Although PD was first described in Brazil, few studies have been conducted for a better understanding of PsHV in the country. One research identified the virus in an *A. aestiva* and a captive *Psittacula krameri* by in situ hybridization in liver samples (GODOY, 2001). Another study used isolation, PCR, sequencing (LUPPI et al., 2011) and genotypic characterization by DNA restriction fragment polymorphism analysis in captive *Amazona* sp, *Pyrrhura* sp, *Pionites* sp, *Aratinga* sp and others captive parrots from a wildlife center (LUPPI et al., 2016). This last study was able to identify PsHV genotype 1 in samples of 13 parrot species. Recently, an abnormal mortality occurred in a breeding facility in the state of São Paulo, with the death of 32.7% (98/300) parrots, with PsHV DNA detected in 61 animals by nested-PCR and sequencing (BARBOSA et al., 2020 *in press*).

3.4.4 Clinical signs

Psittacine herpesvirus can cause severe disease and high mortality even before birds exhibit clinical signs (KALETA, 1990), showing a crop full of food and normal nutritional score. Therefore, PD should be considered as a differential diagnosis whenever a bird dies unexpectedly and when multiple deaths occur within a short period of time (PHALEN, 2016). Signs are usually nonspecific when they occur and the bird rarely survives (hours to two days), shedding large amounts of virus in feces and oropharyngeal secretions before death (TOMASZEWSKI et al., 2001).

Signs may include lethargy, depression, anorexia and greenish or yellowish urates. Regurgitation, bloody diarrhea and neurological signs are less reported (PHALEN, 2005). The virus becomes latent in surviving birds, and can be reactivated and cause disease after stress situations and immunosuppression (KALETA, 1990). Many infections can be subclinical and the host may carry the pathogen. In addition, these birds and those that survive the disease are strong candidates for mucosal papillomas if they are infected with genotype 3 (PHALEN, 2016).

Biliary and pancreatic neoplasia have also been associated with this genotype (STYLES, 2005). Papillomas may appear mainly in the oral cavity and cloaca. Advanced lesions may cause respiratory signs, strain to defecate, bloody faeces, and the papilloma may protrude through the cloaca. Other tumors, when they appear, are clinically characterized as chronic liver disease, including weight loss, overgrown beak and poor feather quality (PHALEN, 2016).

3.4.5 Diagnosis

Aviary history, clinical signs, and laboratory tests help with diagnosis but are not specific and birds usually die before any alterations can be observed. In the clinic, blood tests may reveal increased AST and leukopenia. Hepatomegaly, splenomegaly and nephromegaly may be observed on radiographic examination (PHALEN, 2016). Some birds will only have subtle liver changes, similar to liver lipidosis at necropsy.

When the bird survives longer, macroscopic findings may reveal hepatomegaly, yellowish liver with hemorrhagic areas; splenomegaly with or without areas of hemorrhage, and enlarged kidneys. Pancreatitis and enteritis may be noted less frequently (RITCHIE, 1995). Most birds will have good body condition. Microscopically, moderate to severe hepatic and splenic necrosis may be observed, containing eosinophilic intranuclear inclusions in these organs, even with little inflammatory response. Certain genotypes can cause pancreatic, intestinal and renal necrosis, with inclusion corpuscles also present (PHALEN, 2016).

Definitive diagnosis is made by tissue isolation, immunohistochemistry, PCR or electron microscopy. Cloacal, oropharyngeal swab and blood samples can be collected for a PCR assay (PHALEN, 2016).

3.4.6 Treatment and control

Monovalent vaccines have already been developed for PsHV (PHALEN, 2005), but protection against one of the serotypes does not seem to guarantee protection against the other ones, as two serotypes have been found infecting the same host, proving absence of cross-protection (TOMASZEWSKI; WIGLE; PHALEN,

2006). In addition, vaccines protect the population from disease manifestation but do not prevent infection and shedding of the virus, so birds keep disseminating PsHV in the environment (RITCHIE, 1995). PsHV vaccines, besides being ineffective, are forbidden and not available in Brazil, therefore they are not a control measure that can be used in the country.

Prophylactic use of acyclovir can minimize ongoing outbreaks in an aviary and reduce the mortality rate and should be administered orally for at least seven days (NORTON et al., 1991; PHALEN, 2016). However, these treated birds still infected and can shed PsHV in the future (RITCHIE, 1995).

Hygiene improvement and proper cleaning and disinfection of environmental and cages assist in preventing the spread of disease. Alphaherpesviruses are sensitive to lipolytic agents such as chloroform and ether, and to common disinfectants. *Gallid alphaherpesvirus-1* infectivity is inactivated after 15 minutes of exposure at 55°C, or when exposed for 48 hours at temperatures of 38°C. Under laboratory conditions, GaHV-1 can be inactivated by 5% phenol, 3% cresol and 1% sodium hydroxide solutions in less than one minute of exposure (GARCÍA; SPATZ; GUY, 2013).

A proper quarantine of recently acquired birds is recommended, paying attention to species commonly reported as PsHV latent hosts such as Amazon parrots, *Aratinga* sp., *Cyanoliseus patagonus* and macaws (PHALEN, 2005). PCR is the currently most widely used technique in the diagnosis of PsHV, allowing rapid detection of the virus in tissues and live birds, thus enabling early husbandry actions to prevent the spread of the disease. In addition, PCR allows detection of birds with subclinical infection and is essential in reintroduction programs for birds in the wild to prevent the introduction of the virus into threatened native populations (TOMASZEWSKI et al. 2001)

3.5 AVIAN POX

Avian pox is a disease characterized by the formation of discrete proliferative nodules in the unfeathered areas of birds or proliferative lesions in the mucous areas of the upper respiratory and digestive tract (VAN RIPER III; FORRESTER, 2007). All bird species are considered susceptible to some *Avipoxvirus* strain. More than 278

species from 23 avian orders have been reported as natural hosts (BOLTE; MEURER; KALETA, 1999), being very common in commercial poultry and wild birds (TRIPHATY; REED, 2013).

3.5.1 Etiology

Poxviruses are among the largest and most complex of all animal-infecting viruses. They are enveloped double stranded DNA viruses, containing approximately 300 kb in their genome (KING et al., 2012). They belong to the family Poxviridae, Subfamily Chordopoxvirinae, genus *Avipoxvirus* and contain 10 species recognized by the ICTV that affect birds: *Fowlpox virus, Canarypox virus, Juncopox virus, Mynahpox virus, Pigeonpox virus, Psittacinepox virus, Quailpox virus Turkeypox, Starling virus, Quailpox virus, Sparrowpox virus* (ICTV, 2018).

The various *Avipoxvirus* species are believed to have originated from the same virus, and although they differ in host specificity, being immunologically and antigenically different, they are related in certain characteristics (VAN RIPER III; FORRESTER, 2007). *Fowlpox virus* DNA, for example, is quite similar to *Pigeonpox virus* and *Juncopox virus*, but different from *Quailpox virus, Canarypox* virus and *Mynahpox virus* (TRIPHATY; REED, 2013).

3.5.2 Pathogenesis

Poxvirus transmission between birds occurs either through direct contact with an infected host or indirect contact through a contaminated object, never through intact skin, but through mucous membranes or bruised skin (RITCHIE, 1995). Eleven species of mosquitoes and *Dermanyssus gallinae* can also spread the disease when feeding on an infected bird and later on a susceptible host, and mosquitoes can harbor the virus for many weeks and months (RITCHIE, 1995; TRIPHATY; REED, 2013). Other insects may serve as mechanical vectors of the virus, especially in eye infection (TRIPHATY & REED, 2013).

The spread of the virus through respiratory secretions (since replication occurs in the lungs) (MOCKETT; DEUTER; SOUTHEE, 1990), aerosol in contaminated environments, and artificial insemination (TRIPHATY & REED, 2013) have been

suggested. In addition, some birds may become asymptomatic carriers and develop persistent infections, intermittently eliminating the virus from the gastrointestinal tract (GERLACH, 1994b). The infection may become latent for years and stressors may reactivate the virus. The incubation period for natural infections can range from four days to almost a month, depending on viral load, strain, and host characteristics (RITCHIE, 1995).

The poxvirus initiates its replication in the cytoplasm of skin cells, causing hyperplasia as a cellular response to infection (VAN RIPER III; FORRESTER, 2007). Some poxvirus infections are limited to the site of virus penetration, causing the cutaneous form of the disease. Other viruses reach the bloodstream and cause systemic infection, first settling in the liver and bone marrow, where they replicate again, undergo a second viremia, and cause damage to other organs (RITCHIE, 1995). When the virus is inhaled or ingested, the diphtheric form may appear, concomitant or not with the occurrence of the cutaneous form. The ability to cause hyperplasia of infected tissue is suggestively attributed to a gene belonging to the poxvirus, which encodes a protein similar to epidermal growth factor (TRIPHATY; REED, 2013).

3.5.3 Epizootiology

The occurrence of avian pox is quite common in poultry and wild birds, having worldwide distribution, especially in tropical and subtropical regions (BOLTE; MEURER; KALETA, 1999) (Figure 3.1). Clinical signs are common in canaries, young domestic pigeons, free-range chickens and wild birds (PHALEN, 2005). However, most avian species are considered susceptible to some strain of the virus, regardless of age and gender. More than 278 species from 23 avian orders have been reported as natural hosts of some *Avipoxvirus* strain (BOLTE; MEURER; KALETA, 1999; VAN RIPER III; FORRESTER, 2007), which have adapted significantly to birds. Certain degrees of species-specificity are observed for some strains, especially those of wild birds, and others strains can infect several species producing disease of different virulence (RITCHIE, 1995).

The prevalence of the disease varies in wild birds, according to the species and the time of year, occurring mainly in the rainiest periods when there is high

mosquito activity (RITCHIE, 1995; YOUNG; VANDERWERF, 2008). Recently, an outbreak involving 94 captive native parrots in Brazil with low mortality rate was associated with poxvirus infection, whose DNA was detected by biomolecular techniques in 27 individuals (ESTEVES et al., 2017). Another recent outbreak has been reported in exotic captive birds, also in Brazil, in which 50 individuals died (MURER et al., 2018).

Figure 3.1 - Worldwide distribution of *Avipoxvirus* in captive and wild birds

Source: Adapted from Van Ripper III and Forrester (2007)

Poxviruses are considered factors that negatively affect the distribution and behavioral patterns in native wild bird populations in Hawaii and are relevant when considering conservation efforts for local species (VAN RIPER III; VAN RIPER; HANSEN, 2002). As in Hawaii, invasive and domestic avian species were also considered introducers of poxviruses into the Galapagos Islands, where there is concern about threatened native species (THIEL et al., 2005).

3.5.4 Clinical signs

Poxvirus infections can cause skin, diphtheritic or systemic alterations, and the type of disease and its intensity are influenced by host susceptibility, viral strain, route of exposure to virus, host species and age (TRIPHATY; REED, 2013). The cutaneous form is the most common clinical manifestation of poxvirus, characterized

by the appearance of nodules in the unfeathered parts of the body, such as the Galliformes comb and wattles, the eyelid, around the beak, the legs and the feet (VAN RIPER III; FORRESTER, 2007). Some of these injuries can prevent birds from finding food and water due to lack of vision and difficulty in seizing food (RITCHIE, 1995). However, many birds survive and recover (YOUNG; VANDERWERF, 2008), the lesions regenerate by degeneration and scaling of abnormal proliferated epithelium within up to six weeks (TRIPHATY; REED, 2013). Some birds lose digits and may be blind permanently.

A less common form, the diphtheritic form, is mainly observed in canaries, *Agapornis* sp., mynahs and *Amazona aestiva* (PHALEN, 2005). Necrotic lesions in the mucous membranes of the oral cavity and upper respiratory tract are commonly noticed, and very often result in high mortality, unlike the cutaneous form (RITCHIE, 1995). It is characterized by yellowish diphtheritic lesions and ulcers that may cause mild to severe respiratory signs (GERLACH, 1994b). Canaries usually also develop bilateral blepharitis and conjunctivitis (PHALEN, 2005). Secondary fungal and bacterial lesions may occur on the skin and on the membrane tissue (RITCHIE, 1995). The systemic form is associated with acute depression, ruffled feathers, anorexia, weight loss, dyspnea and high mortality (70-99%), especially in canaries and finches (GERLACH, 1994b).

3.5.5 Diagnosis

Clinical signs of poxviruses are quite suggestive of the disease but may be confused with other illnesses. For definitive diagnosis, the presence of the virus must be confirmed by viral isolation, demonstration of the virus by electron microscopy, molecular methods or, more simply, microscopic identification of Bollinger's intracytoplasmic corpuscles in the histopathology (VAN RIPER III; FORRESTER, 2007). Serological tests can be performed, such as ELISA, immunoprecipitation, viral neutralization, and cross-protection tests (TRIPHATY; REED, 2013).

Various stages of the cutaneous form may be observed at necropsy according to the progression of epithelial hyperplasia. The nodules are elevated, smooth or nodular, small to large, and may ulcerate, causing deformities in the affected areas, such as the eyelid and beak (PHALEN, 2005). In the diphtheric form, slightly elevated

whitish and yellowish plaques can be found in the mucosa of the esophagus, trachea, mouth, and tongue (TRIPHATY; REED, 2013).

The inflammatory process may extend to the infraorbital sinus, pharynx and larynx. Histopathologically, the main feature of the infection is the presence of intracytoplasmic inclusion corpuscles (Bollinger corpuscles), epithelium hyperplasia, and ballooning of infected cells associated with inflammatory infiltrates (RITCHIE, 1995). In the systemic form, fibrinous inflammation of serous membranes, hepatic degeneration or necrosis may be observed, with edema and hyperemia of the lungs, and fibrinopneumonia (VAN RIPER III; FORRESTER, 2007).

3.5.6 Treatment and control

There are no specific treatments for birds infected with poxvirus. Secondary bacterial infections can be treated by removing dead tissue, cleaning the wound and using topical antibiotics (RITCHIE, 1995). Systemic antibiotics are indicated for birds with respiratory and digestive tract lesions. Silver nitrate, iodine, and 1-2% saline solution can be directly applied in the lesions to help to decrease the level of infection (VAN RIPER III; FORRESTER, 2007). Surgical removal of lesions should be avoided as this will only cause scarring (PHALEN, 2005).

Cleaning and disinfection of aviary facilities is essential for the control of poxvirus due to the stability of the virus in the environment. Poxviruses can survive for years in dry organic debris such as skin scabs, feces, blood and soil (RITCHIE, 1995). They have resistance to common disinfectants and variable resistance to ether, chloroform and 1% phenol (TRIPHATY; REED, 2013). They can be inactivated by 1% potassium hydroxide, heating at 50°C for 30 minutes, 60°C for 8 minutes, and 5% phenol (RITCHIE, 1995).

The installation of screens in enclosures or cages helps in reducing the number of vectors (RIPER III; FORRESTER, 2007). Birds should be tested for the agent, positive birds should be isolated, and acquisition of new birds should be monitored with appropriate quarantine (PHALEN, 2005). Vaccination exists for some species, such as canaries, pigeons, turkeys, chickens and quails. In Psittaciformes, the vaccine does not appear to protect against infection but decreases the clinical signs of infected birds (RITCHIE, 1995). In Brazil, there are no recommendations of

pox vaccination for birds, excepting poultry, and Psittaciformes vaccines do not exist in the country.

3.6 PSITTACINE BEAK AND FEATHER DISEASE

Psittacine Beak and Feather Disease (PBFD) was initially recognized in Australian Psittaciformes in the 1970s and observations from 1887-1888 already described clinical signs compatible with the disease, such as a record involving wild *Psephotus haematotonus* (FOGELL; MARTIN; GROOMBRIDGE, 2016). PBFD is a relevant disease for Psittaciformes due to its debilitating, highly contagious, chronic and lethal characteristics (RAIDAL, 2016). The country of origin of the disease is likely to be Australia and the dispersal of Australian species by legal and illegal trades has globally spread the PBFD. All Psittaciformes are currently considered susceptible and wild and captive populations are being affected (RAIDAL et al., 2015).

3.6.1 Etiology

PBFD is caused by a non-enveloped circular single-stranded DNA circovirus belonging to the family Circoviridae, genus *Circovirus*, *Beak and feather disease virus* (BFDV) species. It is one of the smallest viruses known, capable of causing infection (HARKINS et al., 2014; PETERS et al., 2014). Virions are icosahedral in shape, 14 to 16 nm in size and approximately 2 kilobases in length, highly genetically diverse and prone to mutation (JULIAN et al., 2013), but relatively conserved in their antigenic characteristics (RAIDAL et al., 1993).

The family Circoviridae includes two genera, *Circovirus* and *Cyclovirus*, which have 39 and 48 species, respectively. In birds, the number of species reaches 11, including: BFDV, *Canary circovirus, Duck circovirus, Finch circovirus, Goose circovirus, Gull circovirus, Pigeon circovirus, Raven circovirus, Starling circovirus, Swan circovirus* and *Zebra finch circovirus* (ICTV, 2018). BFDV has already been found infecting other avian orders, including wild and captive individuals that showed (Strigiformes, Coraciiformes) (SARKER et al., 2015; SARKER et al., 2016) or not

(Caprimulgiformes, Strigiformes, Coraciiformes, Pelecaniformes, Accpitriformes, Passeriformes) clinical signs (AMERY-GALE et al., 2017)

3.6.2 Pathogenesis

The excretion of circovirus occurs in high concentrations in faeces and feathers, and also in crop secretions (RITCHIE et al., 1991). Transmission occurs horizontally via the oral/aerogenous route. The vertical transmission pathway is suggested in the literature because embryonic eggs positive for circovirus have already been detected (RAHAUS et al., 2008). However, there are no experiments proving vertical transmission, since cloacal secretions and nest contamination could be the source of dissemination for embryos (RAIDAL, 2016). Despite the possible existence of vertical transmission, the resistance characteristics, high excretion concentrations and generalist behavior of circovirus (SARKER et al., 2014b) make horizontal transmission more feasible and relevant for infection of new individuals.

Nest contamination is believed to be an important source of dissemination in Australia, as virions can probably remain activated for years in tree hollows, which can be occupied by different avian species at each breeding season (RAIDAL, 2016). It has also been shown that the virus can be carried by mites (PORTAS et al., 2017), which suggests the likely role of other ectoparasites and insects as BFDV fomites and mechanical vectors (SARKER et al., 2015).

Once in the host, the incubation period of BFDV varies from 21 days to several years, depending on the infective dose, the pathogenicity of the strain, the age of the bird, its immunological status and the stage of feather development (RITCHIE, 1995). After entering into a susceptible host, hematogenous dissemination of BFDV occurs to the cutaneous follicular epithelium and to the cloacal bursa and thymus. BFDV has tropism by rapidly dividing cells such as lymphoid tissue, basal follicular epithelium and intestinal epithelium (WOODS; LATIMER, 2013). In the cutaneous region, BFDV induces necrosis and disruption of the basal epithelium and feather pulp, and thrombosis and hemorrhage in the feather pulp, causing dystrophies in the feathers, in the beak and claws of the host. Studies suggest that BFDV may indirectly induce premature apoptosis and stimulate phagocytic activity of infected cells, which probably induces lymphocellular necrosis and lymphoid depletion in the cloacal

bursa, thymus and spleen (WOODS; LATIMER, 2013). Therefore, many birds become immunosuppressed and susceptible to secondary infections, which cause the death of the host.

3.6.3 Epizootiology

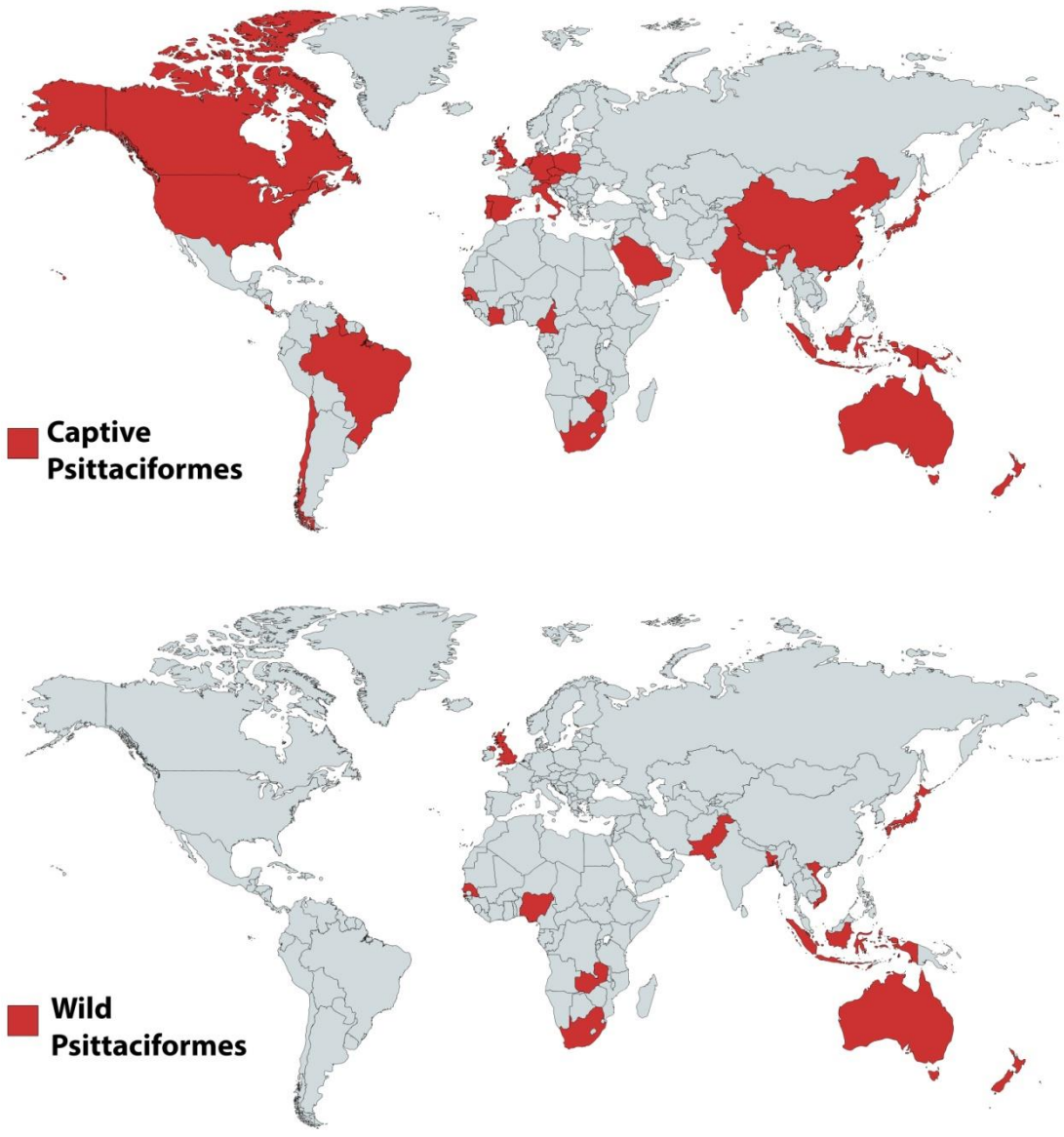
BFDV currently has worldwide distribution, and its spread was possible by the international bird trade (VARSANI et al., 2011) (Figure 3.1). All Psittaciformes of any age and gender are considered susceptible to BFDV (SARKER et al., 2014a) as it has been diagnosed in over 78 native species from all continents (FOGELL; MARTIN; GROOMBRIDGE, 2016). In addition, it is considered a generalist pathogen with frequent host-switching events and sporadic spill-over infections (RAIDAL; PETERS, 2018), and may affect different avian orders, as mentioned above. These characteristics allow rapid viral evolution and the consequent emergence of new variants with different pathogenesis and virulences (SARKER et al., 2014a). The resistance and long permanence of the virus in the environment, associated with the carrier status of an infected bird, allow the spread of the pathogen even in places with low avian density (JACKSON et al., 2015).

The circovirus has been detected in parrots housed on all continents, and in the wild has been detected in Oceania, Africa, Asia and Europe, including invasive species (FOGELL; MARTIN; GROOMBRIDGE, 2016; FOGELL et al., 2018). Different prevalence can be observed in captive and wild birds (FOGELL; MARTIN; GROOMBRIDGE, 2016). The prevalence can range from 41% (32/79) to 94% (15/17) in different wild Australian populations when serological tests are used (RAIDAL et al., 1993).

In Brazil, PBFD was first detected using the in-situ hybridization technique on skin and feather biopsy samples from a captive adult white cockatoo (*Cacatua alba*), which showed classical clinical signs of the disease (WERTHER et al., 1999). Recent studies indicate that the pathogen may be spread in native and exotic captive birds in some areas of the country. Of 120 psittacine pet birds showing clinical signs and evaluated in the state of São Paulo, 41 (34.17%) were positive for BFDV using molecular techniques, being 16 native species (AZEVEDO, 2014). One hundred ninety psittacine native birds from a wildlife center were evaluated in a study in Minas

Gerais, showing a prevalence of 6.3% for BFDV (ARAÚJO et al., 2015). Single BFDV detections have been reported in a *A. aestiva* and a *Psittacara leucophtalmus* from a veterinary hospital (DUARTE et al., 2019), in an *Anodorhynchus leari* from an endangered species conservation program (COELHO et al., 2015) and in a *A. hyacinthinus* from a zoo (HIDASI et al., 2018).

Figure 3.2 – Global distribution of *Beak and Feather Disease Virus* in captive and wild birds. Isolated or several cases were reported in one or more locations in the countries.



Source: Adapted from Fogell, Martin and Groombridge (2016).

Cloacal, oropharyngeal swab and blood samples from 11 wild *A. leari* nestlings were negative for BFDV on PCR (COELHO et al., 2016). However, there are no other *in situ* studies about the pathogen in Brazil and the increasing occurrence of captive circovirosis is of concern when it comes to parrot conservation. The pathogen has already been introduced into endangered wild populations in other countries (REGNARD et al., 2014), causing outbreaks and having as source of infection the introduction and release of invasive species (KUNDU et al., 2012). Therefore, conducting *in situ* epidemiological studies to monitor native Brazilian species is essential.

3.6.4 Clinical signs

Clinical manifestations of PBFD vary according to the age of the bird, being young and subadult birds the most susceptible (before cloacal bursa involution), but individuals of any age can succumb to the disease (RAIDAL, 2016). The hyperacute form occurs in neonatal and young Psittaciformes, which can manifest pneumonia, enteritis, weight loss and death, or die without any clinical sign (RITCHIE, 1995). The acute form of the disease affects young birds in the molting period and is associated with sudden alterations in feather development, including absence, necrosis, fracture, hemorrhage, stress lines, and sheath retention (WYLIE; PASS, 1987). Other clinical signs include apathy, anorexia, green diarrhea, crop stasis and regurgitation, with a high degree of mortality within one week of onset of clinical signs caused by virus-induced liver necrosis (RAIDAL, 2016).

The most common clinical form, the chronic form, affects birds usually older than three years of age (adult), which initially manifest subtle, slow and symmetrical feather abnormalities that evolve with each moulting period, usually with no other associated signs. Beak and claw abnormalities may occur occasionally, especially in cockatoos. The beak becomes elongated, can develop fractures and even avulsion of the ramphoteca. Over time, immunosuppression caused by the disease commonly leads to the acquisition of secondary infections. Birds finally have difficulty feeding, lose weight and die (RAIDAL, 2016). A subclinical form, with no apparent clinical signs, is reported in the literature, making it difficult to detect the disease, facilitating its spread (BERT et al., 2005).

3.6.5 Diagnosis

In vitro isolation of BFDV have not been successful so far (RAIDAL, 2016). The clinical diagnosis of PBFD can be made for the chronic form of the disease, since few diseases resemble bilateral feather dysplasia (RITCHIE, 1995). Macroscopic changes of the internal organs are rarely noted, but when present they vary according to the age of the animal and the types of secondary infections present. Cloacal bursa atrophy may occur in nestlings with the presence of undeveloped folds and thymus atrophy, presenting pale necrotic areas (RAIDAL; CROSS, 1995; RITCHIE, 1995). Hepatomegaly and decreased kidney size can be observed (RAIDAL; CROSS, 1995). In adult birds, the spleen may be small and lymphocyte depleted (RITCHIE, 1995)

Microscopically, varying degrees of diffuse to multifocal necrosis and inflammation can be observed in the basal epithelium of the feathers and follicles of the injured areas, in addition to hemorrhage in the pulp cavity, epidermal hyperplasia and hyperkeratosis. Basophilic intracytoplasmic inclusions can be found within macrophages in the feather pulp, in the follicular and feather epithelium and in the feather sheath. Intranuclear inclusion corpuscles can be seen in the follicular epithelium and feather cells (LATIMER et al., 1991). Changes in primary and secondary lymphoid tissues may range from mild to severe lymphoid necrosis with lymphoid depletion, commonly accompanied by globular intracytoplasmic inclusions in the cloacal bursa, spleen, intestinal and bronchiolar lymphoid tissues (WOODS; LATIMER, 2013). The liver may be congested, with multifocal areas of necrosis and basophilic inclusions may be found within Kupffer cells (RAIDAL; CROSS, 1995). Inclusions can also be found in numerous other organs of infected birds (LATIMER et al., 1991).

The gold standard for serological diagnosis of the disease is indirect hemagglutination (IH), widely used in Oceania (RAIDAL et al., 1993; JACKSON et al., 2015) but is not available in Brazil. PCR has been the main test used on most occasions for pathogen detection, especially after a universal assay has been designated (YPELAAR et al., 1999; FOGELL; MARTIN; GROOMBRIDGE, 2016). Feather, blood, tissue and cloacal swab samples can be used for PCR diagnosis in live birds. The cloacal bursa, liver and spleen may also be collected at necropsy.

Intermittent tests using probes on blood samples from a clinically normal Solomon Cockatoo (*Cacatua ducorpsii*) could detect the virus for 18 months before the bird had feather dystrophies (RITCHIE, 1995). Viruses can be detected in the blood two days after exposure to the pathogen, before clinical signs develop (RITCHIE, 1995; PHALEN, 2005). Feather collection should be performed with caution and in isolated birds, because the chances of environmental contamination are high, due to the high virus load eliminated by feather dust (RAIDAL, 2016).

The presence of antibodies can be a strong indication of disease absence, as asymptomatic animals have been found with antibodies in the wild (RAIDAL et al., 1993). The host, especially as an adult, may develop transient viremia, seroconverting and becoming clinically normal (subclinical infection) (RITCHIE, 1995). However, birds with active disease infection may have low antibody titers, which can increase and decrease (KHALESI et al., 2005), due to damage to the cloacal bursa and thymus (LATIMER et al., 1991).

3.6.6 Treatment and control

There are no specific treatments for birds infected with circovirus. Interferon-α modulators, tumor necrosis factor inhibitors α and interferon-γ have been used, but without evidence of their efficacy in birds (TOMASEK; TUKAC, 2007; RAIDAL, 2016). In addition, recovered birds can remain latently infected and be a source of BFDV dissemination (RAIDAL; CROSS, 1994).

Attempts to create vaccines to Psittaciformes have provided some protection against the disease in Australia, but are still ineffective (RAIDAL et al., 1994; BONNE et al., 2009; PATTERSON et al., 2013), which reflects the lack of commercial products in the world, including in Brazil.

So, the prevention of the disease should be done through some actions in the aviary. The use of appropriate disinfectants and good hygiene, as the virus have an innate resistance in the environment and to common deleterious agents such as ether, chloroform, formaldehyde, 70% alcohol and high temperatures (WOODS; LATIMER, 2013). Iodine and hypochlorite compounds can inactivate viruses, but in unusual concentration (10%), temperature (37ºC) and duration (two hours).

Glutaraldehyde compounds and similar disinfectants are safe and efficient when used in an organic-matter free surface at 2% for 10 minutes (DEH, 2006).

Other preventive actions include diagnostic tests of the population and of recently acquired birds; quarantine establishment and adequate biological material collection to avoid false negative results; proper husbandry to keep birds always in good immune status (RAIDAL, 2016). Quarantine monitoring o captive birds must be performed for at least 63 days, including blood and blood feather samples testing by PCR on days 1, 28 and 56 (DEH, 2006). However, all these actions together have been reported to be inefficient to reduce the threat of PBDV due to mismanagement (PETERS et al., 2014).

Once the virus has been introduced in a population, it is very difficult or even impossible to remove it. PBFD control has been performed using euthanasia of diseased birds and segregation of clinically normal but PCR positive birds (SARKER et al., 2014a). These control actions seem to be more drastic, but the imminent PBFD spread and threats to wild Brazilian and South American birds need to be taken more seriously, and we hope that future public policies can ensure the health protection of our parrot fauna.

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4. SURVEY AND MOLECULAR CHARACTERIZATION OF VIRAL PATHOGENS AND *CHLAMYDIA PSITTACI* IN WILD AMAZON PARROT NESTLINGS IN BRAZIL: IMPLICATIONS FOR CONSERVATION

4.1 ABSTRACT

Brazil is home of a great diversity of birds, containing the largest number of Psittaciformes in the world. Unfortunately, this large diversity is threatened by anthropogenic activities as habitat destruction and illegal trade of wild caught nestlings. In addition, exotic viruses have been detected in captive psittacine birds in Brazil. However, all these negative anthopogenic impacts on the health of Psittaciformes are poorly studied in the country, mainly on wild populations and even concerning endemic pathogens. So, the aim of the present study was to evaluate the presence of *Chlamydia psittaci*, *Psittacid alphaherpesvirus* 1, poxvirus and *Beak and feather disease virus* in wild *Amazona aestiva, A. brasiliensis* and *A. pretrei* nestlings located in four states of Brazil. Blood and cloacal/oropharyngeal swab samples were collected from 170 nestlings; thirty one nestlings was sampled only by collecting blood and 34 nestlings only by collecting swab samples, totaling 235 Amazon parrots sampled. DNA extraction was performed using a commercial kit and PCR was performed for each pathogen in all samples. *Chlamydia psittaci* DNA was detected on swab samples from three *A. aestiva* and one *A. brasiliensis*, and blood from one *A. brasiliensis.* All the other pathogens were not detected. Sequencing was possible only in the *A. brasiliensis* blood PCR product, revealing a high similarity with Brazilian *C. psittaci* strains, belonging to the genotype A. The results observed in the present study have a relevant implication for the conservation of wild parrots and show that wild populations have a low prevalence of endemic pathogens and apparently were not reached by the BFDV, an exotic introduced virus. So, the movement of psittacine birds in Brazil must be carried out with greater responsibility and seriousness, performing proper preventive measures, quarantine and laboratory analysis. Better health protocols should be discussed and established for the reintroduction of birds to the wild, so we can try to guarantee the protection of our parrot fauna.

4.2 INTRODUCTION

Brazil is considered a megadiverse country, containing the third largest diversity of birds in the world, counting on 1,919 species. The largest number of Psittaciformes is also found here. Among the known 411 species, 86 occur in the national territory (PIACENTINI et al., 2015). Unfortunately, Brazil is also in first position when it comes to threatened species, being the Psittaciformes one of the most threatened bird orders, with 25 native species in the Global International Union for Conservation of Nature Red List (IUCN, 2019).

The Amazon parrots have a highlight considering the national species, being in the first place of the Brazil's most trafficked psittacine birds. Their colorful plumage, sociability and ability to talk make them heavily sough after as pets and creates a domestic demand (SNYDER, 2000). The *Amazona* genus comprises 12 species in Brazil and faces, in addition to the illegal trade, another major threat in all biomes: habitat loss, alteration and fragmentation. One third of the native Amazon species is threatened (IUCN, 2019).

Among these species, the red-spectacled Amazon parrot (*Amazona pretrei*) is threatened inside the vulnerable category. The red-tailed Amazon parrot (*Amazona brasiliensis*) has left the IUCN Red List, entering the near threatened status, but it still needs management in the wild. Both species have a restricted distribution and only exist in the Brazilian territory (Figure 4.1). The blue-fronted Amazon parrot (*Amazona aestiva*) is in the least concern category and has a wide natural distribution, including Brazil, Argentina, Bolivia and Paraguay territories (Figure 4.1). But there is a special interest in this species because it is the main target of the illegal trade (SEIXAS; MOURÃO, 2018; IUCN, 2019). In Brazil, there are three conservation projects for these species in an attempt to minimize threats to their populations.

Nevertheless, another challenge to wildlife conservation efforts in the current world scenario is the dissemination of infectious diseases. As parrots are very popular pet animals, the demand created around the world has led to an international movement of over 19 million birds since 1975 (CITES, 2018), which creates the perfect scenario for the spread of pathogens. Disease emergence can be triggered by translocation, introduction of infected animals, pathogens or vectors to new geographic regions, human/domestic animals encroachment, spill-over, *ex situ*

contact and ecological manipulation (DASZAK; CUNNINGHAM; HYATT, 2000). As the Amazon parrots are subjected to at least three of these activities, their health assessment in the wild would be an important addition to their conservation efforts (BERKUNSKY et al., 2017).

Illegal trade in wild caught birds stills a reality in Brazil and only a small part of the nestlings removed from nature is seized by environmental authorities. These birds are mixed in rehabilitation centers with resident birds, and frequently are released to the wild without any health criteria (MARINI; GARCIA, 2005). In addition, cross border movement of birds continues as the result of smuggling and the legal trade of domestically raised birds (PHALEN, 2015). All this movement creates the perfect scenario for disease dissemination for wild and captive animals as trafficked and imported birds are fed improper diets, housed in crowded unhygienic conditions, and mixed with other species (GASKIN; ROBBINS; JACOBSON, 1978; RASO et al., 2004). The globally spread of diseases have caused significantly negative conservation impacts on captive and wild populations (KUNDU et al., 2012; OLSEN et al., 2016). Resistant virus and subclinically persistent infections make controlling these pathogens challenging (PHALEN, 2015).

The *Chlamydia psittaci* and the Psittacid herpesviruses (PsHV) are relevant pathogens that affect captive parrots and have been observed in psittacine birds from rehabilitation centers in Brazil (LUPPI et al., 2011; RASO et al., 2013), including a chlamydiosis outbreak (RASO et al., 2004). A neglected virus in wild birds, the poxvirus, has also caused outbreaks in psittacine species located in a conservation facility in Brazil (ESTEVES et al., 2017). In addition, the Beak and Feather Disease Virus (BFDV), an exotic pathogen introduced in Brazil (WERTHER et al., 1999), has been reported in native Brazilian species in one rehabilitation center (ARAUJO et al., 2015), and in exotic and native pet birds (AZEVEDO, 2014).

The results of all the negative anthropogenic actions for wild Amazon parrot health in Brazil is unknown and there is scarce information in the literature (RASO et al., 2006; RIBAS et al., 2014; VAZ et al., 2017). So, the objective of the present study was to investigate the presence of *C. psittaci* and viral pathogens in wild Amazon parrot nestlings located in four states of Brazil, in an attempt to molecularly characterize the pathogens detected and to discuss the implications of these findings for the conservation of psittacine birds in Brazil.

4.3 MATERIAL AND METHODS

4.3.1 Species of parrots and study area

The study site encompassed three species of parrots in four states of Brazil (Figure 4.1). *Amazona pretrei* nestlings were sampled in a fragmented area of the Southern fields, in the municipality of Pontão, state of Rio Grande do Sul. *Amazona brasiliensis* parrots were studied in the Rasa Island, Gamela Island, Chica Island, state of Paraná, located in the Environmental Protection Area of Guaraqueçaba, which has an extensive area of Atlantic forest. This species was also sampled in Comprida Island, state of São Paulo, another Atlantic forest area. *Amazona aestiva* parrots were studied in two areas located in the municipalities of Campo Grande and Miranda, state of Mato Grosso do Sul. One region is characterized by a fragmented area of Brazilian Cerrado and the other one is located in the Brazilian Pantanal wetlands.

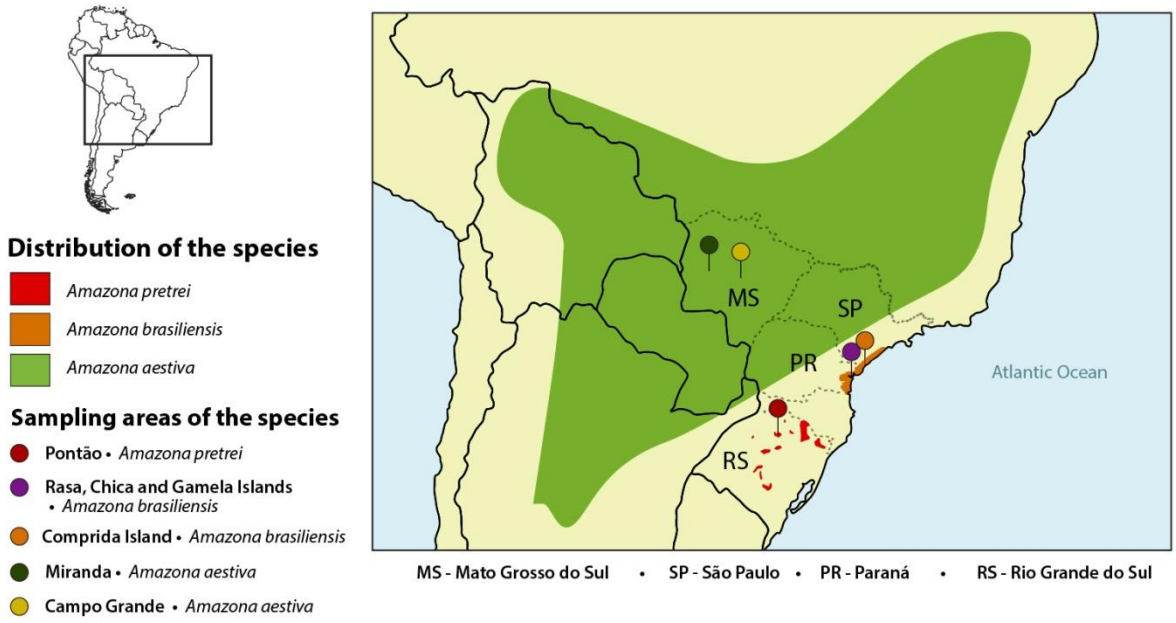
This study was approved by the Ethic Committee on Animal Use of the University of São Paulo (CEUA 9545290116) and by the Brazilian environmental authority (SISBIO 43876-1, 4993-6, 35621-4).

4.3.2 Sample collection

Oropharyngeal and cloacal swab samples and blood were collected from Amazon parrot nestlings in the 2016 to the 2019 breeding seasons (October to January). Some parrots were sampled only by collecting blood or swab samples. Liver and spleen were collected from one wild *A. brasiliensis* nestling that was found recently dead inside one nest in Rasa Island. In addition, samples obtained from 74 *A. brasiliensis* (cloacal/oropharyngeal swab samples from 74 birds and blood from 55 of these birds) and 49 *A. aestiva* (swab/blood samples from 18 birds and only blood from 31 birds) from nestlings in previous field expeditions (2013/2014, 2013 and 2006, respectively) were also used in the present study. All field expeditions were carried out in partnership with the conservation projects, which monitor the nestlings during the breeding seasons in longitudinal studies.

Natural and artificial nests (made of wood or polyvinyl chloride) were accessed using ladders or climbing equipment. The birds were removed from the nests, sampled and then put back in the nests. Swab samples were kept frozen in microtubes containing viral transport media and blood were kept frozen in microtubes until the analyses.

Figure 4.1. Distribution of *Amazona pretrei, Amazona brasiliensis* and *Amazona aestiva* in Brazil and South America and locations where sampling was performed.



Source: Adapted from IUCN (2019).

4.3.3 Laboratory analysis

Genomic DNA extraction was performed using the NucleoSpin Tissue kit® (Macherey-Nagel, Germany) according to manufacturer's instructions.

Each DNA sample was screened using universal/pan PCRs for *C. psittaci* (EHRICHT et al., 2006), PsHV (TOMAZEWSKI et al., 2003), poxvirus (LEE; LEE, 1997) and BFDV (YPELAAR et al., 1999), using the thermal cycler Axygen® Maxygene (Axygen, Union City, California, USA). A longer sequence for *C. psittaci* positive samples was obtained by another PCR analysis (SACHSE et al., 2009). *Chlamydia psittaci* was not investigated in 58 *A. brasiliensis* DNA samples and in 30

A. aestiva samples as they were already negative in previous studies (RASO et al., 2006; VAZ et al., 2017).

The primers used can be found on Table 4.1 and the cycling conditions in Table 4.2. Cycling conditions not included in the table remained the same as the reference cited. Briefly, a 25 µL reaction mix containing 2 µL of genomic DNA, 0.5 µL of each primer (10 pmol), 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, USA) and nuclease-free water qsp was used in the reaction. PCR products, including standard positive and negative controls routinely used in our lab, were analyzed by electrophoresis in a 1.2% agarose gel stained with GelRed® (Biotium, Fremont, California, USA) nucleic acid stain.

Table 4.1. Primers selected and used for detection of viruses and *Chlamydia psittaci* in wild Amazon parrot samples from Brazil.

Pathogen	Gene region / Fragment size	Primer 5'-3' sequence	Reference
Chlamydia psittaci	23S ribosomal / 111bp	f- CTGAAACCAGTAGCTTATAAGC GT r- ACCTCGCCGTTTAACTTAACTCC	Ehricht et al. (2006)
	ompA / 418 bp	f- ACTACGGAGATTATGTTTTCGATCGTGT r- CGTGCACCYACGCTCCAAGA	Sachse et al. (2009)
PsHV-1	UL16/17 / 667 bp	f- TGCGTGGGGTTAAACTCGGAAC r- CGACTACACGAGCCTAACATC	Tomaszewski, Kaleta and Phalen (2003)
Poxvirus	4b / 576 bp	f- CAGCAGGTGCTAAACAACAA r- CGGTAGCTTAACGCCGAATA	Lee and Lee (1997)
Circovirus	VP1 / 717 bp	f- AACCCTACAGACGGCGAG r- GTCACAGTCCTCCTTGTACC	Ypelaar et al. (1999)

Table 4.2. Cycling conditions performed to detected viruses and *Chlamydia psittaci* by PCR in wild Amazon parrot samples from Brazil.

Pathogen	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Reference
Chlamydia psittaci	96ºC/1 min	94ºC/30s	50ºC/60s	72ºC/30s	72ºC/4 min	Sachse et al. (2009)
Poxvirus	94ºC/5 min	94ºC/30s	50ºC/60s	72ºC/60s	72ºC/7 min	Lee and Lee (1997)
Circovirus	94ºC/5 min	94ºC/30s	60ºC/30s	72ºC/90s	72ºC/7 min	Ypelaar et al. (1999)

4.3.4 Phylogenetic analysis

The PCR fragments found in positive samples were purified from the gel using a commercial kit (NucleoSpin Gel and PCR Clean-Up, Macherey Nagel, Düren, North Rhine-Westphalia, Germany) according to the manufacturer's instructions and sequenced in sense and antisense direction by Sanger sequencing (The Human Genome and Stem Cell Research Center, Institute of Biosciences, University of São Paulo, Brazil). The primers were trimmed out of the sequences using Mega X software and the sequences were aligned with reference ones available on GenBank. Alignment was performed using MAFFT version 7 with the FFT-NS-I algorithm (KATOH; STANDLEY, 2013). The best model to construct a neighbor joining tree was performed using Mega X (TAMURA et al., 2011). The Tamura-Nei model was chosen to create the tree tested by bootstrapping with 1000 replicates.

4.4 RESULTS

A total of 235 Amazon parrot nestlings were sampled and the kind and number of samples obtained are shown in Table 4.3.

Table 4.3. Number of Amazon parrot nestlings sampled in the states of Rio Grande do Sul (RS), Paraná (PR), Mato Grosso do Sul (MS) and São Paulo (SP), Brazil.

Amazon species	**State**	**Swab samples**	**Blood**	**Tissue**	**Total samples collected**	**Number of birds****
Amazona pretrei	RS	4	4	0	8	4
Amazona brasiliensis	PR/SP*	138	95	1	234	138
Amazona aestiva	MS	62	59	0	121	93
Total		204	158	1	363	235

*Just two parrots were sampled in the state of São Paulo (cloacal/oropharyngeal swab sample)
**Fifty eight *A. brasiliensis* and 30 *A. aestiva* were only evaluated for viral pathogens, as *Chlamydia psittaci* was investigated on these birds in previous studies (RASO et al., 2006; VAZ et al., 2017).

Three *A. aestiva* cloacal/oropharyngeal swab samples (4.8%, 3/63) were positive for *C. psittaci* in the state of Mato Grosso do Sul. Two *A. brasiliensis* samples (one cloacal/oropharyngeal swab sample and one blood) (2.5%, 2/80) were positive for *C. psittaci* in the state of Paraná. The total *Chlamydia* prevalence found for all the parrots evaluated was 3.4% (5/147). None of the nestling samples tested yielded positive PCR results for PsHV, poxvirus and circovirus.

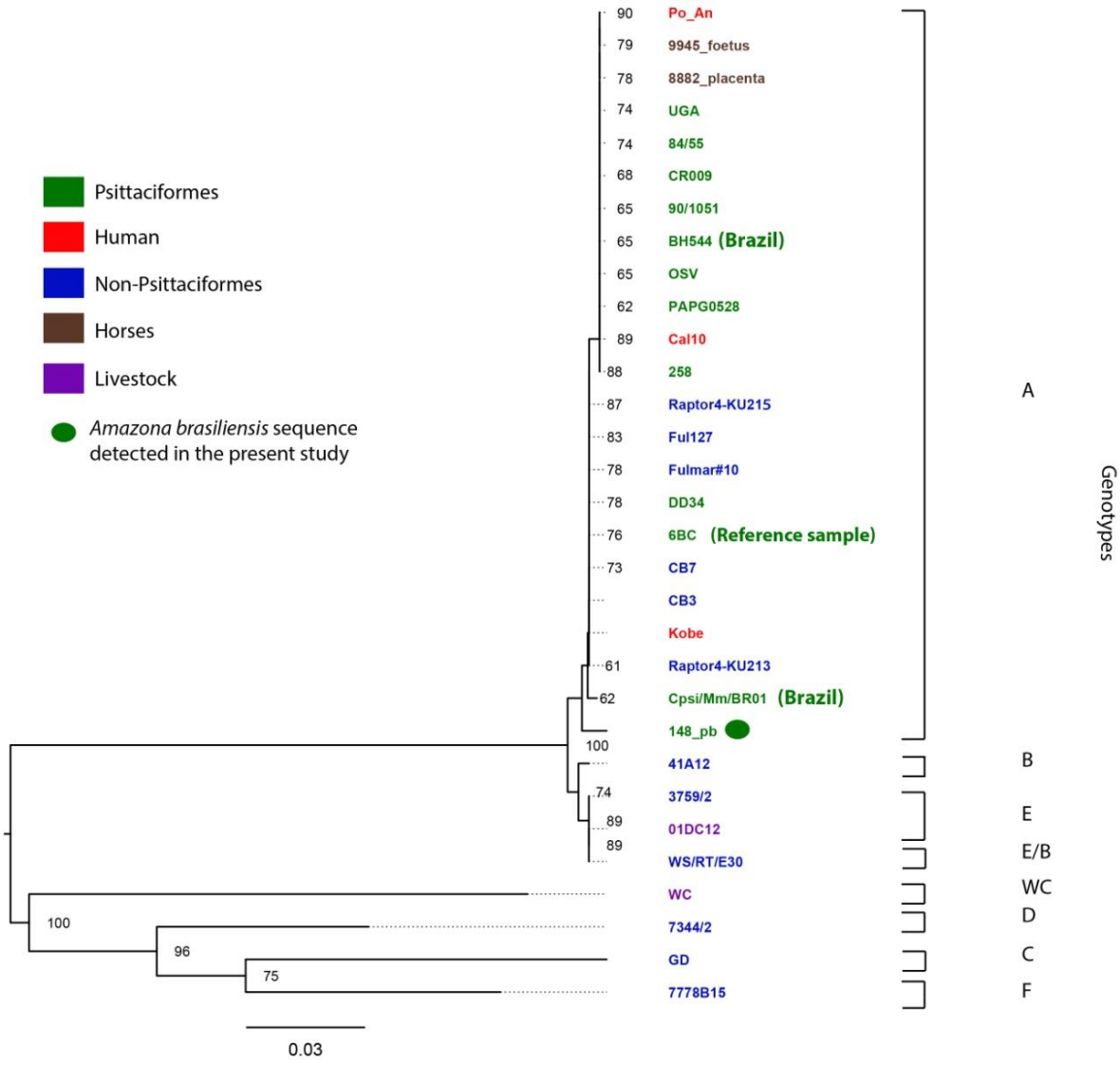
Chlamydia psittaci nucleotide sequencing was possible only in the *A. brasiliensis* blood sample, which was analyzed and aligned with reference ones available on GenBank (Table 4.4). The phylogenetic analysis can be seen in Figure 4.2. The 401 bp sequence obtained was confirmed to be a *C. psittaci* fragment DNA as it had a high percent identity (99.25%) with other *C. psittaci* sequences, clustering within the genotype A (Figure 4.2).

Table 4.4. *Chlamydia psittaci* strains and Genbank accession numbers used in the present study.

Strain	**Host common name**	**Host scientific name**	**Origin**	**Genbank Accession number**
01DC12	Pig	*Sus scrofa*	United Kingdom	HF545614
258	Parrot	-	China	KF611904
3759/2	Pigeon	*Columba livia*	Belgium	AY762611
41A12	Turkey	*Meleagris gallopavo*	Belgium	AY762609
6BC	Parrot	-	USA	NC_017287
7344/2	Pigeon	*Columba livia*	Belgium	AY762610
7778B15	Turkey	*Meleagris gallopavo*	Belgium	AY762612
84/55	Amazon parrot	*Amazona* sp.	USA	CP003790
8882_placenta	Horse	*Equus caballus*	Australia	NZ_PJQA00000000
90/1051	Amazon parrot	*Amazona* sp.	Belgium	AY762608
9945_foetus	Horse	*Equus caballus*	Australia	NZ_PJPY00000000
BH544	Blue fronted Amazon parrot	*Amazona aestiva*	Brazil	MH138296
Cal10	Human	*Homo sapiens*	USA	AEZD01000001
CB3	House sparrow	*Passer domesticus*	China	NZ_JMEI01000017
CB7	Vinous-throated Parrotbill	*Paradoxornis webbianus*	China	NZ_JMBZ01000015
Cpsi/Mm/BR01	Monk parakeet	*Myiopsitta monachus*	Brazil	JQ926183
CR009	Crimson rosella	*Platycercus elegans*	Australia	NZ_LZRX00000000
DD34	Parrot	-	USA	AFVL01000002
Ful_127*	Fulmar bird	*Fulmarus glacialis*	Faroe Islands	CP033059
Fulmar#10*	Fulmar bird	*Fulmarus glacialis*	Faroe Islands	AM050561
GD	Duck	-	USA	AF269261
Kobe*	Human	*Homo sapiens*	Japan	AB468956
OSV	Amazon parrot	*Amazona* sp	Netherlands	DQ230095
PAPG0528	Red-fronted parrot	*Poicephalus gulielmi*	Taiwan	PAPG0528
Po_An	Human	*Homo sapiens*	Australia	NZ_LZRG00000000
Raptor5-KU213*	Asian barred owlet	*Glaucidium cuculoides*	Thailand	KP893667
Raptor5-KU215	Asian barred owlet	*Glaucidium cuculoides*	Thailand	KP893668
UGA	Cockatiel	*Nymphicus hollandicus*	USA	AWXQ01000002
WC	Bovine	*Bos Taurus*	Belgium	CP003796
WS/RT/E30	Duck	*Anas platyrhynchos*	Belgium	AY762613

*DNA sequences with high percent identity (99.25%) for the *Chlamydia psittaci* sequence detected in Brazil from a wild *Amazona brasiliensis* sample.

Figure 4.2. A mid-point rooted, neighbor joining phylogeny of the DNA sequences of the *Chlamydia psittaci* outer membrane protein gene alignment (1000 bootstrap replicates). The length of the DNA sequences included in the final analysis ranged from 380 to 401 nucleotides. Bootstrap support of nodes is shown if it exceeds 60%.



4.5 DISCUSSION

Anthropic activities have been a trigger for dissemination of diseases in psittacine birds as shown in previous studies, including the international introduction of pathogens to wild and captive naïve populations (KUNDU et al., 2012), and outbreak appearance in wild and captive birds (REGNARD et al., 2014). Despite that, the impact of these actions on the health of wild Brazilian parrots is unknown, as a large-scale assessment has never been performed. Therefore, this study analyzed three wild Amazon parrot species in four states of Brazil for selected viral pathogens and *C. psittaci*. These results showed no viral detection and low prevalence of *C.*

psittaci in the wild populations, which are still apparently unreached from the global spread of relevant psittacine pathogens (FOGELL et al., 2018).

Chlamydia psittaci is a bacteria considered endemic in Brazilian psittacine birds, as it has been detected in wild *A. aestiva* (2/32, 6.25%) and *Anodorhynchus hyacinthinus* (16/45, 35.5%) populations in Pantanal, Mato Grosso do Sul, using a semi-nested PCR (RASO et al., 2006). This low prevalence in Amazon parrots is in accordance with the findings of the present study (3/63, 4.8%) in the nestlings evaluated from the same region of Pantanal. Similar results were also observed in wild *A. brasiliensis* nestlings in Rasa Island, Paraná, using a semi-nested PCR (1/117, 0.8%) (RIBAS et al., 2014) and a PCR (0/58) (VAZ et al., 2017), which are in accordance with the findings of the present study for the species (2/80, 2.5%). Among 11 wild *Anodorhynchus leari* nestlings sampled in northeast Brazil, no *C. psittaci* DNA was detected in the samples (COELHO et al., 2016). Unfortunately, no *C. psittaci* sequences from these studies are available for comparison with our sequence. So, even though some psittacine populations have the bacteria circulating, the overall prevalence seems to be very low and in some of them the circulating genotypes are not known.

Recently, Australian wild cacatuids were evaluated for the presence of *C. psittaci*, which DNA was detected in one sample of *Cacatua sanguinea* using Next Generation Sequencing, giving a prevalence of 1.81% (n=55) (SUTHERLAND et al., 2019), similar to our study. The strain belongs to the genotype A, as the sequence reported in the present study, being among the few sequences of parrots from wild available on Genbank (RASO et al., 2012; SUTHERLAND et al., 2019). In other Latin American countries, only serologic surveys were performed for *C. psittaci,* in Bolivia using 34 wild *A. aestiva* samples (DEEM et al., 2005) and in Peru using 35 wild *Aratinga weddelli* and 13 wild *Brotogeris sanctithomae* samples (GILARDI et al., 1995), which did not find any antibodies against *C. psittaci*.

The sequence detected in the *A. brasiliensis* had a higher nucleotide identity (99%) to the *C. psittaci* reference sequence 6BC (Genbank accession number NC_017287) and to the two Brazilian *C. psittaci* strains found in *Amazona aestiva* (Genbank accession number MH138296) and *Myiopsitta monachus* (Genbank accession number JQ926183), being also phylogenetically close related with them

(Figure 4.2). Besides that, the highest percentage of nucleotide identity (99.25%) was observed to *C. psittaci* found in birds and humans from Europe and Asia (Genbank accession numbers CP033059, KP893667 and AB468956). The *A. brasiliensis* sequence showed at least two nucleotide substitutions when compared to the other sequences within the genotype A, which could have been introduced via recombination or mutation (READ et al., 2013) and led to its branch separation from the other sequences (Figure 4.2). This is not surprising considering the island isolation of the *A. brasiliensis* population evaluated, which could have led to the subtle genetic diversity observed.

The Pacheco's Diseases (PD) was first recognized in captive parrots in Brazil (PACHECO; BIER, 1930), and only later was seen in a large number of psittacine birds exported from South America to Europe and North America (SIMPSON; HANLEY; GASKIN, 1975; MILLER; MILLAR; NAQI, 1979). A nested PCR screening was used to evaluate the presence of PsHV in 112 wild clinically healthy Hyacinthy macaw (*Anodorhynchus hyacinthinus*) nestlings in Pantanal, state of Mato Grosso do Sul, being 14 individuals (12.4%) positive (ALLGAYER, 2009). These findings are noteworthy, considering that Amazon parrots account for the majority of PsHV cases, including probably more susceptible species when compared to macaws (GASKIN; ROBBINS; JACOBSON, 1978). However, the incubation period of PsHV is five to seven days and the virus can be recovered from cloacal and oropharyngeal samples about seven days post-exposure (GASKIN; ROBBINS; JACOBSON, 1978; PHALEN, 2016). This period should be considered in the diagnosis and interpretation of negative results.

In another study with nestling macaws, Karesh et al. (1997) observed positive antibody titers for PsHV in six of nine (66.7%) wild *Ara macao* and *Ara ararauna* from Peru, using complement fixation test. The distinct techniques performed (nested PCR and complement fixation test) may explain the discrepant results among the nestling macaws sampled in the studies above and the Amazon nestlings of the present study - even both of them occurring in the same environment (Pantanal).

Even knowing the PD was first identified in Brazilian parrots, it is not known which genotype could be circulating in the natural Amazon populations. In captivity, only genotype 1 was found in 18 Amazon individuals, which were sampled in life or

after showing sudden death in Brazil (LUPPI et al., 2016). There are four genotypes of PsHV, all can cause deaths in these species and differences in pathogenicity can be observed even within the same genotype (TOMASZEWSKI; KALETA; PHALEN, 2003). Therefore, the introduction of any genotype in wild parrots could be a concern and have potential to occur with accidental or deliberate releases of captive psittacine birds to the wild.

Besides one study detected PsHV in wild Hyacinth macaws, no sequence from wild South American psittacine birds is available on Genbank and only sequences obtained in captive birds are available (LUPPI et al., 2016). The only herpesvirus sequence available on Genbank from a wild psittacine bird is a new herpesvirus detected in a *Cacatua sanguinea* from Australia, Cacatuid Herpesvirus-2 (CaHV-2), close related to the PsHV-1. The prevalence of herpesviruses in this Australian study was very low (1.81%, 1/55) (SUTHERLAND et al., 2019), similar to the present study. This close relationship shows a common ancestor between both alphaherpesviruses, the PsHV-1 and CaHV-2 of the genus *Iltovirus* (SUTHERLAND et al., 2019). However, the current available information in the literature does not allow knowing if they can be both found in other wild Australian and Brazilian species, and how psittacine herpesviruses evolves with their hosts has yet to be more studied.

Poxviruses were not detected in the present study. Similar negative results were already reported in 29 *A. vinacea* while in captivity and once analyzed after their release to the wild in Brazil, using the same primers in a conventional PCR (SAIDENBERG et al., 2015). However, poxvirus outbreaks have been reported in captive native (ESTEVES et al., 2017) and exotic (MURER et al., 2018) Psittaciformes in Brazil, showing low (3/94, 3.2%) and high mortality rates (50 deaths), respectively. Esteves et al. (2017) detected poxvirus DNA on conjunctiva/cloacal swabs collected from four *A. aestiva* showing no clinical signs, and on cutaneous lesions obtained from one *A. brasiliensis*. Natural avianpox infections have been identified in more than 200 avian species of 23 orders, especially within the Psittaciformes order, one of the main groups affected (at least 46 species) (BOLTE; MEURER; KALETA, 1999; VAN RIPER III; FORRESTER, 2007). *Psittacinepox* is considered an important pathogen for aviculturists as it can result in high losses in a short time (TRIPATHY; REED, 2013). However,

epidemiology, pathogenesis and host range of poxviruses in wild birds remains a knowledge gap in the literature and more research should be stimulated.

No circovirus was detected in the wild birds sampled in the present study. Similar results have been reported for a small population of *A. leari* sampled (n=11) in northeast Brazil (COELHO et al., 2016). Based on these findings, it is likely that wild parrot populations can still unreached by the worrisome global spread of the BFDV (FOGELL; MARTIN; GROOMBRIDGE, 2016). PBFD has worried avian veterinarians because of its high dissemination capacity. The pathogen is very stable in the environment and infected birds excrete heavy amounts of the virus in feather dander and faeces (RAIDAL; SABINE; CROSS, 1993), which guarantee the highly infectious characteristic of the virus. Besides its effects on bird feathers, which became unable to fly and consequently easy preys, the BFDV has an immunosuppressive effect, making birds more susceptible to secondary opportunistic diseases (RAIDAL; PETERS, 2018).

Australia is the most likely originating country of the BFDV (HARKINS et al., 2014), where it is the dominant pathogen of wild Psittaciformes (RAIDAL; SARKER; PETERS, 2015). The BFDV was recognized as a key threatening process in 2001 for critically endangered and endangered species like the orange-belied parrot (*Neophema chrysogaster*) and the Norfolk Island green parrot (*Cyanoramphus cookii*), respectively (DEH, 2005). A Threat Abatement Plan was developed to ensure that the BFDV does not become a risk factor for the conservation of other species and does not pose greater risks to key targeted endangered species (DEH, 2005). Nowadays the virus still a threat for two more species in Australia, the swift parrot (*Lathamus discolor*) and the Carnaby's cockatoo (*Calyptorhynchus latirostris*) (DEP, 2015).

The BFDV has been globally spread by the international legal or illegal pet trade (FOGELL et al., 2018). The virus has been introduced and detected in wild invasive and/or native birds in at least 11 countries, including the Asian, African and European continents (FOGELL; MARTIN; GROOMBRIDGE, 2016; FOGELL et al., 2018). It has caused outbreaks in wild endangered birds as the Echo parakeet (*Psittacula eques*) in the Republic of Mauritius, which has an estimated 450 wild individual's population (KUNDU et al., 2012).

Brazil is home of the greater diversity of Psittaciformes in the world, counting on 86 species, being 24 endemic and some of them occurring in a very restricted area (PIACENTINI et al., 2015; IUCN, 2019). Seventeen of this species are included in the national red list (ICMBIO, 2018). In Brazil, the BFDV was first diagnosed in an imported *Cacatua alba* (WERTHER et al., 1999), and more recently it has been detected in exotic and native pet species (AZEVEDO, 2014). The virus has also been observed in one bird from a captive-breeding program for an endangered species, the *A. leari* (COELHO et al., 2015), which occur in a very small area in Brazil with an estimate population of 250-1000 individuals (IUCN, 2019). In addition, a molecular investigation performed in native species from a wildlife rehabilitation center revealed a prevalence of 7.8% in *A. aestiva* samples (ARAUJO et al., 2015). These centers are responsible for receiving native and exotic pet and wild native birds, including parrots seized from illegal trade, and releasing them to the wild after recovered.

These findings are very concern and there is an imminent risk for the introduction of the BFDV to wild Brazilian psittacine populations, as thousands of birds are released to the wild every year without any health criteria/quarantine. In addition, wild birds can have contact with captive ones from aviaries, which can also escape to the wild. Considering these data and the negative BFDV results reported for wild parrots in the present study, very little has been done in mitigating disease threats and to improve the protection of the Brazilian parrot fauna. There are no proper health protocols for psittacine bird's reintroduction, as the one determined by the Brazilian environmental authorities (IBAMA, 2008), which needs plenty of improvement. Release protocols developed together with environment authorities (SÃO PAULO, 2017) does not include quarantine or laboratory exams for a proper avian health evaluation. The importation of ornamental birds also lacks proper health exams, since only Avian Influenza and NewCastle virus tests are performed according to normative instructions (MAPA, 2018). So, health protocols must be revised and proper elaborated. Once introduced in a captive or wild population, it is very difficult or even impossible to eradicate the BFDV, and many birds have to be euthanized to achieve this. So, prevention methods are the best methods of control (RAIDAL; PETERS, 2018).

The present study showed the first large scale health assessment of wild psittacine birds in Brazil. Subclinical infectious of *C. psittaci* was found in a low

prevalence, which was already demonstrated in previous research (RASO et al., 2006). The sequence here obtained and analyzed revealed that genotype A is circulating in wild *Amazon* parrot populations, which have been reported in captive birds in Brazil (VILELA et al., 2019).

Our study group is making further epidemiologic studies to provide more healthy data on pathogens of wild and captive parrots in the country, as it is early to assess the real impact of diseases on psittacine species conservation. However, no viruses were detected in the nestlings evaluated, reinforcing the need to test the health status of parrots before being released into the wild.

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5. HEALTH ASSESSMENT OF SEIZED *Amazona aestiva* NESTLINGS FROM ILLEGAL TRADE IN TWO STATES OF BRAZIL

5.1 ABSTRACT

The blue-fronted Amazon parrot (*Amazona aestiva*) is a South American psittacine bird, being the main parrot species caught from wild to supply the illegal trade in Brazil. Trafficked parrots are submitted to poor husbandry conditions, stress, and this unbalanced bird movement enhances the risk of disease dissemination. *Chlamydia psittaci*, *Psittacid alphaherpesvirus 1*, poxvirus and *Beak and feather disease virus* have been detected in captive *A. aestiva,* including birds from wildlife rehabilitation centers. However, rare studies have been carried out in the Brazil to evaluate the risks of the illegal wildlife trade for the health of psittacine birds, which also threats the public health. So, the aim of the present study was to screen recently seized *A. aestiva* nestlings from illegal trade from two states of Brazil and submitted to a wildlife rehabilitation center. Cloacal swab samples were collected from 90 nestlings and blood were collected from 30 of these seized birds from three different origins. PCR was performed for the four pathogens in all samples. None of the reactions yielded positive results. These results may reflect the low prevalence that has been observed for some of the pathogens evaluated in wild populations. The isolation of the birds and sampling performed in recently arrived birds are also hypotheses for the results observed, as no screening was performed in these animals to evaluate housing long term effects on their health. So, preventive measures should never be neglected in animals seized from illegal trade and should be further discussed and taken seriously in a megadiverse country as Brazil.

5.2 INTRODUCTION

The blue-fronted Amazon parrot (*Amazona aestiva*) is a South American native psittacine species, mainly occurring in Brazil, but also in Bolivia, Argentina and Paraguay territories. In Brazil, the species is distributed in almost all biomas, including Caatinga, Cerrado and Pantanal in the North, Center-West, Southeast and Northeast regions. Its extensive range makes the *A. aestiva* be included in the least

concern conservation status, but its population trend appears to be decreasing (IUCN, 2019).

The main threats to *A. aestiva* populations in all environments where it occurs are habitat loss and the illegal national/international trade in wild caught nestlings (IUCN, 2019). It is estimated that poaching of wild nestlings for the pet trade in Neotropical parrots occurs on an average of 30% of the nests (WRIGHT et al., 2001). Ten-year records show that the *A. aestiva* is among the 10 most received bird in three wildlife rehabilitation centers located in the city of São Paulo (SÃO PAULO, 2017), being the psittacine bird more frequently found in wildlife rehabilitation centers (SEIXAS; MOURÃO, 2000; IBAMA, 2016). Hundreds of nestlings are seized by environmental authorities every year, and it is estimated that the double or triple of wild parrots are caught for the national and international illegal trade, but are not reported because of the lack of inspection or they come to death during transportation, capture and handling (IÑIGO-ELIAS; RAMOS, 1991). The illegal trade is considered an environmental crime in Brazil according to the federal laws 5197/1967 and 9605/1998 and federal decree 6514/2008, which provide punishments as detention and fines (CPITRAFI, 2003). However, law enforcement and wildlife inspection are often lacking in most countries of the world, including Brazil (OIE, 2012).

It is known that birds caught in nature for the illegal trade pass through severe poor conditions of transport, housing, feeding and temperatures. Stress and consequently immunosuppression are inevitable conditions in these cases and the nestlings become vulnerable to diseases (RASO et al., 2004). Birds seized from different locations are housed together in rehabilitation centers, being also in contact with resident animals that once could have been pet animals (OIE, 2012; MORA-CHAVARÍA et al., 2017). Most of the seized birds go to release programs and most of times are released to nature without any health criteria or biosecurity (MARINI; GARCIA, 2005), as Brazil lacks proper normative instructions including national standard health screening protocols. Considering this unbalanced movement of birds, the risk of disseminating and even introducing pathogens to wild populations is imminent.

Rare studies have been carried out in the world evaluating the role of the illegal wildlife trade on the spread of infectious diseases (DAUT et al., 2016), mainly on Brazilian birds during the period housed on *ex situ* facilities (GODOY; MATUSHIMA, 2010; RASO et al., 2013). Information generated by these studies can improve the health care of captive animals and promote changes of husbandry (VANSTREELS et al., 2010). In addition, negative impacts of all these anthropic activities are not only restricted to avian health, but also extended to public health (RASO et al., 2014), as these animals are exposed to hunters, traders, sellers, consumers, veterinarians, biologists and keepers along the trade chain, markets and rehabilitation centers (OIE, 2012).

Avian chlamydiosis and psittacosis are important diseases for psittacine birds and humans, respectively, caused by the bacteria *Chlamydia psittaci*. In many countries they must be reported within 48 hours for public or animal health agencies as they are considered notifiable diseases (VANROMPAY, 2013). Chlamydiosis outbreaks have been observed in *A. aestiva* nestlings seized from the illegal trade in Brazil with a mortality rate of 96.5% (56/58) (RASO et al., 2004). In addition, psittacine birds acquired from the illegal trade have been related to a psittacosis domiciliary outbreak in seven people with severe atypical pneumonia (RASO et al., 2014).

Nevertheless, other relevant psittacine pathogens have been observed in *A. aestiva* housed in Brazilian wildlife rehabilitation centers, as the Psittacid Herpesvirus 1 (PsHV) of the Pacheco's Disease (PD) (LUPPI et al., 2011), and the exotic virus Beak and Feather Disease Virus (BFDV) (ARAUJO et al., 2015). Avianpox infections have been observed within the Psittaciformes order (BOLTE; MEURER; KALETA, 1999), with a recent outbreak reported in a conservation facility in Brazil, involving four *A. aestiva* and one *A. brasiliensis*, besides other exotic psittacine birds (ESTEVES et al., 2017).

The conditions of vulnerability to which *A. aestiva* is subjected in Brazil, its susceptibility to *C. psittaci* and viral pathogens, the risk of spreading and introducing diseases to wild populations and the lack of health information on trafficked birds is a great concern for avian veterinarians in the country. So, the aim of the present study was to screen recently seized *A. aestiva* nestlings from illegal trade from two states of Brazil and housed in a rehabilitation center.

5.3 MATERIAL AND METHODS

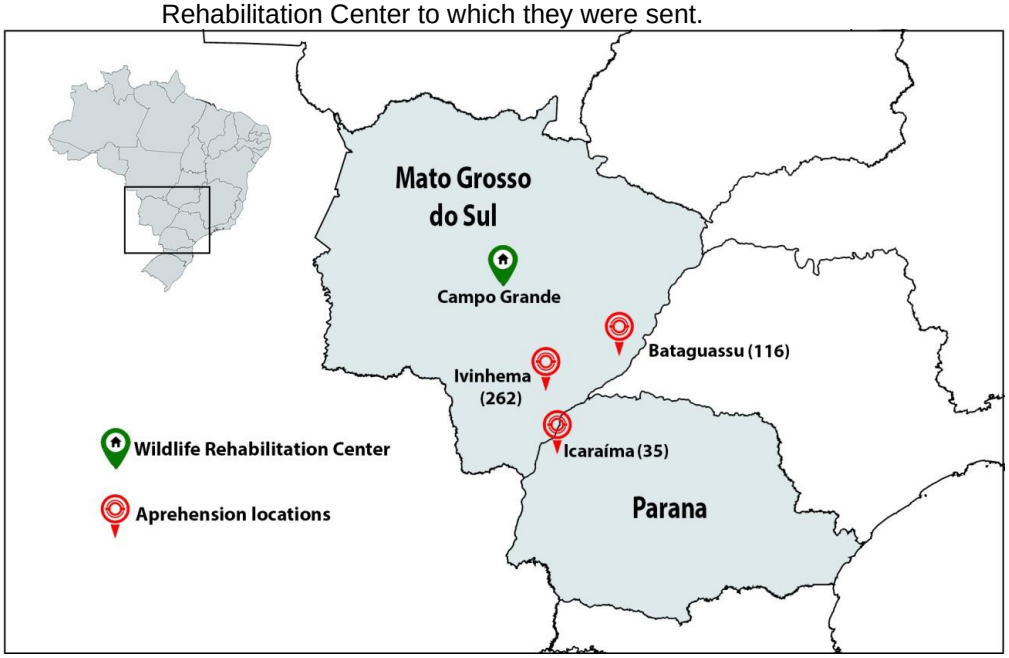
5.3.1 Sampling

This study was approved by the Ethic Committee on Animal Use of the University of São Paulo (CEUA 9545290116) and by the Brazilian environmental authority (SISBIO 43876-1).

Samples for the present study were collected at the Wildlife Rehabilitation Center (WRC) located in the city of Campo Grande, state of Mato Grosso do Sul. In 2015 (september-october), 413 *A. aestiva* nestlings were seized from the illegal trade in three different locations of two states in Brazil and submitted to the WRC. The number of parrots and locations of seizures are shown in Figure 5.1.

Birds were different ages, from recently hatched to fully fledged nestlings (about 5 to 50 days). They were housed together inside 24 boxes and separated by date of seizure in a room within the WRC. The number of parrots per box ranged from nine to 25, with an average of 17 nestlings per box. The parrots were being fed twice a day with a hand-feeding formula for psittacine nestlings and once a day the boxes were cleaned.

Figure 5.1 - Map of Brazil showing number of seized *Amazona aestiva* nestlings from illegal trade and their respective locations, including the Wildlife Rehabilitation Center to which they were sent.



Source: Created with mapchart.net

Cloacal swab samples were randomly collected from approximately 20% of the nestlings from each box, totalizing 90 nestlings, an average of 21.58% of animals sampled. Blood were collected from the superficial ulnar vein of 30 of these parrots and stored inside tubes containing EDTA. Swab samples were stored inside tubes containing PBS and all samples were kept frozen until analysis.

5.3.2 Laboratory analysis

DNA was extract from all samples using the NucleoSpin Tissue kit® (Macherey-Nagel, Düren, North Rhine-Westphalia, Germany) according to manufacturer's instructions.

Polymerase chain reaction (PCR) analysis were performed to screen all the samples for *C. psittaci* (EHRICHT et al., 2006) (111bp fragment of the 23S RNAr gene); Psittacid herpesvirus 1 (TOMAZEWSKI; KALETA; PHALEN, 2003) (667 bp fragment of the UL 17/16 open reading frame gene); poxvirus (LEE; LEE et al., 1997) (576 bp fragment of the p4b gene); and BFDV (YPELAAR et al., 1999) (717 bp fragment of the open reading frame 1 gene) using the thermal cycler Axygen® Maxygene (Axygen, Union City, California, USA). Positive and negative controls were included in all reactions.

The PCR products were analyzed in a 1.2% agarose gel electrophoresis stained with GelRed® (Biotium, Fremont, California, USA) nucleic acid stain.

5.4 RESULTS AND DISCUSSION

None of the nestling samples were positive for any of the pathogens evaluated.

Evidence of negative impacts that the illegal trade imposes to the health of birds have been reported in the literature, but little studies have been carried out on these animals. According to previous studies involving *A. aestiva*, common Psittaciformes pathogens could be found in birds of this species seized from illegal trade and submitted to rehabilitation centers, including endemic and exotic viruses (RASO et al., 2004; LUPPI et al., 2011; ARAUJO et al., 2015).

In the present study, *C. psittaci*, PsHV, poxvirus and BFDV DNAs were not detected in cloacal samples and blood collected from parrots seized from the illegal trade analyzed by PCR. It is known that the prevalence of some these pathogens can be very low in wild populations (RASO et al.; 2006; ALLGAYER, 2009; VAZ et al., 2017), and circovirus was investigated just in a small wild population of *Anodorhynchus leari* (COELHO et al., 2016), showing negative results. So, the results here presented could be due the wild origin of the nestlings, which kept similar disease patterns even after being probably exposed to poor conditions during the illegal pet trade. Another hypothesis could be the isolation of the parrots, which were kept segregated and were sampled as soon as they reached the center, not having contact with the resident animals or being subject to local contamination.

This is supported by other studies performed on psittacine birds housed for a longer period in WRCs, reporting *C. psittaci* outbreaks and higher prevalence than the one here observed. Vilella et al. (2019) investigated *C. psittaci* on 242 *A. aestiva* showing hepatic disease, seized from illegal trade and submitted to a WRC, over a two years period. The *post-mortem* PCR tests revealed a high prevalence of 71.7% (152/242). A high mortality rate caused by *C. psittaci* in Brazilian *A. aestiva* nestlings (56/58, 96.5%) (RASO et al., 2004) and in adult Mexican psittacine birds (11/19, 57%) (ORNELAS-EUSEBIO et al., 2016) from the illegal trade and with longer captivity have been also reported. So, according to our and previous studies, housing effects in WRCs and period of captivity are determinant in disease spread and infection in birds seized from illegal trade, even more than the trafficking activity itself.

Studies conducted in captive psittacine species from conservation facilities, observed a low prevalence for *C. psittaci*, similar to the present study, as reported in *Anodorhynchus leari* (3/39, 7.6%) using a conventional PCR analysis (COELHO et al., 2015) and in *Amazona vinacea* (2/29, 6.9%), using a nested-PCR (SAIDENBERG et al., 2015) These results are not surprising as conservation facilities are usually environments with better husbandry and biosecurity measures.

The need to establish a biosecurity routine in wildlife rehabilitation facilities were also demonstrated by Saidenberg et al. (2015), who found *C. psittaci* in birds that were part of reintroduction projects. The *A. vinacea* individuals were properly treated and retested negative twice by nested-PCR for *C. psittaci*, therefore remaining candidates to be reintroduced. This was a relevant conduct, because although *C.*

psittaci is endemic in South American psittacine birds (RASO et al., 2006; RIBAS et al., 2014), which genotype/strain is circulating in these populations and the prevalence in many other species and in other environments are not known.

The introduction of new animals in aviaries and in natural habitats and the stressful conditions of these activities to the birds, including the illegal trade chain, can trigger clinical diseases and pathogen excretion of latently infected birds (RASO et al., 2004). So, quarantine periods including pathogen diagnosis could prevent the spread of diseases to captive and wild bird populations (SAIDENBERG et al., 2015) and even to human beings (ORNELAS-EUSEBIO et al., 2016; FERREIRA et al., 2017), as there is evidence of highly virulent *C. psittaci* strains emerging globally (BRANLEY et al., 2016). Although the samples of the present study were negative for *C. psittaci*, the WHC constantly receives new animals and disease surveillance would be an ideal practice to promote animal, human and environment health.

Regarding Pacheco's disease, its first world description was in captive psittacine birds in Brazil (PACHECO; BIER, 1930). However, until recently, just four reports had identified the virus in captive birds in Brazil being isolated cases (GODOY, 2001), epidemiological studies (LUPPI et al., 2011; LUPPI et al., 2016; FIEDLER; RASO, 2019) or a worrisome outbreak showing a high mortality rate (61/98, 62.24%) (BARBOSA et al., 2020 *in press*).

In Latin America, serological studies have been performed in wild *A. aestiva* (DEEM et al., 2005) and Amazon species (STONE; MONTIEL-PARRA; PEREZ, 2005) in Bolivia and Mexico, respectively, revealing a seroprevalence of 25% (6/24) and 0% (0/25). These low prevalences observed for PsHV are important data, as PsHV has been found in WRC (LUPPI et al., 2011) that for many times can release birds to the wild, in which the virus apparently is not very common. Naive populations can be highly susceptible for PD, and massive outbreaks with high mortality rates have been reported (GASKIN; ROBBINS; JACOBSON, 1978; MILLER; MILLAR; NAQI, 1979; BARBOSA et al., 2020 *in press*).

Besides poxviruses were not observed in the nestlings, avian pox has been detected in *A. aestiva* from a conservation facility in Brazil (ESTEVES et al., 2017). Rarely poxvirus is detected in a bird showing no classical clinical signs, but conjunctiva/cloacal swabs from four *A. aestiva* showing no lesions were positive using a PCR assay (ESTEVES et al., 2017). However, these birds were diagnosed

during a poxvirus outbreak in a conservation facility, in which 27 of 94 birds were sick. Another outbreak has been recently reported in a breeding facility in Brazil, including death of 50 psittacine exotic birds (MURER et al., 2018), showing the relevance of this pathogen in the country, which are often neglected. Saidenberg et al. (2015) did not find poxviruses in twenty-nine healthy captive *A. vinacea* adults evaluated by the same conventional PCR here performed, which is similar to our study using healthy parrots.

There is just one study reporting circovirus in tissues of birds from a WRC in Brazil, with a general prevalence of 6.3% (12/190) (ARAUJO et al., 2015). These observations are a result of the general husbandry of this WRC, which usually receives native/exotic parrots from many locations and origins, including mixed breeding facilities, creating the perfect scenario for circovirus spread.

Although WRCs has intense movement and manipulation of animals (OIE, 2012), BFDV detection in these facilities in Brazil is surprising, as they should only receive native species, being mostly wild caught birds seized from illegal trade. However, *A. aestiva* is the psittacine bird most submitted to WRCs by citizens (IBAMA, 2016), and this could be a route of BFDV introduction in the centers as health history and general husbandry of these pet animals are not known. Native and exotic pet parrots showing clinical PBFD have been observed in Brazil, whose cases were confirmed by laboratory analysis (AZEVEDO, 2009). In addition, if other psittacine birds of a WRC are positive or not for BFDV is also not known. So, as there are many health risks for psittacine birds from trafficking regarding BFDV, the negative results here found provide a good prognosis for the flock evaluated.

The Food and Agriculture Organization (OIE) and the World Health Organization (WHO) stated the movement and manipulation of domestic and wild animals as a big trigger for emerging disease (OIE, 2012). Although the parrots of the present study were subjected to illegal trade, *C. psittaci* and viral pathogen DNAs were not detected. However, these results were observed under certain circumstances of flock isolation and samples being collected upon the arrival of the birds at the WRC. No screening was performed later on these *A. aestiva* nestlings to evaluated housing long term effects on their health, as studies have demonstrated that longer captivity can considerably affects disease spread and infection. So, preventive measures must be ongoing activities in psittacine birds introduced in a

flock and should be further discussed and taken seriously in a megadiverse country as Brazil.

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6. FINAL CONSIDERATIONS

Considering the lack of health studies in the area, the present study showed a broad investigation of the occurrence of *C. psittaci* and viral pathogens in wild Amazon parrot populations and a health screening of *A. aestiva* nestlings seized from the illegal trade. The Psittaciformes is one of the most endangered avian orders in the country and contains key-species for the conservation of nature. Parrots are among the most trafficked birds and are constantly moved and manipulated, which are intense triggers for emerging diseases and pathogen dissemination.

The results revealed a small prevalence of *C. psittaci* in two wild populations (*A. aestiva* and *A. brasiliensis*) and no detection of *Psittacid alphaherpesvirus 1*, poxviruses and *Beak and feather disease virus* in the nestlings.

Few *C. psittaci* DNA sequences are available in the literature from wild psittacine birds in the world. We reported here a *C. psittaci* close related to the ones that have been found in captive parrots in Brazil, within the genotype A, which have been the only genotype detected in the country. This genotype A is the most involved in human psittacosis acquired from birds, and future manipulation of these animals should be performed using proper individual protection equipment as masks and gloves.

The negative results for the viral pathogens here observed provide implications for the conservation of psittacine birds in Brazil, especially regarding PsHV and BFDV. South America is the likely origin of PsHV, however, it has been infrequently reported in Brazil and the prevalence seems to be very low in wild parrot populations. These birds must remain protected as even which PsHV genotype that could be circulating in the wild is not known.

The BFDV, an exotic pathogen, is recognized as a key-threatened process for endangered species in Australia and has reached wild endangered populations in other continents. Recently, it has been detected in captive native Brazilian birds and there is a worrisome risk of its introduction to the wild, as no health criteria are compulsory to release birds in Brazil, and the existent health protocols are obsolete.

So, new health protocols should be established for the release of birds to the wild and they should be strictly followed. Preventive measures are the best ones for

the BFDV, as it doesn’t have a specific treatment. Once introduced in a population, it is almost impossible to eradicate the circovirus, and euthanasia of positive birds should be discussed and performed.

Our study also collaborated to compare the illegal trade activity to longer captivity effects on the health of parrots. A flock seized from illegal trade can have a low prevalence of certain pathogen or even be free of it when arriving at a wildlife rehabilitation center, as studies revealed low infection rates in the wild. However, the longer these birds stay in some of those centers, the greater the chance of being infected with these pathogens, due to poor husbandry, local contamination, intense movement of birds or contact with exotic species.

Despite the significant number of parrots sampled and the different assessment areas used in this study, further virus/population studies are still needed to clarify pathogen impacts on psittacine species in Brazil, contributing to the conservation efforts of these birds and their environments.