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Molecular Survey of Pathogens in Wild Amazon Parrot Nestlings: Implications for Conservation

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Abstract: South America presents the greatest Psittacidae diversity in the world, but also has the highest numbers of threatened parrot species. Recently, exotic viruses have been detected in captive native psittacine birds in Brazil, however, their impacts on the health of wild parrots are still unknown. We evaluated the presence of *Chlamydia psittaci*, *Psittacid alphaherpesvirus* 1 (PsHV-1), avipoxvirus and beak and feather disease virus (BFDV) in wild *Amazona aestiva*, *A. brasiliensis* and *A. pretrei* nestlings and in wild caught *A. aestiva* nestlings seized from illegal trade. Samples were collected from 205 wild nestlings and 90 nestlings from illegal trade and pathogen-specific PCR was performed for each sample. *Chlamydia* DNA prevalence was 4.7% in *A. aestiva* and 2.5% in *A. brasiliensis* sampled from the wild. Sequencing revealed that the *C. psittaci* sample belonged to the genotype A. PsHV-1, avipoxvirus and BFDV DNA was not detected. These results have conservation implications since they suggest that wild parrot populations have a low prevalence of the selected pathogens and, apparently, they were not reached by the exotic BFDV. Stricter health protocols should be established as condition to reintroduction of birds to the wild to guarantee the protection of Neotropical parrots.

Keywords: wild parrots; *Chlamydia psittaci*; *Psittacid alphaherpesvirus* 1; avipoxvirus; beak and feather disease virus; conservation threats

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1. Introduction

Psittacidae diversity in South America is the greatest in the world and Brazil is the country with the largest number of species. Among the 411 known species, 86 occur in the national territory [1]. Unfortunately, Brazil is also in the first position when it comes to threatened species, with Psittaciformes being one of the most threatened, containing 25 native species in the Global International Union for Conservation of Nature (IUCN) Red List [2].

Amazon parrots are prominent among the national species, being the first among the most trafficked psittacine birds in Brazil. *Amazona* genus comprises 12 species in Brazil and these birds are threatened mainly by the illegal trade and loss of their habitat. Currently, one third of the native Amazon species is threatened [2].

Among these species, the red-spectacled Amazon parrot (*Amazona pretrei*) is threatened within the vulnerable category. The red-tailed Amazon parrot (*Amazona brasiliensis*) has left the IUCN Red List, entering the near-threatened status. Both species have a restricted distribution and exist only in Brazilian territory. The blue-fronted Amazon parrot (*Amazona aestiva*) is also in the near-threatened category and has a wide distribution, including Brazil, Argentina, Bolivia, and Paraguay territories. However, there is a special interest in this species because it is the main target of the illegal trade [2,3].

Nonetheless, another current challenge to wildlife conservation efforts is the dissemination of infectious diseases. As parrots are extremely popular pets, the demand created around the world has led to an international movement of over 19 million birds since 1975 [4], which triggers the spread of pathogens. Disease emergence can be triggered by translocation; introduction of infected animals, pathogens or vectors to new geographic regions; human or domestic animals' encroachment, spill-over, ex situ contact and ecological manipulation [5]. Amazon parrots are subjected to at least three of these situations [6], therefore, their health assessment in the wild is an important addition to their conservation efforts.

The illegal trade of wild birds is still a reality in Brazil, and only a small part of the nestlings removed from nature is apprehended by environmental authorities. These birds are mixed in rehabilitation centres with resident or pet birds and are often released into the wild without any health criteria [7]. In addition, national and international cross border movement of birds continues as the result of smuggling and legal trade of domestically raised birds [8], creating the perfect scenario for disease dissemination in wild and captive animals, as trafficked and imported birds are fed with improper diets, housed in crowded unhygienic conditions, and mixed with other species [9,10]. The global spread of diseases has caused a significantly negative conservation impact on captive and wild populations [11,12]. Highly resistant viruses in the environment and persistent subclinical infections make controlling these pathogens a challenge [8].

Chlamydia psittaci and the Psittacid herpesvirus 1 (PsHV-1) are relevant pathogens that affect parrots and have been observed in captive psittacine in Brazil [13,14], including occasional outbreaks [10]. A neglected virus in wild birds, the avipoxvirus, has also caused an outbreak in psittacine species located in a facility in Brazil [15]. In addition, the Psittacine Beak and Feather Disease (PBFD) caused by a circovirus, is an exotic pathogen introduced in the country [16], that has been reported in exotic and native pet birds [17,18].

The results of all the negative anthropogenic actions for the health of wild Amazon parrots in Brazil are unknown and information is scarce in the literature [19–21]. The aim of the present study was to investigate the presence of *C. psittaci* and viral pathogens DNA in wild Amazon parrot nestlings and in wild caught nestlings recently apprehended from illegal trade in Brazil, and to discuss the implications of the results for the conservation of psittacine birds.

2. Materials and Methods

The study comprised three species of parrots in four states of Brazil (Figure 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 on three islands (Ilha Rasa, Ilha Gamela and Ilha Chica), in the state of Paraná, located in the Guaraqueçaba Environmental Protection Area, which has an extensive area of Atlantic Forest. This species was also sampled in Comprida Island, state of São Paulo, another Atlantic Forest area within the Ilha Comprida Environmental Protection Area. *Amazona aestiva* parrots were sampled in Miranda, state of Mato Grosso do Sul, located in the Brazilian Pantanal wetlands.

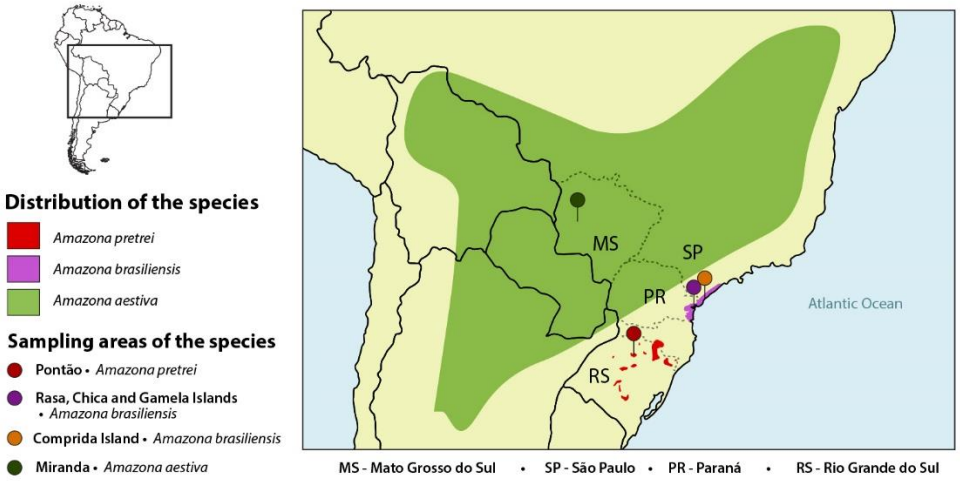

Figure 1. Distribution of *Amazona pretrei, Amazona brasiliensis* and *Amazona aestiva* in Brazil and South America and sampling areas.

Oropharyngeal and cloacal swab samples and/or blood samples were collected from Amazon parrot nestlings in field expeditions during the breeding seasons (October to January) from 2013 to 2018. All samples were collected by trained professionals. Natural and artificial nests were accessed using ladders or climbing equipment. The birds were removed from the nests, examined, sampled, and then placed back in the nests. Swab samples were kept frozen in microtubes containing viral transport media, and blood samples were kept frozen in microtubes until the analyses. One *A. brasiliensis* was found recently dead inside a nest and was necropsied for sample collection. Liver and spleen fragments were collected and kept frozen until laboratory analysis.

Additionally, in 2015, 413 wild caught *A. aestiva* nestlings were seized from the illegal trade by environmental officials in three different locations (A—262 birds, B—116 birds, C—35 birds) in two states of Brazil (Mato Grosso do Sul and Paraná). The nestlings apprehended were submitted to the Wildlife Rehabilitation Center (WRC) located in the city of Campo Grande, Mato Grosso do Sul. Birds were different ages, ranging from recently hatched to fully fledged nestlings (about 5 to 50 days). They were housed inside boxes in a proper room, separated by bird size and by origin. Biological samples were collected in the first 72 h after the birds were seized. Cloacal swab samples were randomly collected from approximately 20% of the nestlings from each box, totalizing 90 nestlings, 21.6% of the nestlings received in that year. Blood was collected from the brachial vein in 30 of these parrots and all samples were kept frozen until analysis.

Genomic DNA extraction was performed using the NucleoSpin Tissue kit® (Macherey-Nagel, Germany) according to manufacturer's instructions. Each sample was digested in 200 µL of lysis buffer and proteinase K (20 mg/mL) at 56 °C for 12 h prior extraction. DNA was extracted from 326 samples (swab/blood samples and a fragment of liver/spleen) from 205 birds. All samples were screened for the presence of chlamydial DNA using a conventional PCR targeting 111bp of the Chlamydiaceae 23S rRNA gene with primers from Enrich et al. [22]. A 25 µL reaction mix containing 3 µL of genomic DNA, 10 pmol of each primer, 12.5 µL of buffer (DreamTaq Green PCR Master Mix, Thermo Fisher Scientific, Waltham, MA, USA) and nuclease-free water qsp was used in the reaction. Target DNA was amplified performing a conventional PCR using an initial denaturation of 60 s at 96 °C, then 40 cycles of 30 s at 94 °C, 60 s at 50 °C and 30 s at 72 °C, followed by a final extension of 4 min at 72 °C. The samples were also screened for the presence of viruses using conventional PCRs for PsHV-1 [23], avipoxvirus [24] and BFDV [25]. Following this, positive samples in the *Chlamydiaceae* PCR were evaluated using a second PCR assay to amplify a fragment of *C. psittaci omp*A gene [26]. The primer sequences used for all agents can be found in the Table S1.

Negative and positive standard controls were used in all PCR reactions for each agent. Nuclease-free water was used as negative control. The *C. psittaci* genotype A, Cpsi/Mm/BR01 DNA was used as positive control (GenBank accession number JQ926183) for *Chlamydiacea* and *omp*A PCR assays. BFDV (strain from a *Psittacus erithacus*), herpesvirus (PsHV-1 genotype 3 from an *Ara ararauna*) and Pox vaccine (Pox pigeon, Biovet, Brazil) DNA were used as positive controls for the other PCR assays. All reactions were carried out using the thermal cycler Axygen® Maxygene (Axygen, Union City, CA, USA). The products were analysed by electrophoresis in a 1.5% agarose gel stained with GelRed® (Biotium, Fremont, CA, USA) nucleic acid stain.

Amplified products from the *omp*A PCR assays were purified from the agarose 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 dual-direction by Sanger sequencing (Genome Research Center, University of São Paulo, São Paulo, Brazil). The chromatograms were analysed for quality using MEGA X software, and sequences were compared with data available on GenBank through a BLAST search. The nucleotide alignment was performed using MAFFT version 7 with the FFT-NS-I algorithm [27]. A neighbor joining tree was constructed using Mega X [28]. The Tamura-Nei model was chosen to create the tree tested by bootstrapping with 1000 replicates.

3. Results

A total of 205 Amazon parrot nestlings from wildlife were sampled as shown in Table 1. All the birds showed no clinical signs that could suggest infection by any of the pathogens here investigated. Liver and spleen fragments were collected from one wild *A. brasiliensis* nestling that was found recently dead inside one nest at Rasa Island, but pathogens DNA were not detected in those samples. None of the nestling samples tested yielded positive PCR results for PsHV-1, avipoxvirus or BFDV.

Table 1. Number of wild Amazon parrot nestlings sampled according to breeding season in the states of Rio Grande do Sul (RS), Paraná (PR), Mato Grosso do Sul (MS) and São Paulo (SP), Brazil.

Amazon Species	State	2013/2014	2015	2016	2017	2018	Total Samples (Swab and/or Blood)	Number of Birds Sampled for Selected Virus **/Positive (%)	Number of *Chlamydia* Positive/Total Birds (%)
A. pretrei	RS	0	0	4	0	0	8	4 (0%)	0/4 (0%)
A. brasiliensis	PR/SP	74 *	0	28 #	15	21	230	138 (0%)	2/80 (2.5%)
A. aestiva	MS	17	17	23	3	0	89	63 (0%)	3/63 (4.8%)
Total							327	205 (0%)	5/147 (3.4%)

* Just 16 swabs were tested for *Chlamydia* in this period. Total number of birds tested for *Chlamydia* = 147. # Liver and spleen fragments sampled from one carcass. ** PsHV-1, avipoxvirus and BFDV.

Chlamydia prevalence found for all the parrots evaluated was 3.4% (5/147); these samples were from parrots sampled between 2015–2018. From the *Chlamydia*-positive samples, 4.8% (3/63) were collected from *A. aestiva* (cloacal swab samples), and 2.5% (2/80) were collected from *A. brasiliensis* (one cloacal/oropharyngeal swab sample and one blood sample). The *A. pretrei* nestlings evaluated were negative (0/4, 0%). *Chlamydia psittaci* nucleotide sequencing was possible only in the blood sample from an *A. brasiliensis* nestling (Cpsi/Ab/BR02; GenBank accession number MT741095). This partial *ompA* gene sequence was analysed and aligned with reference sequences available on GenBank (Table S2). The phylogenetic tree is presented in Figure 2. The sequence obtained was confirmed as *C. psittaci* as it had a high percentage of identity (99.25%) with other *C. psittaci* sequences, clustering within the Genotype A.

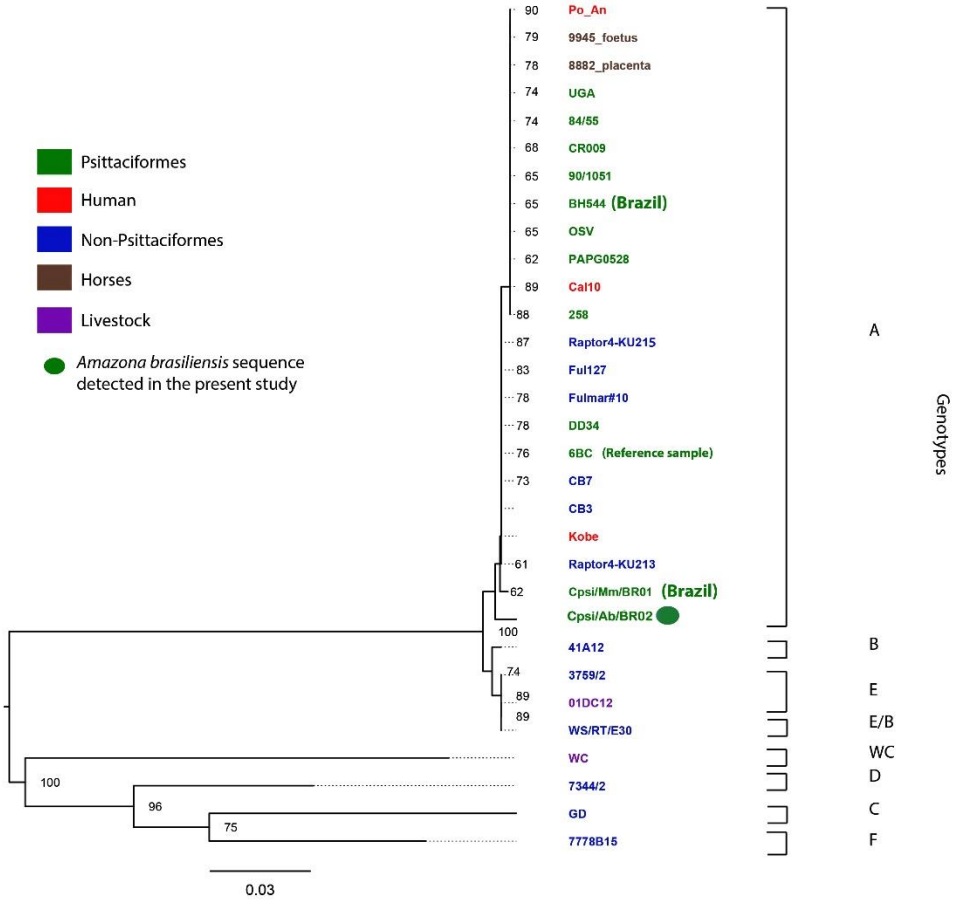

Figure 2. A mid-point rooted, neighbor joining phylogeny of the partial DNA sequences of the *Chlamydia psittaci* outer membrane protein gene alignment (1000 bootstrap replicates). Bootstrap support of nodes is shown if it exceeds 60%. (Cpsi/Ab/BR02, GenBank accession number MT741095).

Regarding the nestlings from illegal trade, all samples (90 swabs and 30 blood samples) collected in the first 72 h after apprehension of the birds were negative for the agents analysed (*Chlamydia psittaci*, PsHV-1, avipoxvirus and BFDV).

4. Discussion

Anthropogenic activities have been a trigger for dissemination of diseases in psittacine birds as shown in previous studies around the world [5], including the international introduction of pathogens to wild and captive naïve populations [11], and outbreaks in wild and captive birds [29]. Nevertheless, the impact of these actions on the health of wild Brazilian parrots is unknown, as a large-scale assessment has never been performed. The results of this study showed low prevalence of *C. psittaci* and no viral detection in the wild Amazon parrot nestlings sampled, which apparently have not yet been reached by the global spread of relevant psittacine pathogens, which is not the case of BFDV in other countries [30].

C. psittaci is a bacterium considered endemic in Brazilian psittacine birds. It was first detected in wild *A. aestiva* (2/32, 6.3%) in Pantanal, Mato Grosso do Sul, in 2006, using a semi-nested PCR and complement fixation test [19]. This prevalence is in accordance with our results (3/63, 4.8%) in *A. aestiva* nestlings evaluated from the same region of Pantanal. Other studies in *A. brasiliensis* nestlings in Rasa Island, Paraná, showed 0.8% [20] and 0% [21] of *C. psittaci* prevalence which are in accordance with our results for this species (2/80, 2.5%). Unfortunately, no *C. psittaci* sequences from these previous studies are available for comparison. Therefore, even though some parrot populations have the

bacteria circulating, the overall prevalence seems to be stable over the years and, in some of them, the circulating genotypes are unknown. Conventional PCR is widely used in research of pathogens in captive birds; however, a minor limitation is that this assay can have less sensitivity than real time PCR and maybe low copy number samples can be missed. The sampled birds here were very young fledglings and possibly were not even infected. This fact was demonstrated in a previous study [21], in which the combined serology and PCR results were negative for *Chlamydia*, demonstrating that the nestlings were not infected in the sample collection time. Therefore, we believe that the chlamydial prevalence in these populations is indeed low. In other Latin American countries, only serologic surveys are available for *C. psittaci* in the wild: *A. aestiva* in Bolivia [31] and *Aratinga weddelli* and *Brotogeris sanctithomae* in Peru [32]. However, no antibodies against *C. psittaci* were detected.

The amplified *C. psittaci ompA* fragment clustered with previously described *ompA* Genotype A. Based on BLAST analyses, our sequence had 99% identity with the reference sequence 6BC (NC_017287) and with two Brazilian *C. psittaci* sequences: one from a long-term captive *A. aestiva* (Genbank MH138296) and one from a wild caught monk parakeet (*Myiopsitta monachus*) sampled after being seized from poachers in Southern Brazil (Genbank JQ926183) [33]. Furthermore, the highest percentage of nucleotide identity (99.25%) was observed with *C. psittaci* found in birds and humans from Europe and Asia (Genbank CP033059, KP893667 and AB468956). The sequence obtained here and from previous studies in Brazil suggest that Genotype A can be the main circulating genotype in wild psittacine birds in the country. Moreover, Genotype A is most frequently associated with psittacosis cases in humans. In Brazil, the potential of monk parakeets (*Myiopsitta monachus*) in transmitting *C. psittaci* to humans has already been documented [34].

In the present study, no BFDV, PsHV and avipoxvirus DNA was detected in wild and in smuggled nestlings. Nevertheless, the wild caught nestlings seized from the illegal trade were not sampled later to evaluate housing long term effects on their health.

Pacheco's disease (PD) is caused by PsHV-1 and it was first recognized in parrots in Brazil [35], and only later it was seen in many psittacine birds exported from South America to Europe and North America [36]. Even so, there are only sporadic reports on PD occurrence in parrots in Brazil [14] and there is no information on the genotypes circulating in the country. So far, in captive birds, only Genotype 1 was found in 18 Amazon individuals [37]. Negative results for avipoxvirus were also reported in 29 captive *A. vinacea* using the same primers in a conventional PCR [38]. However, avipoxvirus outbreaks have been reported in captive native [15] and exotic [39] Psittaciformes in Brazil, showing low and high mortality rates, respectively. BFDV has been recently detected in captive exotic and native species [17,18] in Brazil. Based on the initial findings of the present study, it is likely that wild parrot populations can still be unreached by the global spread of BFDV [40]. Because of the high dissemination capacity and the immunosuppressive effects, BFDV has worried avian veterinarians around the world [41]. Hence, further research must be done to provide more detailed data on prevalence, diversity of genotypes and host range of these viruses in wild and captive psittacine birds in the South America.

Wildlife rehabilitation centers are responsible for receiving injured or apprehended wild animals, which are mainly represented by native species seized from illegal trade or illegally maintained as pets by owners or by irregular breeders and, occasionally, exotic species from irregular captivity. Frequently, these places release native birds to the wild after being recovered. This situation is concerning and there is an imminent risk of introduction of pathogens to wild Brazilian psittacine populations, as thousands of birds are released to the wild every year without any health criteria/quarantine. Considering these data and the negative results for BFDV reported here in wild parrots, little has been done in mitigating health threats and to improve the protection of the Brazilian parrot fauna. Therefore, the elaboration of a national health program for relevant pathogens that affects psittacine birds is extremely urgent. Once introduced in a captive or wild population, it is difficult or even impossible to eradicate the BFDV, and many birds would have to be euthanized to achieve

this. Thus, there is no doubt that prevention methods are the best approach to control the spread of this virus [41].

5. Conclusions

Our study reveals a longitudinal pathogens assessment of wild psittacine fledglings showing low chlamydial prevalence and no detection of some important parrot viruses. Even though the incidence of exotic viral diseases is increasing in captive psittacine in Brazil, it is still early to assess the real impact in wildlife parrots. Unfortunately, the capture and sampling of adult Amazon parrots is not an easy procedure in natural conditions, which could provide more robust data about the health status of these populations. Further, the infectious diseases control in psittacine from captivity or from illegal trade must be carried out carefully before releasing the birds into the wild avoiding the dissemination of pathogens that have the potential to negatively impact the conservation of Neotropical parrots.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13060272/s1, Table S1: primer sequences used for detection of viruses and *Chlamydia psittac*i in wild Amazon parrot from Brazil. Table S2: GenBank accessions, host species, origins, and *Chlamydia psittaci* strains used for comparation in this study.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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