Short Communication

Pacheco's Disease Outbreak with High Mortality in Brazilian Captive Psittacine Birds

Carla Meneguin Barbosa[1*], Fabio Gregori[2], Cairo Monteiro[1], Thais Helena Martins Gamon[1], Luciano Matsumiya Thomazelli[1], Tânia de Freitas Raso[2] and Edison Luiz Durigon[1]

[1]Biomedical Sciences Institute of University of São Paulo; [2]School of Veterinary Medicine and Animal Science of University of São Paulo.

Abstract | *Psittacid Alphaherpesvirus* 1 (PSHv-1) is the causative agent of Pacheco's disease that infects psittacine birds, highly transmissible and presenting high mortality rate. Here we describe the investigation of 98 sudden deaths of birds, belonging to 8 Brazilian native psittacine species, divided in two outbreaks, one year apart between 2016 and 2017, in a breeding farm in São Paulo State, Brazil. PSHv-1 was detected in 6 species (61 birds) through Nested-PCR, that tested negative for Influenza, Paramyxovirus, Flavivirus, and Adenovirus. Genetic sequencing and phylogenetic analysis support these results.

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***Correspondence** | Carla Meneguin Barbosa, Biomedical Sciences Institute of University of São Paulo, Brazil; **Email:** carlameneguinb@hotmail.com
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Introduction

Psittaciformes order sum 113 species listed as critically endangered, endangered or vulnerable by the International Union for Conservation of Nature (IUCN, 2022). Besides their ecological importance, psittacines are also the most common exotic species sold as pets, with two-thirds of the known species in this group known to occur in the pet trade (parrots, conures and cockatoos) (Turral *et al.*, 2017).

According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2021), over 15 million parrots have been traded since 1975 and this market may be responsible for the spread of numerous bird diseases such as Polyomavirus (Phalen *et al.*, 1997), Circovirus (Varsani *et al.*, 2011; Katoh *et al.*, 2008), Adenovirus (Hulbert *et al.*, 2015). Many confiscated birds from illegal trade are sent to rescue centers, zoos, or even relocated to their habitats, and may pose a risk to transmission of many disease agents, if quarantine and screening protocols are not consistently applied (Saidenberg *et al.*, 2015).

Psittacid alphaherpesvirus 1 is a species of the Iltovirus genus belonging to the Alphaherpesvirinae subfamily of the Herpesviridae family (ICTV, 2022). Known as Pacheco's Disease, affects members of the Psittacine family, causing acute hepatitis and sudden death

(Simpson *et al.*, 1975). Those enveloped ds-DNA viruses may remain latent for a long period, causing symptoms when the immunity declines (Grinde, 2013). Parrots from the *Amazona spp.* genus are frequently diagnosed as carriers of the virus, while new world psittacines are more susceptible and often show higher mortality rates (Turral *et al.*, 2017).

The disease was described in many countries, including USA (Simpson *et al.*, 1975); Japan (Katoh *et al.*, 2011), South Africa (Horner *et al.*, 1992), UK (Gough and Alexander, 1993), Australia (Gabor *et al.*, 2013) and Mexico (Turral *et al.*, 2017).

In Brazil, outbreaks were reported both in Southeast Brazil region in seized parrots (Saidenberg *et al.*, 2015) from a reintroduction program and in parrots kept in captivity at Zoo and in a triage center for wild animals (Luppi *et al.*, 2011).

Here, we report the occurrence of *Psittacid alphaherpesvirus* 1, found during an investigation of 98 sudden deaths of captive psittacines in a breeding farm in São Paulo State, Brazil, between 2016 and 2017.

Materials and Methods

Animals and sampling

A psittacine breeding farm in Mairiporã City in São Paulo State, Brazil (Figure 1) registered abnormal mortality rates (32,7%, n=98/300) during February, March and April of the years of 2016 and 2017. All of dead birds were adults in reproductive age, belonging to these species: hyacinth macaws (*Anodorhynchus hyacinthinus*) (n=8/98), red-and-green macaws (*Ara chloropterus*) (n=12/98), blue-and-yellow macaws (*Ara ararauna*) (n=13/98), golden parakeets (*Guaruba guarouba*) (n=5/98), jenday conures (*Aratinga jandaya*) (n=3/98), sun parakeets (*Aratinga solstitialis*) (n=3/98), golden-capped parakeets (*Aratinga auricapillus*) (n=6/98), and turquoise-fronted amazon (*Amazona aestiva*) (n=48/98). The animals were kept in cages, placed side by side inside two hangars that allowed the entrance of other wild birds and synanthropic animals. There wasn't any quarantine protocol. Most cases were limited to sudden death, while a few animals showed depression and lack of appetite followed by severe dyspnea and ataxia, leading to death in a few hours. Fragments of brain, trachea, proventriculus, liver, spleen, large intestine and feathers were separately collected using different sets of instruments in cryotubes containing 'Viral Transport Medium' (VTM). Samples were preserved in dry ice and then kept in ultra-low freezer (-80ºC) until processed.

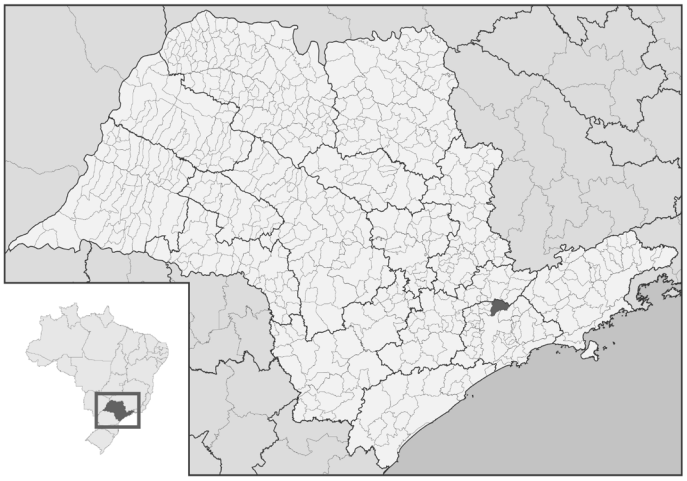

Figure 1: *Map depicting the localization of Mairiporã City São Paulo State, Brazil. (© 2006 Raphael Lorenzeto de Abreu under Creative Commons BY 2.5).*

Virus detection

Each fragment of the clinical samples was macerated individually using the MagNA Lyser™ (Roche Life Science, Indianapolis, IN, USA) Instrument and clarified at 3000 g for 30 min at 4ºC. The resulting supernatant was then used to perform the viral screening. For herpesvirus detection, only trachea, intestine, liver, and spleen samples were tested. The nucleic acids were obtained using automated extractor MagMax™ and the Mag MAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. Routine conventional PCR protocols were conducted to check the presence of Influenza (Anthony *et al.*, 2012), Paramyxovirus (Tong *et al.*, 2008), Flavi virus (Moureau *et al.*, 2007), and Adenovirus (Katoh *et al.*, 2008), using primers and thermocycling conditions according to the respective articles. For herpesvirus detection, a nested-PCR was performed using primers (VanDevanter *et al.*, 1996) targeting DNA polymerase gene (UL30). The first round PCR consisted of 1X PCR Buffer™, 0.4 mM of each DNTP, 2 mM $MgCl_2$, 0.4 µM of each primer (DFA+, ILK+, and KG1-), 1.5 U Platinum Taq DNA Polymerase (Invitrogen™, Waltham, MA, USA), 5 µL of extracted DNA and nuclease-free water to obtain a final volume of 25 µL. The reaction was submitted for 95°C for 5 min, 45 cycles of 96°C for 5 sec., 48°C for 8 sec and 72°C for 12 sec, with last extension at 72°C for 2 min. The second-round amplification mix consisted of 1X PCR

Buffer™, 0.4 mM of each DNTP, 2 mM $MgCl_2$, 0.4 µM of each primer (IGV+, IYG-), 1.5U Platinum Taq DNA Polymerase (Invitrogen™, Waltham, MA, USA), 5 µL of the PCR product, and nuclease-free water to obtain a final volume of 25 µL. The thermocycling conditions were the same of previous step.

Sequencing

Positive samples presenting either 315 bp (PCR) or 215 bp (nested-PCR) under conventional gel electrophoresis were purified with ExoSAP-IT™ PCR Product Cleanup (USB Products Affymetrix, Waltham, MA, USA) and submitted to bidirectional DNA sequencing with BigDye™ 3.1 (Applied Biosystems), according to the manufacturer's protocols. Sequences were resolved in an ABI PRISM™ 3130XL DNA Sequencer (Thermo Fisher Scientific, Waltham, MA, USA).

Data analysis and phylogeny

DNA polymerase gene (UL30) partial nucleotide sequences were aligned with other avian herpesvirus representatives retrieved from GenBank, belonging to either Iltovirus or Mardivirus genus, with ClustalW 1.8 software (Thompson *et al.*, 1994) available in BioEdit 7.1.3.0 software (Hall, 1999). The phylogenetic tree was generated with the neighbor-joining distance algorithm and the maximum composite likelihood substitution model with 500 bootstrap replicates using Mega X software (Kumar *et al.*, 2018). Deduced amino acid identities of the generated sequences were calculated with Bioedit 7.1.3.0 software (Hall, 1999).

Results and Discussion

Necroscopic findings

During clinical samples collection, many birds showed swollen dark brown liver with a number of necrotic spots, and small intestine was also swollen fraught with mucous. No other noticeable post-mortem lesions have been found.

PCR virus detection

From the 98 tested birds, 61 were positive in nested-PCR test for Herpesviruses from which 23 were further confirmed by nucleotide sequencing (Table 1), in at least one tissue sample. All of tested samples resulted negative for Influenza, Paramyxovirus, Flavivirus, Adenovirus, and Circovirus.

Table 1: *Frequency of positives for Herpesviruses in the Nested-PCR reaction per total of dead birds of each species.*

Avian Species	Nested-PCR positives
Anodorhynchus hyacinthinus	1/8 (12.5%)
Ara chloropterus	9/12 (75.0%)
Ara ararauna	7/13 (53.8%)
Guaruba guarouba	2/5 (40%)
Aratinga jandaya	0/3 (0)
Aratinga solstitialis	0/3 (0)
Aratinga auricapillus	1/6 (16.7%)
Amazona aestiva	41/48 (85.4%)
Total	61/98 (62,24%)

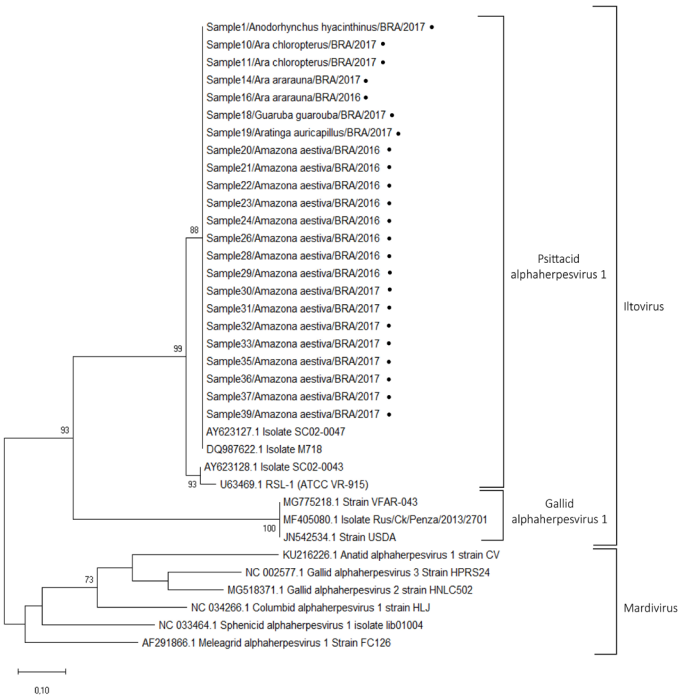

Figure 2: *Nucleotide neighbor-joining distance tree (maximum composite likelihood substitution model) for the partial DNA polymerase gene (UL30) showing the avian herpesvirus species belonging to Iltovirus genus and representatives from Mardivirus genus. Strains detected in the present study are followed by black dots. The numbers at each node are bootstrap values greater than 70% from 500 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.*

Sequence and phylogenetic analyses

The phylogenetic tree of the DNA polymerase gene (UL30) partial nucleotide sequences demonstrates that the 23 Brazilian strains segregate with the Psittacid alphaherpesvirus 1 representatives, while the topology was maintained, with the remaining groups (Psittacid alphaherpesvirus 2, Gallid alphaherpesvirus 1 and Mardivirus genus) distinct from each other and supported by high bootstrap values (Figure 2). It was found 100% of nucleotide and amino acid identities

among the partial herpesvirus fragments generated in this study. Once sequences length is lower than 200 bp, they could not be submitted to Genbank, but they are available as Supplemental Figure 1.

Psittacid Alphaherpesvirus 1 is the causative agent of Pacheco's disease that infects psittacine birds, highly transmissible and presenting mortality rate close to 100% (Tomaszewski *et al.*, 2001). When present, symptoms are nonspecific, such as respiratory inflammation, diarrhea and neurological signs, leading to death only a few hours later (Bistyák *et al.*, 2007).

Here we describe the mortality of 98 birds, belonging to 8 species, divided in two outbreaks, one year apart between 2016 and 2017. Interestingly, parrots and macaws presented higher mortality rates and multiple factors may be involved, such as the number of specimens of each species in the farm, their age, nutritional status, introduction of new specimens, or host sensibility to the specific genotype.

Studies show that *Psittacid Alphaherpesvirus* 1 may remain latent in birds for years (Tomaszewski *et al.*, 2001). Hence, the occurrence of the disease may be due to (i) the contact with free wild psittacines, (ii) introduction of infected animals in the farm, (iii) contact of naive and infected animals, either showing clinical symptoms or asymptomatic birds intermittently eliminating the virus between latency stages.

The sudden death of the animals limited the necroscopic findings, restricted to necrotic spots on the liver, swollen intestines and filled with mucus. Similarly, these same characteristics were described in accomited birds in a Hungarian zoo (Bistyák *et al.*, 2007). These authors additionally described, during autopsy in birds with rapid disease course, remarkably swollen liver, with dark brownish-red coloration, containing considerable amount of blood, and exhibiting numerous surface and parenchymal hemorrhages.

PCR reaction demonstrated to be a useful tool for the PSHv-1 detection posteriorly confirmed through sequencing. Primers and conditions used in this study would potentially detect any Herpersviridae family member. Many samples resulted positive in one or more different organs samples (data not shown) including liver, spleen and intestines. Therefore, this approach minimizes chances of false-negative diagnosis, especially considering the possibility of subclinically or latently infected animals.

Besides Herpesvirus, samples were also tested Influenza, Paramyxovirus, Flavivirus and Adenovirus, once these viruses may cause similar symptomatology, but none were found. It was suggested to *Amazona Vinacea* a screening protocol that encompass at least Influenza, Paramyxovirus type 1, *Salmonella* spp., *C. psittaci*, Psittacine herpesvirus, *E. coli*, and endoparasites (Saidenberg *et al.*, 2015). Due to their insidious characteristics, other causes like intoxications and mycotoxins and even other agents, should be considered and may explain the remaining undiagnosed deaths found in this study.

Although generated sequence fragments are relatively small and related to a conserved gene region (viral DNA polymerase), it was possible to notice they were identical, with 100% amino acid identity among them. At first, this suggests a common exposure to the same agent, corroborated by the provision of the facilities and management of avian species practiced in the farm, which favors viral dissemination. Further analysis of other genetic regions, including UL16 ORF, could provide more consistent data on circulating viral genotypes (Luppi *et al.*, 2016; Tomaszewski *et al.*, 2003).

Phylogenetic tree (Figure 2) showed that all of the samples detected in this study clustered together in a *Psitacide alphaherpesvirus* 1. The tree topology maintained the relationship with *Gallid alphaherpesvirus* 1, grouped in the Iltovirus genus clade, while, regarding Mardivirus genus, no evidence was found among the analyzed samples.

In conclusion, *Psittacid alphaherpesvirus* 1 is the cause of the high mortality outbreak observed in this Brazilian psittacine breeding farm. Considering that no commercial vaccine is available to this disease, continuous monitoring of viral disease is a key to provide useful information on developing preventive measures. Once the agent rapidly spreads to many psittacine species, strict quarantine protocols, cages and fomites disinfection, and routine animal management should also be associated to avoid similar situations in captive birds destined to pet trade and the ones there will be reintroduced to the wild life.

Further investigations are needed to determine the other potential reservoirs for the virus introduction and a more comprehensive genetic sequencing to determine whether effectively there are differences among viruses infecting the host species.

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Authors's Contribution

All the authors contributed equally and approved the manuscript for submission.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.hv/2022/9.32.37

Conflict of interest

The authors have declared no conflict of interest.

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