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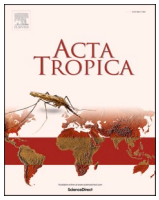

Herpesvirus and adenovirus surveillance in threatened wild West Indian (*Trichechus manatus*) and Amazonian manatees (*Trichechus inunguis*), Brazil

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ABSTRACT

The family Trichechidae (order Sirenia) comprises three species: African (*Trichechus senegalenses*), West Indian (*T. manatus*), WIM)], and the Amazonian manatees (*T. inunguis*, AMM). Whereas WIM inhabits both riverine and coastal systems in the western Atlantic, AMM is the only exclusively freshwater sirenian, endemic to the Amazon River Basin. The study of infectious agents is essential to species conservation, especially considering that both species are classified as Vulnerable by the IUCN Red List and as Endangered by the Brazilian Red List. The current knowledge about viral agents in sirenians is scarce. Herpesviruses and adenovirus are DNA viruses able to infect and cause disease in a wide range of hosts. Herein, we used panPCR protocols to survey herpesvirus and adenovirus in blood samples of wild WIM ($n = 23$) and AMM ($n = 26$) under human care in Brazil. Herpesvirus DNA was detected in one juvenile female WIM (1/23; 4.3%; 95% CI -4.7 – 13.3) from Ceará state and in four AMM (two juvenile females, a juvenile male, and an adult female; 4/26; 15.4%; 95% CI 0.5 – 30.3) from Amazonas state. The two different gammaherpesvirus DNA polymerase sequence types identified (one per species, a sequence type in a WIM and another one in three AMM) were highly similar (99% nucleotide identity) to Trichechid herpesvirus 1, reported in West Indian manatees of Florida (USA), and 100% identical when translated into amino acids. A herpesviral glycoprotein B sequence was identified in two AMM. None of the samples was positive to adenovirus. To the authors' knowledge, this is the first herpesvirus detection in manatees from South America, expanding the herpesvirus geographical range, and the first in WIM and AMM worldwide. Our findings suggest (i) that West Indian and Amazonian manatees are possibly the natural hosts of the detected herpesvirus, and (ii) coevolution of that gammaherpesvirus with *Trichechus*. Future studies are necessary to characterize the obtained virus and elucidate potential pathological effects (if any) in these species.

1. Introduction

The superorder Afrotheria is considered an earlier branch of placental mammals (Wellehan et al., 2008). First proposed in 1980 (Dejong et al., 1981; Goodman, 1982), it includes anatomically and biologically diverse species subdivided into Afroinsectiphilia (orders Tubulidentata [aardvark], Afroscoricida [tenrecs and golden moles], and Macroscelidea [elephant shrews]) and Paenungulata (orders Sirenia [manatees and dugong], Hyracoidea [hyraxes], and Proboscidea [mammoths and elephants]) (Kuntner et al., 2011). The order Sirenia comprises the extinct Steller's sea cow (*Hydrodamalis gigas*), and the dugong (*Dugong dugon*), African manatee (*Trichechus senegalensis*), West Indian manatee (*T. manatus*), and Amazonian manatee (*Trichechus inunguis*) (Sharko et al., 2019). The West Indian and the Amazonian manatee are the only two sirenians that occur in Brazil (Cantanhede et al., 2005).

The West Indian manatee inhabits both riverine and coastal systems in the tropical and subtropical Western Atlantic coast, in the Caribbean

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Sea and Gulf of Mexico, from the Bahamas to Brazil - where it occurs discontinuously in the northern and northeastern Brazilian coasts (Reynolds et al., 2009). The Amazonian manatee is the only exclusively freshwater sirenian and is endemic to the Amazon River basin, in South America - from the river headwaters of Colombia, Peru and Ecuador up to the Marajó Island, Brazil (Best, 1984; Cantanhede Alves-Gomes, 2005).

The study of infectious agents is essential to species conservation (Smith et al., 2009). West Indian and Amazonian manatees are classified as Vulnerable by the IUCN Red List and as Endangered and Vulnerable by the Brazilian Red List (Deutsch et al., 2008; Marmontel et al., 2016; ICMB, 2018). Major threats to these species' survival in Brazil include low genetic variability, habitat degradation and loss, hunting, interaction with fisheries, stranding of calves in the northeast region, collision with watercraft, pollution and natural disasters (Deutsch et al., 2008; Meireles, 2008; Balensiefer et al., 2020). The current knowledge about viral agents in sirenians is scarce, limited to the report of gammaherpesvirus (Wellehan et al., 2008; Ferrante et al., 2017) and papillomavirus in West Indian manatees of Florida, USA (Bossart et al., 2002; Ghim et al., 2014), and serological studies of morbillivirus in Florida and Belize (Duignan et al., 1995; Sulzner et al., 2012).

Herpesviruses are enveloped double-stranded DNA viruses classified into the order *Herpesvirales*, which comprises the families *Malacoherpesviridae, Alloherpesviridae*, and *Herpesviridae* (Davison et al., 2009). The members of the family *Herpesviridae*, subdivided into the subfamilies *Alpha, Beta* and *Gammaherpesvirinae*, are able to cause latent infections in their natural hosts and disease in a wide variety of reptiles, birds and mammals (Gatherer et al., 2021). Adenovirus (order *Rowavirales*, family *Adenoviridae*) are nonenveloped double-stranded DNA viruses able to establish persistent infections (Harrach et al., 2011; Kosulin et al., 2016; Benkő et al., 2022). Of note, both herpesvirus and adenovirus are able to co-evolve with their natural host, generally showing low pathogenicity (Kaján et al., 2020), and can reactivate under immunosuppressive conditions (Woźniakowski and Samorek-Salamonowicz, 2015; Kosulin et al., 2016). Although herpesviruses have not been linked to clinical disease in manatees, they are considered important infectious agents in other Afrotheria (i.e., elephants) (Long et al., 2016). To this date no adenovirus has been described in sirenians, neither in other Afrotheria; however, they are considered important infectious agents in other marine mammals such as pinnipeds and cetaceans, in which they may cause hepatitis and, and has been described in cetaceans with diarrhea (Goldstein et al., 2011; Rubio Guerri et al., 2015; van Beurden et al., 2017).

Thus, in order to assess the baseline health and expand the current knowledge on viral diseases in these poorly studied and threatened species, we surveyed selected viral agents (herpesvirus and adenovirus) in blood samples from wild manatees undergoing rehabilitation or under permanent human care in Brazil.

2. Material and methods

2.1. Sampling

We analyzed blood samples of 23 West Indian manatees (14 males and nine females; eight neonates, seven calves, five juveniles and three adults), collected between 2002 and 2021, by AQUASIS (Associação de Pesquisa e Preservação de Ecossistemas Aquáticos, 3º41'34.5″S, 38°37′33.4′'W), a wildlife rescue center located in Caucaia municipality, Ceará state, northeastern Brazil, and kept in a blood bank. Twenty-one individuals were rescued alive in Ceará and Rio Grande do Norte states, and had different outcomes (released, still undergoing rehabilitation, permanent resident or death); their samples were collected from the pectoral arteriovenous plexus (ventral surface of the pectoral flipper). Two individuals were found stranded dead at the beach (Codes 2 and 3) in Ceará state and were necropsied according with standard procedures (Geraci et al., 2005), and blood samples were collected through intracardiac puncture.

Furthermore, we also analyzed blood samples from 26 Amazonian manatees (13 males and 13 females; 13 juveniles, eight adults and five calves) either undergoing rehabilitation or kept under permanent human care at INPA (Instituto Nacional de Pesquisas Amazônicas, 3º5'42.9″S, 59º59'18.3″W), another wildlife rescue center located in Manaus, Amazonas state, Brazil.

The blood samples of the West Indian manatees were collected during periodic health assessments or during necropsy. The blood samples from the Amazonian manatees were taken in a health assessment procedure in February 2022.

The institution, age, sex, rescue site, clinical history, herpesvirus and adenovirus status, and outcome of the tested individuals are described in Supplementary Table 1.

2.2. Molecular methods

Total DNA from blood samples was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Extractions were tested for herpesvirus with a broad-spectrum nested PCR that partially amplifies a 215–315 bp fragment of the DNA polymerase (DPOL) gene of a broad variety of species belonging to the subfamilies *Alpha, Beta* and *Gammaherpesvirinae* (Vandevanter et al., 1996). Subsequently, all DNA extractions were also tested with a nested PCR that amplifies a 500 bp fragment of the glycoprotein B (gB) of the subfamily *Gammaherpesvirinae*, as described by Ehlers et al. (2008), given that these viruses usually establish latency in lymphoid cells, and that this technique is able to detect gammaherpesvirus not amplified with the Vandevanter et al. (1996) protocol (Exposto Novoselecki et al., 2021; Navas-Suárez et al., 2021; Nicolas de Francisco et al., 2021). Finally, we also used the nested PCR protocol described by Li et al. (2010), in an attempt to amplify a 224 bp fragment of the adenovirus DNA polymerase gene in the above-mentioned samples. Adequate positive and no template controls were added to all reactions.

Amplicons of the expected size were purified with ExoSap-IT (USB Corporation, Ohio, USA) and directly sequenced in both directions with an ABI 3730 DNA Analyser. The quality of the obtained sequences was visually assessed with BioEdit. Subsequently, the consensus sequences were constructed based on the alignment of the forward and reverse sequences performed on Mega 7.0 (Kumar et al., 2016). Following primer removal, the consensus sequences were compared by BLAST search to those kept at the GenBank/EMBL/DDBJ database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The evolutionary models for the phylograms were selected with JModel test (version 2.1.10). The percentage of identity with the closest sequences was obtained based on p-distance ([1 – *p-distance*)*100]). Herpesviral DPOL and gB maximum likelihood phylograms were done with MEGA 7.0 based on the deduced amino sequences obtained in this study, herpesvirus sequences from West Indian manatees of Florida, sequences from other Afrotheria, and representative herpesvirus species recognized by the International Committee on Viral Taxonomy (ICTV) (Fig. 1). The Le Gasquel model with a discrete gamma distribution and invariant sites was used to construct for the phylograms. A bootstrap of 1000 replications was selected. All bootstrap frequency values less than 70 were omitted. Representative herpesviral sequences were submitted to GenBank under accession numbers ON733328 (DPOL West Indian manatee 02S0112/75), ON733329 (DPOL Amazonian manatee INPA#101), and ON733330 (gB Amazonian manatee INPA#101).

The observed herpesvirus proportion of positives in West Indian and Amazonian manatees attended, respectively, by the AQUASIS and INPA wildlife rescue centers, was compared to those previously described in the genus *Trichechus* by Wellehan et al. (2008) and Ferrante et al. (2017) using Kruskal Wallis test with a p value significance < 0.05.

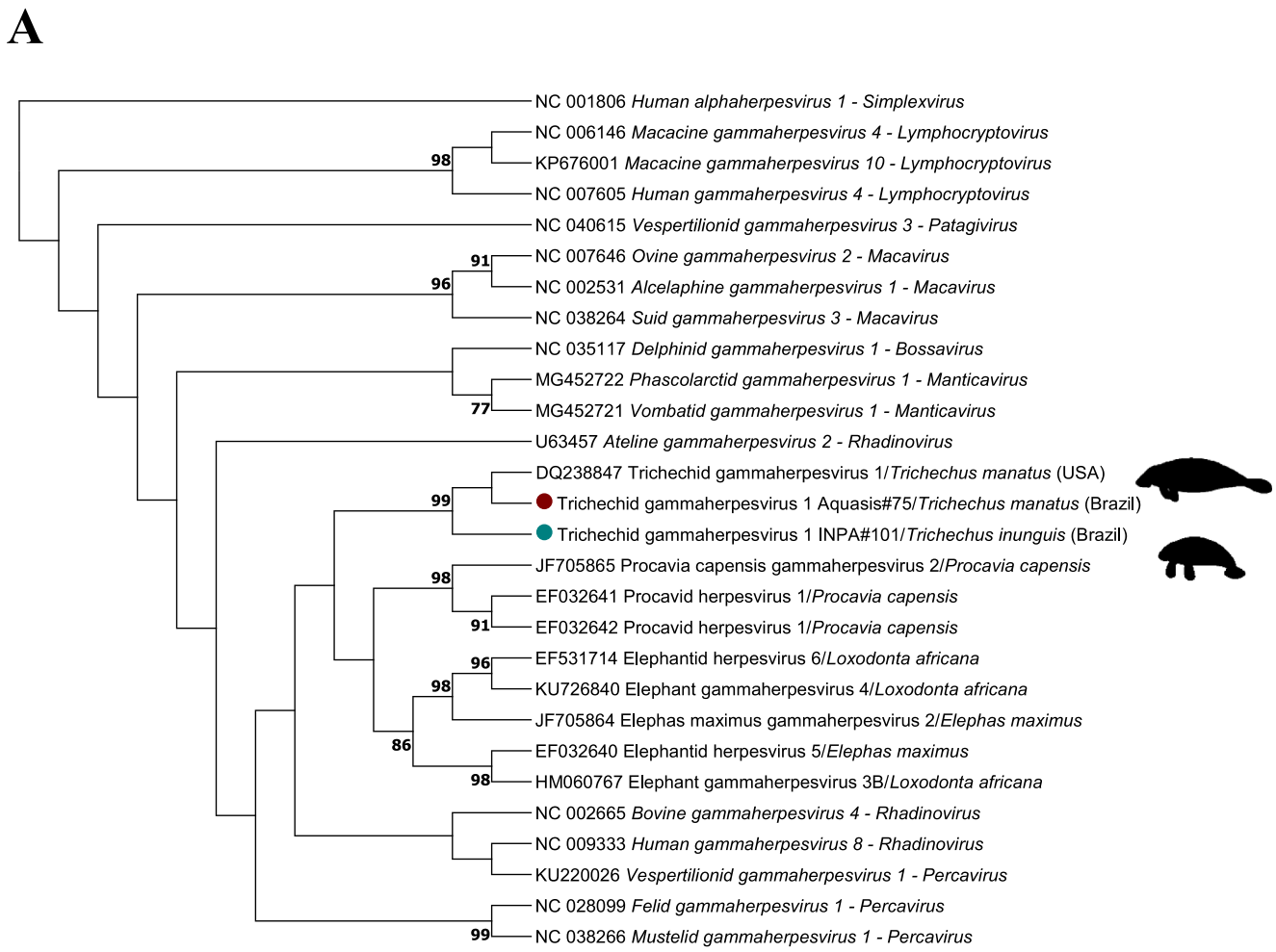

Fig 1. Maximum likelihood phylograms of the alignment of: (A) the deduced DNA polymerase amino acid herpesvirus sequences obtained in West Indian (*Trichechus manatus*) and Amazonian (*T. inunguis*) manatees (red and blue dots, respectively), in West Indian manatees (*T. manatus latirostris*) of Florida, in other Afrotheria, and representative herpesvirus species recognized by the International Committee on Viral Taxonomy (ICTV), and (B) a representative deduced herpesvirus glycoprotein B sequence obtained in Amazonian manatee (blue dot), the closest sequences from GenBank, and representative herpesvirus species recognized by the ICTV. *Human alphaherpesvirus 1* and *Human alphaherpesvirus 3* sequences were selected as outgroup for DNA polymerase and glycoprotein B phylograms, respectively. A bootstrap value of 1000 replications was selected for both trees and all bootstrap frequency values less than 70 were omitted. The evolutionary Le Gasquel model with a discrete gamma distribution and invariant sites was selected according to the JModel test (version 2.1.10) results.

3. Results

3.1. Herpesvirus

The overall herpesvirus proportion of positives (combining the results of DPOL and gB PCRs) in manatees attended by the wildlife rescue centers evaluated in this study was 1/23 (4.3%; 95% CI −4.7 – 13.3) in West Indian manatees and 4/26 (15.4%, 95% CI 0.5 – 30.3) in Amazonian manatees. Herpesvirus DNA was detected in the DPOL PCR in one juvenile female West Indian manatee (1/23; 4.3%; 95% CI −4.7 – 13.3) from AQUASIS and in three Amazonian manatees (one juvenile female, one juvenile male and an adult female; 3/26; 11.5%; 95% CI −1.6 – 24.7) from INPA (Supplementary Table 1). The two different DPOL gammaherpesvirus nucleotide sequence types identified (one in each species) were very highly similar (99% nucleotide identity) to Trichechid herpesvirus 1 (TrHV1), reported in West Indian manatees of Florida, USA (GenBank accession nº DQ238847), and 100% identical when translated into amino acids. Additionally, two Amazonian manatees (one juvenile female and an adult female; 2/26; 7.7%; 95% CI −3.3 – 18.7) from INPA were positive to gB PCR for herpesvirus, including one adult female that was also positive in the DPOL protocol. The retrieved identical gB nucleotide sequence type presented the highest nucleotide and amino acid identities (74% and 80%, respectively) with *Pontoporiid gammaherpesvirus 1*, detected in a franciscana dolphin (*Pontoporia blainvillei)* of Brazil (GenBank accession nº MZ209259). The clustering of the manatee herpesvirus with herpesvirus sequences from other species the superorder Afrotheria was not supported by high bootstrap values (Fig. 1A) in the DPOL phylogenetic tree analysis. The obtained gB unique sequence cluster with recognized herpesviral species into the genus *Rhadinovirus* (Fig 1B).

The TrHV-1-positive West Indian manatee is a permanent resident of AQUASIS, deemed unfit for release upon its rescue as a neonate due to malformations (scapular disquenesia and partial fold of the caudal flipper), that had been in the center for over four years when sampled. In spite of such malformations, the animal is in good body condition and has no history of clinical diseases since its arrival. All herpesvirus-positive individuals from INPA were in good body condition and had no history and/or clinical signs of disease. The AQUASIS individuals that were found dead did not present any macroscopic signs of herpesviral infection on necropsy (e.g., mucocutaneous lesions, pneumonia).

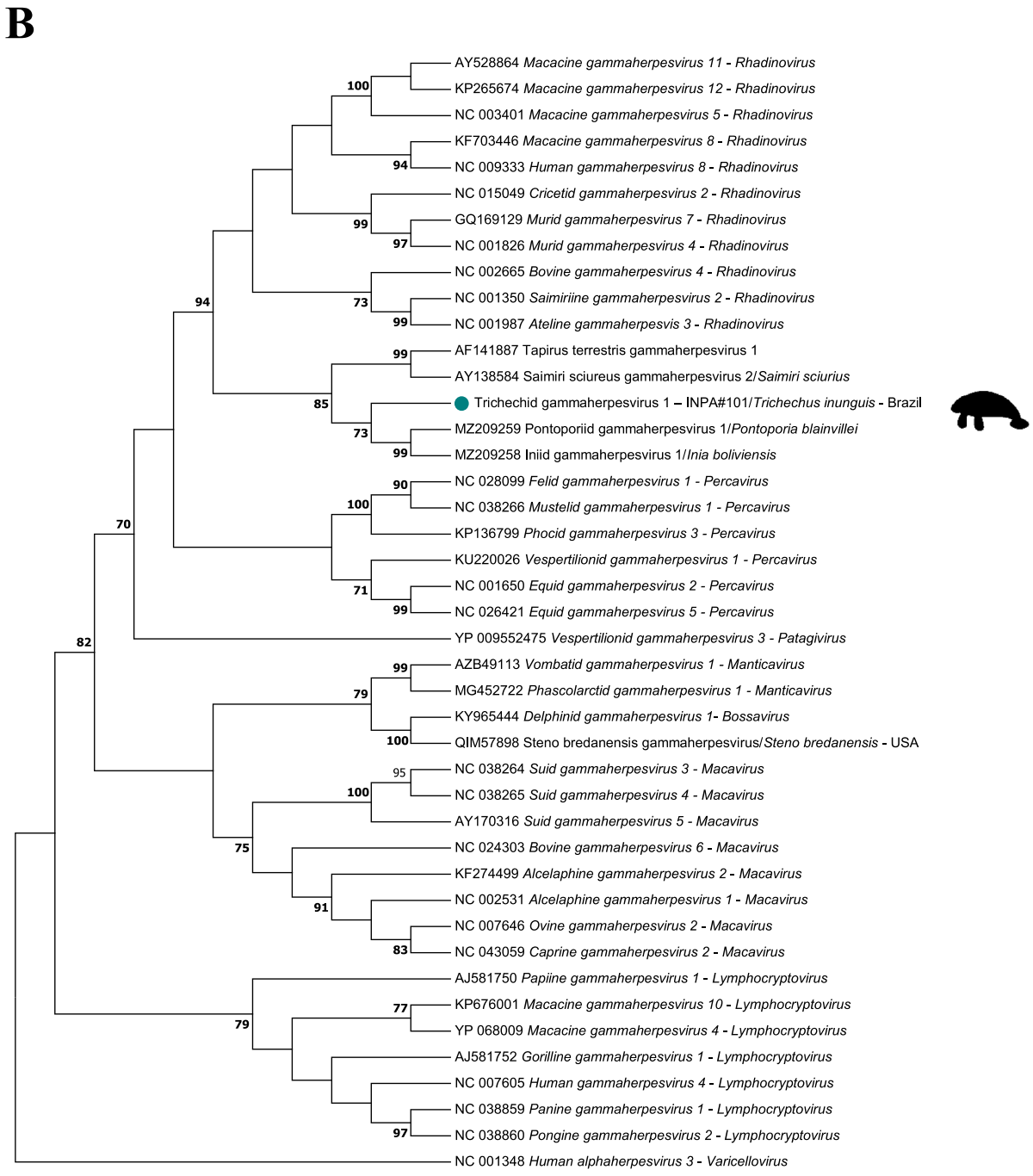

Fig 1. (*continued*).

3.2. Adenovirus

None of the samples was positive to adenovirus PCR.

4. Discussion

Herpesviruses have been previously described in the superorder Afrotheria, including different beta and gammaherpesvirus species in Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*), alpha and a gammaherpesvirus in rock hyraxes (*Procavia capensis*), and the gammaherpesvirus TrHV1 in West Indian manatees of Florida (Wellehan et al., 2008; Galeota et al., 2009; Latimer et al., 2011). Herein, we detected gammaherpesvirus in blood samples of 4.2% of the West Indian manatees (1/23) and 15.4% (4/26) of the Amazonian manatees under human care. Herpesvirus in manatees (the gammaherpesvirus Trichechid herpesvirus 1 - TrHV1) was first described in a survey in skin lesions and/or blood samples of 47 captive or free-ranging wild West Indian manatees of Florida, at a proportion of positives of 17/36 (47%) in skin biopsies and 8/31 (26%) in blood (Wellehan et al., 2008). Almost two decades later, a TrHV-1 real-time quantitative probe hybridization PCR assay was used to quantify TrHV-1 in manatee peripheral blood mononuclear cells (PBMCs), finding that wild clinically healthy TrHV-1-positive West Indian manatees sampled in Florida (45%; 19/42) presented an average baseline load of 40.9 copies/100 ng DNA (Ferrante et al., 2017).

We found no statistically significant differences among the overall herpesvirus-occurrence (TrHV1-positive individuals) found herein in blood samples of manatees under human care (4.2% for West Indian

manatees and 15.4% for Amazonian manatees) and those previously reported by Wellehan et al. (2008) and Ferrante et al. (2017) in complete blood and peripheral blood mononuclear cells of wild West Indian manatees sampled in Florida (26% [8/31] and 21.4% [9/42], respectively) using PCR. Further studies assessing and comparing the occurrence of TrHV-1 in larger sample sizes, individuals with varying clinical histories (i.e., clinically healthy, presenting herpesvirus-compatible clinical signs [e.g., skin lesions], immunosuppressed), different manatee species, subspecies and populations, and their correlation (or not) with immunosuppressive events known to affect these animals (e.g., cold stress and brevetoxicosis) (Walsh et al., 2005), are required to understand such findings.

To the authors' knowledge, this is the first herpesvirus detection in manatees from South America, and the first in Amazonian manatees worldwide. Although further studies are needed to confirm it, the high DPOL similarity (99% nucleotide and 100% amino acid identity) to the TrHV-1 reported in West Indian manatees of Florida (USA) by Wellehan et al. (2008) suggests (i) that West Indian and Amazonian manatees are possibly the natural hosts of the detected herpesvirus, which is consistent with host-virus coevolution and codivergence (Jackson, 2005; Wellehan et al., 2004, 2008), and (ii) gammaherpesvirus coevolution with *Trichechus* - as previously hypothesized for gammaherpesvirus in giant armadillos (*Priodontes maximus*) and South American river dolphins (Navas-Suárez et al., 2021; Exposto Novoselecki et al., 2021), herein based on the hypothetical Plio-Pleistocene origin and diversification of the genus *Trichechus*. In light of such findings, we propose renaming this herpesvirus as trichechid gammaherpesvirus 1. The gB sequences retrieved from two Amazonian manatees were very divergent to those available in GenBank, likely due to the lack of previous gB sequences for TrHV1. According to the gB phylogram the manatee herpesvirus is classified into the genus *Rhadinovirus*. Unfortunately, there were no other Afrotheria sequences available for this gene to elucidate if they cluster with the trichechid gammaherpesvirus 1. Unfortunately, although there are other Afroteria sequences aside from those of manatees available for DPOL gene, the length of the obtained DPOL sequences were likely too short to clarify this question.

Gammaherpesviruses establish their latency in lymphoid cells, which favors their detection in blood samples, and can promote lymphoproliferative malignancies (Widen et al., 2012; Pellet and Roizman, 2013; Osterrieder et al., 2014). While under care, these manatees are more exposed to stress and factors that may reactivate latent infections, and promote viral infections among individuals and different populations. Unfortunately, the molecular technique (endpoint PCR) employed herein is not able to differentiate between latent or active infections. Future studies using mRNA analyses or real time PCR may assist on this issue. The route of infection for the herpesvirus-positive animals is unknown; however, it is not possible to exclude the role of water as a mechanical transmitter, as observed for other herpesviruses (Dayaram et al., 2017). Neonatal infection during pregnancy (vertical transmission) is also a possibility (Sanmartín et al., 2016; Bhatta et al., 2018).

Herpesvirus infections have a reportedly significant impact in free-ranging wild mammals (gammaherpesvirus *Otarine herpesvirus 1* in pinnipeds; California sea lions (*Zalophus californianus*) and South American fur seals (*Arctocephalus australis*) (Johne et al., 2002; King et al., 2002; Tomaszewski et al., 2003; Dagleish et al., 2013; Work et al., 2015), and in wild populations under human care (e.g., zoos and rehabilitation centers; *Elephant endotheliotropic HV* in Asian elephants (*Elephas maximus*), *Human alphaherpesvirus 1* and *2* in Neotropical primates), promoting potentially fatal disease (Assis-Casagrande, 2014). Our herpesvirus-positive animals were apparently healthy upon sample collection and had no clinical history of disease.

None of the tested animals was positive to adenovirus. Of note, most adenoviruses are thought to be host specific and coevolve with their hosts (Kaján et al., 2020). To the authors' best knowledge, no adenoviruses have been detected in members of the superorder Afrotheria (Harrach et al., 2019), which includes manatees. Nevertheless, further surveillance is advised, once these viruses have been detected in other marine mammals (Rubio Guerri et al., 2015; Chiappetta et al., 2017).

5. Conclusions

Herein we described the first detection of a gammaherpesvirus in Amazonian manatees. The analyses of the obtained DPOL sequence types suggest that this agent has possibly coevolved with the genus *Thichechus*. Future herpesvirus studies in healthy and diseased wild manatees and individual undergoing human care are necessary to characterize TrHV1, in order to determine its complete genome, occurrence and prevalence, investigate potential pathological effects (if any) related to this virus, and further assess the coevolution hypothesis.

CRediT authorship contribution statement

Ana Carolina Ewbank: Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Aricia Duarte-Benvenuto:** Methodology, Investigation, Resources, Writing – review & editing. **Roberta Zamana-Ramblas:** Methodology, Investigation, Writing – review & editing. **Irene Sacristán:** Methodology, Formal analysis, Writing – review & editing. **Samira Costa-Silva:** Investigation, Resources, Writing – review & editing. **Vitor L. Carvalho:** Investigation, Resources, Writing – review & editing. **Daniela Magalhães Drummond de Mello:** Investigation, Resources, Writing – review & editing. **Vera Maria Ferreira da Silva:** Investigation, Resources, Writing – review & editing. **José Luiz Catão-Dias:** Writing – review & editing, Visualization, Project administration, Funding acquisition. **Carlos Sacristán:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The gammaherpesvirus nucleotide sequence types identified herein are available at GenBank under accession numbers ON733328 and ON733329 (DPOL), and ON733330 (gB).

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Authorization System - SISBIO (License nº SISBIO 72608–1) and the Brazilian Institute of Environment and Renewable Natural Resources – IBAMA (SisGen A2B0851 and ACEDD3F).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106740.

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