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**VÍRUS GIGANTES DO PANTANAL: CARACTERIZAÇÃO MORFOLÓGICA E
GENÔMICA DE NOVOS ISOLADOS**

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**VÍRUS GIGANTES DO PANTANAL: CARACTERIZAÇÃO MORFOLÓGICA E
GENÔMICA DE NOVOS ISOLADOS**

Versão final

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Orientador: Prof. Jônatas Santos Abrahão

Coorientadores: Dra. Nidia Esther Colquehuanca Arias e
Dra. Victória Fulgêncio Queiroz

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RESUMO

Desde a descrição do primeiro vírus gigante de ameba em 2003, o conhecimento sobre a virosfera vem se expandindo significativamente. O posterior isolamento de vários outros vírus gigantes de ameba, caracterizados por partículas com dimensões e morfologias distintas, bem como por grandes genomas, contribuiu para a compreensão da abundância e diversidade dessas entidades. Nesse contexto, o objetivo deste trabalho foi relatar a descoberta de dois vírus gigantes denominados de *Marseillevírus pantanense* e *Naiavírus pantanense* a partir de amostras do Pantanal brasileiro. Nas prospecções, foram utilizadas como plataformas celulares de isolamento as espécies *Acanthamoeba castellanii* e *Vermamoeba vermiformis*, e após observação de efeito citopático, as amostras foram preparadas para várias técnicas de microscopia. Nas imagens obtidas por microscopia eletrônica de varredura e transmissão, foi possível visualizar e analisar as partículas de *M. pantanense* e *N. pantanense*. Foi observado que as partículas de *M. pantanense* possuíam simetria icosaédrica com aproximadamente 190 nm de diâmetro. Análises gênicas e filogenéticas revelaram o agrupamento do *M. pantanense* no grupo I ou linhagem A da família *Marseilleviridae*. Já o *Naiavírus* apresenta partículas pleomórficas e assimétricas, atingindo até 1.824 nm de comprimento (média de 1.350 nm) com uma estrutura constituída por uma região denominada de cabeça e outra referida como a cauda. Além disso, o *Naiavírus* é o primeiro vírus gigante de ameba conhecido a apresentar um envelope externo, que envolve o capsídeo, e uma cauda flexível capaz de se esticar, dobrar e se grudar a superfícies próximas. A descoberta tanto do *Naiavírus* quanto de uma nova cepa de *Marseillevírus* destaca a importância dos estudos de prospecção para o isolamento viral, visto que essa técnica é essencial para complementar abordagens de metagenômica, que visam melhor compreensão da virosfera. Mesmo após mais de duas décadas da descrição do primeiro vírus gigante de ameba, essas entidades ainda surpreendem a comunidade científica com suas adaptações estruturais únicas.

Palavras-chave: Vírus Gigantes, Prospecção, Pantanal, *Marseillevírus*, *Naiavírus*.

ABSTRACT

Since the description of the first giant amoeba virus in 2003, knowledge about the virosphere has expanded significantly. The subsequent isolation of several other giant amoeba viruses, characterized by particles with distinct sizes and morphologies as well as large genomes, has contributed to understanding the abundance and diversity of these entities. In this context, the aim of this study was to report the discovery of two giant viruses named *Marseillevirus pantanense* and *Naiavirus pantanense* from samples collected in the Brazilian Pantanal. During the surveys, the species *Acanthamoeba castellanii* and *Vermamoeba vermiformis* were used as cellular platforms for viral isolation, and after observing cytopathic effects, the samples were prepared for various microscopy techniques. In images obtained by scanning and transmission electron microscopy, it was possible to visualize and analyze the particles of *M. pantanense* and *N. pantanense*. It was observed that *M. pantanense* particles exhibited icosahedral symmetry with an approximate diameter of 190 nm. Genomic and phylogenetic analyses revealed that *M. pantanense* clusters within group I or lineage A of the family *Marseilleviridae*. *Naiavirus*, in turn, displays pleomorphic and asymmetric particles reaching up to 1,824 nm in length (average of 1,350 nm), with a structure consisting of a region referred to as the head and another referred to as the tail. In addition, *Naiavirus* is the first known giant amoeba virus to present an external envelope surrounding the capsid, as well as a flexible tail capable of stretching, bending, and attaching to nearby surfaces. The discovery of both *Naiavirus* and a new strain of *Marseillevirus* highlights the importance of prospecting studies for viral isolation, as this technique is essential to complement metagenomic approaches aimed at achieving a better understanding of the virosphere. Even more than two decades after the description of the first giant amoeba virus, these entities continue to surprise the scientific community with their unique structural adaptations.

Keywords: Giant Viruses, Prospecting, Pantanal, *Marseillevirus*, *Naiavirus*.

LISTA DE ABREVIATURAS E SIMBOLOS

Mpb: Megapares de base

Kpb: kilopares de base

NCLDV: Vírus grandes nucleocitoplasmáticos de DNA (do inglês “*Nucleo-cytoplasmic large DNA viruses*”)

MCP-DJR: Proteína principal do capsídeo (do inglês “*Major Capsid Protein – Double Jelly-Roll*”)

ICTV: Comitê Internacional de Taxonomia de Vírus (do inglês “*International Committee on Taxonomy of Viruses*”)

APMV: *Acanthamoeba polyphaga* mimivírus

nm: nanômetro(s)

hpi: hora(s) pós infecção

ORFs: Janelas abertas de leitura (do inglês, “*Open Reading Frames*”)

ORFans: genes sem homologia em bancos de dados

tRNAs: RNAs transportador

tRNA-Glu: RNA transportador do aminoácido glutamato

tRNA-Gln: RNA transportador do aminoácido glutamina

%: porcentagem

SUMÁRIO

1 – INTRODUÇÃO.....	11
2 – JUSTIFICATIVA	14
3 – OBJETIVOS.....	15
3.1 – Objetivo geral.....	15
3.2 – Objetivos específicos.....	15
4 – METODOLOGIA, RESULTADOS E DISCUSSÕES SERÃO APRESENTADAS NO FORMATO DE ARTIGOS PLUBLICADOS PRECEDIDOS DE UM BREVE RESUMO.....	16
4.1 – Artigo 1 – A Marseillevirus isolate from the Brazilian wetlands.....	17
4.2 – Artigo 2 - Naiavirus: an enveloped giant virus with a pleomorphic, flexible tail.....	26
5 – DISCUSSÃO.....	38
6 – CONCLUSÃO.....	41
7 – REFERÊNCIAS BIBLIOGRÁFICAS.....	42
8 – PRODUÇÕES, EVENTOS CIENTÍFICOS E APRESENTAÇÕES	47
8.1 - Participações em eventos científicos.....	47
8.2 - Formação complementar.....	48
8.3 - Atividades de extensão.....	49
8.4 – Outros artigos científicos publicados durante o Mestrado.....	49

1 - INTRODUÇÃO

Os vírus são as entidades biológicas mais abundantes do planeta Terra, e estima-se que haja em toda a biosfera aproximadamente 10^{31} partículas virais (Hendrix et al. 1999; Mushegian, 2020). Essas entidades são ubíquas e estão distribuídas em diversos ambientes, como solos, geleiras, desertos, oceanos e rios (Bergh et al. 1989; Mokili et al. 2012; Zablocki et al. 2016) participando ativamente na evolução e principalmente no controle biológico das comunidades microbianas (Wommack & Colwell, 2000).

Constituindo a grande complexidade da virosfera, há os vírus gigantes de ameba. Eles possuem partículas que podem chegar a micrômetros (Abrahão et al. 2018) e simetrias variadas, como ovoides (Philippe et al. 2013; Andreani et al. 2018), esféricos (Legendre et al. 2015), icosaédricos (Boyer et al. 2009), pseudo-icosaédricos (La Scola et al. 2003) e complexas (Abrahão et al. 2018). Além disso, seus genomas podem ultrapassar 2,5 Mpb (Philippe et al. 2013; Koonin & Yutin, 2019) codificando proteínas desconhecidas ou que antes foram somente associadas a organismos celulares.

Os vírus gigantes de ameba, fazem parte do agrupamento dos Grandes Vírus Nucleocitoplasmáticos de DNA (NCLDV) e taxonomicamente pertencem ao domínio *Varidnaviria*, reino *Bamfordvirae* e filo *Nucleocytoviricota* (ICTV, 2026). Eles compartilham uma série de genes que estão relacionados aos processos de replicação e estrutural (Abrahão, 2025), como por exemplo, a proteína principal do capsídeo que contém um domínio double-jelly-roll (MCP-DJR) (ICTV, 2026).

Os nucleocitovírus de amebas possuem como hospedeiro as amebas de vida livre que já foram detectadas em solos, água doce, geleiras, águas residuais e água salgada (Rodríguez-Zaragoza, 1994; Marciano-Cabral & Cabral, 2003; Samba-Louaka et al. 2019). Dentre esses hospedeiros, o principal modelo utilizado para isolar os vírus gigantes de amostras ambientais é a *Acanthamoeba castellanii* (Siddiqui & Khan, 2012; Sun et al. 2020). Além dessa, outras amebas também são utilizadas, como *Acanthamoeba polyphaga*, *Acanthamoeba griffini* (La Scola et al. 2003; Abrahão et al. 2018; Dornas et al. 2015), *Vermamoeba vermiformis* (Reteno et al. 2015; Geballa-Koukoulas et al. 2022) e recentemente *Naegleria clarki* (Arthofer et al. 2024).

Em 2003, o primeiro vírus gigante de ameba foi descrito, isolado em *A. polyphaga*, mas capaz de infectar outras espécies de *Acanthamoeba* (La Scola et al., 2003). Ele foi denominado

de *Acanthamoeba polyphaga mimivirus* (APMV) e possui partículas com simetria pseudo-icosaédrica de até 750 nm e um genoma de aproximadamente 1,2 Mpb (La Scola et al. 2003; Raoult et al. 2004; Xiao et al. 2005; Zauberman et al. 2008). Subsequentemente, ainda utilizando como modelo celular amebas do gênero *Acanthamoeba* diversos outros nucleocitovírus foram isolados, como outros Marseillevírus (Boyer et al. 2009), megavírus (Arslan et al. 2011), moomouvírus (Yoosuf et al. 2012), Pandoravírus (Philippe et al. 2013), pithovírus (Legendre et al. 2014), cedratvírus (Andreani et al. 2016), Tupanvírus (Abrahão et al. 2018), Mollivírus (Legendre et al. 2015), Pacmanvírus (Andreani et al. 2017), Medusavírus (Yoshikawa et al. 2019) e Yaravírus (Boratto et al. 2020). Em *V. vermiformis* já foram isolados os Faustovírus (Reteno et al. 2015), kaumoebavírus (Bajrai et al. 2016), Orpheovírus (Andreani et al. 2018), Yasminevírus (Bajrai et al. 2019), clandestinovírus (Rolland et al., 2021), fadolivírus (Andreani et al. 2021), e os recém descobertos usurpativírus (Andreani et al. 2025) e Ushikuvírus (Bae et al. 2025). Já na *Naegleria clarki* foi isolado o naegleriavírus (Arthofer et al. 2024).

Todas essas descobertas junto a diversos outros vírus identificados por metagenômica demonstram o quanto essas entidades são diversas e estão presentes em todos os ambientes do mundo. E nesse cenário, o Brasil ganha destaque para o isolamento de vírus gigantes, uma vez que possui um vasto território com grande diversificação biótica e abiótica resultando em seis biomas megadiversos (Amazônia, Cerrado, Pantanal, Mata Atlântica, Caatinga e Pampa) que juntos abrigam um alto percentual das espécies conhecidas no mundo (Alho, 2008).

O Pantanal, por exemplo, possui múltiplos ecossistemas, devido ao fenômeno das inundações sazonais que transformam habitats terrestres em aquáticos e vice-versa, o que resulta na abundância e riqueza de diversos organismos, assim como em complexas relações ecológicas inter e intraespecíficas (Alho, 2007). Foi nesse bioma, a partir de amostras coletadas em lagos alcalinos da Nhecolândia que o Tupanvírus com partículas nunca antes vista na virosfera foi isolado (Abrahão et al. 2018).

Além disso, centenas de vírus gigantes que infectam ameba a partir de amostras de lama, água, solo e esgoto (Boratto et al. 2022; Machado et al., 2024) já foram isolados no território brasileiro, como o sambavírus (Campos et al. 2014), o Brazilian Marseillevírus (Dornas et al. 2016), o Golden Marseillevírus (Dos Santos et al. 2016), os Pandoravírus pampulha, tropicalis e kadiweu (Pereira Andrade et al. 2019; Andrade et al. 2018) o cedratvírus getuliensis (Silva et al. 2018), o Faustovírus mariensis (Borges et al. 2019) e Yaravírus (Boratto et al. 2020).

A caracterização biológica e genômica dessas descobertas contribui cada vez mais com o entendimento da abundância e riqueza dos vírus gigantes nos ecossistemas do Brasil. Além disso, compreender as interações que essas entidades possuem entre si, como competição, mutualismo, sinergia, facilitação, antagonismo (Andreani et al. 2025) e até mesmo as relações que possuem com seus hospedeiros é de grande importância, visto que acabam esclarecendo os processos virais, complexidade evolutiva e diversidade (Abrahão, 2025).

2 - JUSTIFICATIVA

Desde o isolamento do *Acanthamoeba polyphaga mimivirus*, a procura por novos vírus gigantes que infectam amebas fez intensificar os estudos de prospecção viral em amostras ambientais. Como resultado, diversos outros vírus com morfologias distintas e genomas peculiares foram descobertos ao redor do mundo. Esses conhecimentos, nos permitem compreender melhor as entidades presentes na virosfera e consequentemente sua abundância, riqueza e relações que possuem entre si e com seus hospedeiros.

Neste contexto, o Brasil ganha destaque para os estudos voltados à prospecção e caracterização de vírus gigantes, uma vez que possui um extenso território que abrange seis biomas megadiversos com espécies e condições abióticas únicas. Dentre esses biomas há o Pantanal com seus diversos habitats resultante de ciclos de inundações regulares, que acabam o tornando um ambiente promissor para o isolamento de novos vírus. Exemplo é o Tupanvírus isolado até então somente no Brasil a partir de amostras com condições bem extremas (alcalinidade) que só o Pantanal possui.

Além disso, investigações realizadas em outras diferentes regiões do país já resultaram no isolamento de vários vírus gigantes de ameba a partir de amostras de solo, água, lama e esgoto, evidenciando a riqueza viral ainda pouco explorada nos ecossistemas brasileiros. Dessa forma, as descobertas mais recentes acabam por demonstrar que mesmo após duas décadas do isolamento do primeiro vírus gigante de ameba, as prospecções em amostras ambientais vêm demonstrando resultados prósperos no campo da virologia. Por isso, o presente trabalho teve como objetivo relatar a prospecção e o isolamento de dois vírus gigantes a partir de amostras coletadas da região do Pantanal brasileiro bem como a sua caracterização biológica e genômica.

3 - OBJETIVOS:

3.1 - Objetivo geral

Isolar, identificar e caracterizar vírus gigantes que infectam amebas a partir de amostras ambientais coletadas no Pantanal brasileiro.

3.2 - Objetivos específicos

- Prospectar vírus gigantes em amostras da região do Pantanal utilizando modelo de *A. castellanii* e *V. vermiformis*;
- Analisar as etapas do ciclo de multiplicação dos isolados utilizando microscopia eletrônica de transmissão, microscopia eletrônica de varredura, microscopia de imunofluorescência e tomografia eletrônica;
- Caracterizar genomicamente um dos novos isolados;
- Realizar análises genômicas e filogenéticas de um dos novos isolados.

4 – METODOLOGIA, RESULTADOS E DISCUSSÕES SERÃO APRESENTADAS NO FORMATO DE ARTIGOS PLUBLICADOS PRECEDIDOS DE UM BREVE RESUMO

4.1 – Artigo 1: A Marseillevirus isolate from the Brazilian wetlands

Este artigo foi publicado no periódico Archives of Virology, da editora Springer Nature, e descreve a prospecção e caracterização de um novo isolado de Marseillevírus, denominado Marseillevírus pantanense. Este vírus foi isolado de amostras coletadas no bioma Pantanal obtidas junto ao Projeto Navio, coordenado pelo Dr. Luiz Carlos Junior Alcantara, da Fiocruz. Este vírus foi isolado pela Dra. Nidia Esther Colquehuanca Arias em 2024, no Laboratório de Vírus da UFMG. O autor da presente dissertação realizou os ensaios para caracterização biológica e genômica deste vírus. Este trabalho foi desenvolvido no contexto do projeto intitulado “Vírus gigantes de amebas: análises evolutivas e prospecção de antimicrobianos”, com apoio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico, processo: 303680/2022-9, coordenado pelo Prof. Jonatas Abrahão e também foi registrado no Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado, sob o número A2291C9. Os resultados deste estudo ressaltam a grande importância do isolamento de mais vírus para ampliar a compreensão da abundância e distribuição de vírus gigantes no bioma Pantanal.



A Marseillevirus isolate from the Brazilian wetlands

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Abstract

Marseilleviruses are giant viruses that infect amoebas and have been isolated from various environments. Here, we describe a new strain, isolated from a water sample collected from the Paraguay River in the Brazilian Pantanal, which we have named "Marseillevirus pantanense". Transmission and scanning electron microscopy revealed icosahedral particles approximately 190 nm in diameter. Genomic and phylogenetic analyses show that *M. pantanense* has a 379,900-bp genome with 496 open reading frames, including 12 ORFans and two tRNAs. Furthermore, phylogenetic analysis places *M. pantanense* within Marseillevirus lineage A, group I. The findings of in this study underscore the importance of isolating new viruses to expand our understanding of the abundance and distribution of giant viruses in Brazilian biomes.

Keywords Marseillevirus · Pantanal · Giant viruses · Amoeba · Virus isolation

Introduction

Since the discovery of *Acanthamoeba polyphaga mimivirus* in 2003 [1], our understanding of the virosphere has expanded significantly. The subsequent isolation of several

giant viruses – characterized by particles with distinct morphologies and sizes, as well as large genomes – has contributed to a better understanding of their diversity and abundance. Among them are the marseilleviruses, which, since their first isolation in 2009 [2], have been found in a variety of sample types, predominantly those derived from freshwater sources [3–10]. Amoebas of the genus *Acanthamoeba* appear to serve as environmental hosts for these viruses, given the consistent success in isolating them in the laboratory using this host as a platform [2, 3, 7, 8].

Morphologically, marseilleviruses have icosahedral particles with diameters ranging from 180 to 250 nm [2, 11], and all known isolates are classified within the phylum *Nucleocytoviricota*, order *Pimascovirales*, and family *Marseilleviridae* [12]. Marseillevirus genomes are circular, double-stranded (ds) DNA molecules. Among the isolated marseilleviruses, genome sizes range from 348 to 404 kbp, with a GC content of 42.9% to 45.2%, and they have between 386 and 515 predicted genes [10, 11]. Most of these genes are ORFans (genes that have no homologs in public databases) or encode uncharacterized proteins (proteins that have homologs in public databases but whose function is not known), which is a common feature of giant viruses [2, 13]. However, although rare, some marseilleviruses encode translation-related factors [14], including tRNAs

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and aminoacyl-tRNA synthetases (aaRSs), as observed in Tokyovirus, Marseillevirus cajuinensis, and in metagenomic sequences from Loki's Castle [8, 10, 15].

Since 2011, our group has conducted numerous surveys of samples from Brazilian territory to isolate amoeba-infecting viruses. The Brazilian Pantanal, for example, is the largest continuous floodplain on the planet [16] and is therefore characterized by unique ecological features, hosting remarkable biodiversity and species richness. Here, we describe a new Marseillevirus isolate, named Marseillevirus pantanense, obtained from a water sample collected in the Brazilian Pantanal.

Materials and methods

Sample collection and virus isolation and purification

Water samples were collected in 2023 from the Paraguay River and surrounding areas in Mato Grosso do Sul State, Brazil, in partnership with the Expanded Navigation Project for Intensive and Optimized Surveillance (NAVIO). Using 15-mL conical tubes, 10 mL of water was collected from various locations along the river and subsequently aliquoted into microtubes.

Virus prospection was performed by inoculating crude and 1:10 diluted water samples (in phosphate-buffered saline (PBS)), into 96-well plates (Kasvi, Brazil) containing 4×10^4 *Acanthamoeba castellanii* (ATCC 30010) cells in peptone-yeast extract-glucose (PYG) medium. The medium was supplemented with penicillin (100 U/mL; Cellofarm, Brazil), streptomycin (100 µg/mL; Sigma-Aldrich, USA), amphotericin B (0.25 µg/mL; Cultilab, Brazil), ciprofloxacin (0.004 mg/mL; Sigma-Aldrich, USA), vancomycin (0.004 mg/mL; Inlab, Brazil), and doxycycline (0.020 mg/mL; Sigma-Aldrich, USA). The inoculated amoebae were monitored for cytopathic effects over seven days, with three sequential passages [17]. Samples were collected from wells in which the cells exhibited a cytopathic effect and analyzed by transmission electron microscopy (TEM).

For virus purification, 1×10^7 *Acanthamoeba castellanii* cells in 10 T-175 flasks containing PYG medium supplemented with penicillin (100 U/mL; Cellofarm, Brazil), streptomycin (100 µg/mL; Sigma-Aldrich, USA), and amphotericin B (0.25 µg/mL; Cultilab, Brazil) were inoculated with the virus at a multiplicity of infection (MOI) of 0.1. The cells were incubated at 30 °C, and after a cytopathic effect was observed, the supernatants were collected and ultracentrifuged twice at $13,682 \times g$ through a 20% sucrose cushion for 1 hour at 5 °C. The resulting pellet containing purified viral particles was resuspended in 800 µL of PBS,

and viral titers were determined using the endpoint dilution method described by Reed and Muench [18].

Transmission electron microscopy (TEM)

For TEM analysis, 7×10^6 *Acanthamoeba castellanii* cells were infected with the viral isolate at an MOI of 0.1 in T-175 flasks. Once a cytopathic effect was observed, the contents of the flasks were centrifuged for 10 minutes at $1308 \times g$ (Sorvall RT6000B). The pellet was then washed with 0.1 M sodium phosphate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for at least 2 hours at room temperature with homogenization (Benfer BHS 300). The pellet was then washed again with 0.1 M sodium phosphate buffer and resuspended in the same buffer.

Subsequently, samples were fixed for 2 hours in 2% osmium tetroxide, incubated overnight in 2% uranyl acetate at 2–8 °C, dehydrated through a graded ethanol series, treated with acetone, and embedded in EPON or Spurr resin. Ultrathin sections were analyzed using a Tecnai G2-12 FEI Spirit Biotwin 120 kV electron microscope at the Microscopy Center of the Universidade Federal de Minas Gerais (CM-UFMG) [10, 17].

Scanning electron microscopy (SEM)

For SEM analysis, 1×10^6 *Acanthamoeba castellanii* cells infected with the viral isolate at an MOI of 10 were used. Once a cytopathic effect was observed, the cells were centrifuged. The resulting pellet was applied to round glass coverslips coated with poly-L-lysine and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 2 hours at room temperature. The samples were washed three times with 0.1 M cacodylate buffer and post-fixed with 1.0% osmium tetroxide for 1 hour at room temperature. After this second fixation, the samples were washed three more times with 0.1 M cacodylate buffer and immersed in 0.1% tannic acid for 20 minutes. After a final wash with cacodylate buffer, samples were dehydrated through a graded ethanol series (35–100%). Critical-point drying using CO₂ was then performed, followed by mounting on stubs and coating with a 5-nm gold layer. Imaging was conducted using a FEI Quanta 200 electron microscope (Thermo Fisher) at CM-UFMG.

Immunofluorescence microscopy (IF)

For immunofluorescence (IF) analysis, 1×10^6 *Acanthamoeba castellanii* cells were infected with the viral isolate at an MOI of 10. At 0, 1, 3, 6, and 8 hours postinfection (hpi), cells were harvested by centrifugation. The resulting pellets

were resuspended in PBS, spread onto glass microscope slides, and fixed with methanol at -20°C . After drying, five slides were stained with whole-particle anti-MsV polyclonal antibodies produced in mice (diluted 1:400 in PBS with 0.05% Tween-20 and 3% bovine serum albumin [BSA]) for 1 hour at 37°C . Samples were subsequently incubated with anti-mouse secondary antibodies (also diluted 1:400 in PBS with 0.05% Tween-20 and 3% BSA) and Evans blue (Sigma; 0.5 g/L) for 1 hour at room temperature. Fluorescently labeled particles were visualized using a Zeiss Apo-Tome Axio Imager Z2 microscope with Apotome 2. Image processing was performed using Zen Lite software (Zeiss Microscopy) at CM-UFMG.

Sequencing, genome assembly, gene prediction, annotation, and functional categorization

The genome of the purified virus was extracted using a Mag-MAX CORE Nucleic Acid Purification Kit and sequenced on an Illumina MiSeq platform with a single-end library prepared using an Illumina DNA Prep kit (Illumina Inc., San Diego, CA, USA). Genome assembly was performed using SPAdes (version 3.15.5) with default parameters [19, 20]. The resulting scaffold was compared to sequences in the National Center for Biotechnology Information (NCBI) database using BLASTn. Open reading frames (ORFs) were predicted using Prodigal (version 2.6.3) [21]. tRNA coding sequences were identified using tRNAscan-SE (version 2.0) [22] with default parameters and ARAGORN [23]. ORF annotation was carried out using a BLASTp search of the NCBI database, HHpred (searching PDB_mmCIF70_Mar, Pfam-A_V37, SCOPe70_2.08, and UniProt-SwissProt-viral70_3_Nov_2021 databases), and InterProScan. Functional categorization of the predicted proteins was performed based on orthologous groups of nucleocytoplasmic viruses (NCVOGs) [24, 25].

Synteny and phylogenetic analysis

To perform synteny analysis, genome sequences of different Marseillevirus isolates were obtained from the NCBI GenBank database. Only Marseillevirus genomes that were isolated (excluding those reconstructed from metagenomic data) and that were complete and available in GenBank as of March 2025 were selected [10]. Because these viruses have circular genomes, the sequences were edited manually to start at the major capsid protein (MCP) gene to facilitate interpretation of the results [10]. Following sequence selection, synteny analysis was performed using the MAUVE program (version 20150226) with default parameters [26]. The genomic sequences from GenBank that were used in the analysis included Marseillevirus

cajuinensis (OR991738.1), Melbournevirus (NC025412.1), Lausannevirus (HQ113105.1), Tunisvirus (KF483846.1), Brazilian Marseillevirus (KT752522.1), and Golden Marseillevirus (KT835053.1). A database of DNA polymerase delta protein sequences was used for alignment with the MUSCLE algorithm (version 5.1) [27]. Phylogenetic trees were constructed by the maximum-likelihood method using IQ-TREE software (version 1.6.12) with 1,000 bootstrap replicates to assess branch support [28, 29]. The best-fit evolutionary model was selected using ModelFinder, implemented within IQ-TREE. The resulting phylogenetic trees were visualized and edited using iTOL software (version 6) [30]. Finally, Proksee software [31] was used to produce a schematic representation of circular genome of the isolated virus, including predicted open reading frames and GC content.

Results

During a 2023 expedition conducted in partnership with the NAVIO Project and the Brazilian Navy in the Brazilian Pantanal, 978 samples were collected from the Paraguay River in the communities of Porto da Manga, Porto Morrinho, Porto Esperança, Forte Coimbra, and Porto Murtinho (Fig. 1A). These samples were used to inoculate *Acanthamoeba castellanii* cells, and after three consecutive blind passages, a cytopathic effect characterized by cell rounding and lysis was observed in one sample collected from the city of Porto Murtinho, Mato Grosso do Sul, suggesting the presence of a virus.

For confirmation, the sample was prepared for TEM and SEM. The images revealed icosahedral particles approximately 190 nm in diameter, similar to those of members of the family *Marseilleviridae* (Fig. 1B and D). TEM images also revealed a viral factory (VF) occupying the periphery of the infected cell, which had an electron-lucent appearance, and some electron-dense particles representing possibly mature virus particles were also observed (Fig. 1C). Although only one sample tested positive for Marseillevirus, other giant viruses were also isolated from these samples, including a mimivirus (with and without a viroplasm), a moulmouvirus, and a Pandoravirus.

Acanthamoeba castellanii was inoculated with the viral isolate at an MOI of 10 for analysis of the replication cycle by IF microscopy using anti-Marseillevirus (MsV) polyclonal antibodies and Evans blue (Fig. 2). Thirty minutes after infection (considered 0 hours postinfection [hpi] in our analyses), viral particles were observed on the surface of the amoebae, suggesting that they were still in the adsorption stage. At this time point, particles were also detected within vacuoles, indicating viral entry into the host cells. By 3 hpi,

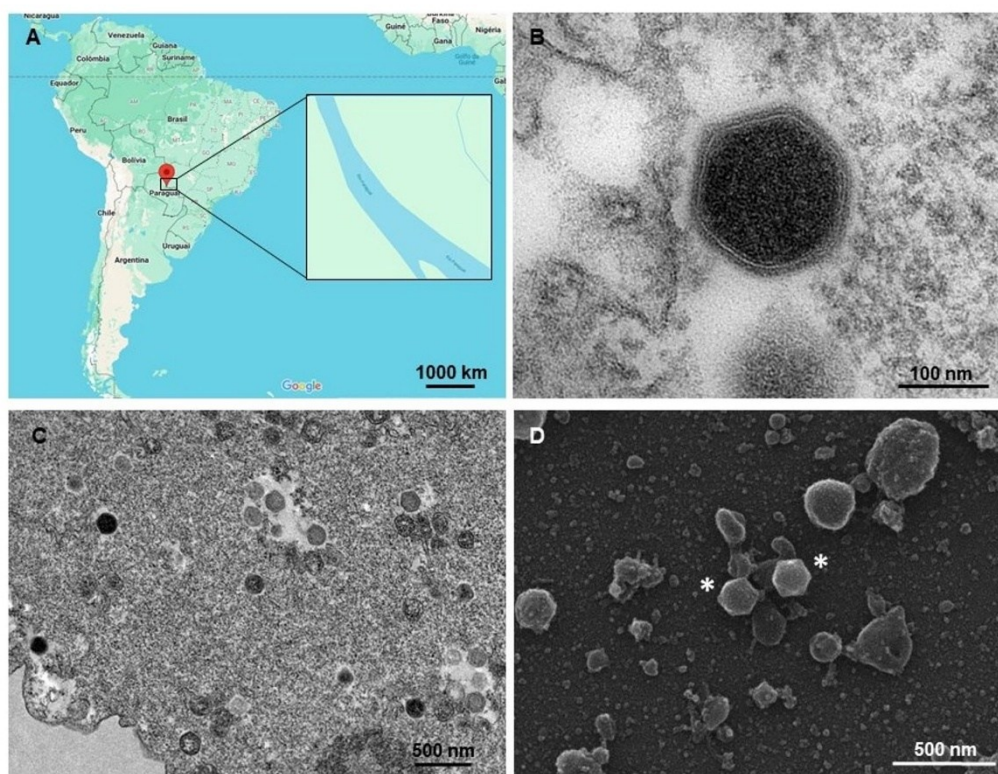


Fig. 1 Isolation of a new marseillevirus from the Pantanal region of Brazil. **A** Map showing the location of the Paraguay River in Brazil, where sample collection was performed. **B** TEM image showing an electron-dense icosahedral virus particle. **C** TEM image of a viral

factory within the cytoplasm of *Acanthamoeba castellanii*. Numerous virus particles at different stages of maturation are visible. **D** SEM image of purified icosahedral Marseillevirus particles, approximately 190 nm in diameter (indicated by asterisks)

viral proteins were localized within a large vacuolar area, predominantly at the periphery of the infected cell. At 6 hpi, viral structural proteins appeared dispersed throughout the cell, forming clusters in the cytoplasm. This observation may indicate that newly formed viral particles were clustering inside vesicles, as described previously [32]. By 8 hpi, the amoebae had been lysed, releasing viral particles.

Complementing the morphological analysis, the viral isolate was further characterized through genome sequencing. Next-generation sequencing generated 1,178,375 reads. Of the 637 contigs assembled, only one contig with appropriate length and coverage matched the Marseillevirus genome, comprising 372,900 bp with 188× coverage (Fig. 3A). The provisional GenBank accession number is 2975624.

Comparison of this genome sequence with sequences in the NCBI database revealed an average nucleotide identity of 98% with the isolate Marseillevirus cajuinensis, confirming

that the isolate belongs to the family *Marseilleviridae*, and we named this virus "Marseillevirus pantanense". The circular double-stranded DNA (dsDNA) genome of *M. pantanense* has a GC content of 45% and contains 496 predicted open reading frames (ORFs) encoding proteins (Fig. 3A). No tRNAs were detected using tRNAscan-SE; however, two tRNA sequences were identified using ARAGORN – a tRNA-Glu (tcc), with an intron of 785 bp, and a tRNA-Gln (gtc), with an intron of 1,379 bp.

Gene annotation indicated that 56 of the 496 predicted proteins are associated with DNA replication, recombination, and repair (Fig. 3B). This category includes DNA polymerase, which is commonly used as a phylogenetic marker for giant viruses, helicases, DNA ligases, various nucleases (HNH and restriction endonuclease), histones, a chaperone, DNA topoisomerase, and methyltransferases, among others. The genome also encodes proteins involved in diverse

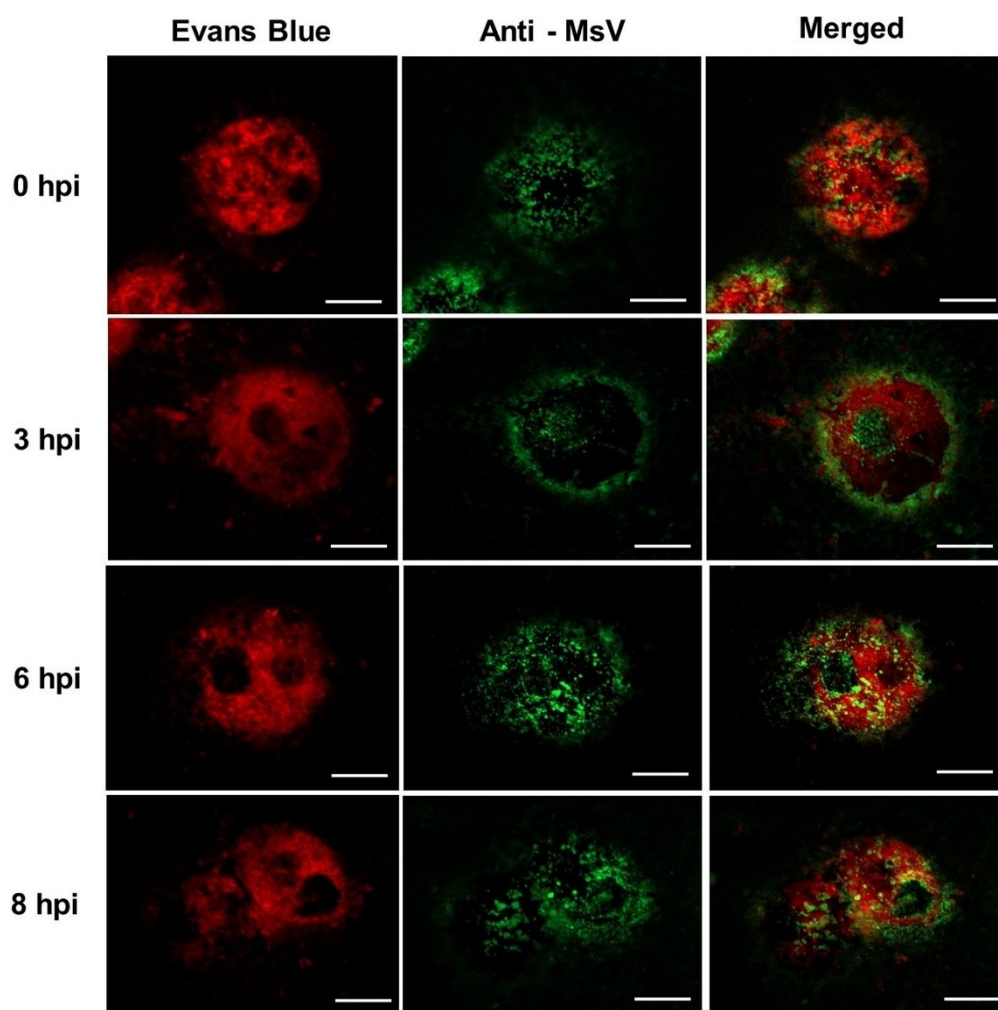


Fig. 2 Viral replication cycle in *Acanthamoeba castellanii* assessed by IF and confocal microscopy (MOI of 10). Scale bar: 10 μ m. Viral structural proteins (green) were detected using mouse polyclonal

antibodies and anti-mouse IgG secondary antibodies conjugated with Alexa Fluor 488. Amocbae were counterstained with Evans blue (red)

metabolic processes such as transcription and RNA processing, as well as lipases, and proteases (Fig. 3B). Within the category "virion structure and morphogenesis" are the major capsid protein (MCP) and DNA-packaging ATPase, both of which are essential for formation of viral progeny.

Most of the proteins (212; 42.7%) of *M. pantanense* were classified as uncharacterized (Fig. 3B). Additionally, 12 proteins were identified as ORFans, as they showed no significant similarity to any sequences in the databases used

for gene annotation (BLASTp, HHpred, and InterProScan). The ORFans ranged in size from 132 to 651 amino acids.

Using the complete genome sequence of *M. pantanense* and those of *M. cajuinensis*, Melbournevirus, Lausannevirus, Tunisvirus, Brazilian Marseillevirus, and golden Marseillevirus, which were obtained from the GenBank database, collinearity among these sequences was analyzed using MAUVE software. Since the genomes of *Marseilleviridae* representatives are circular, the gene encoding the

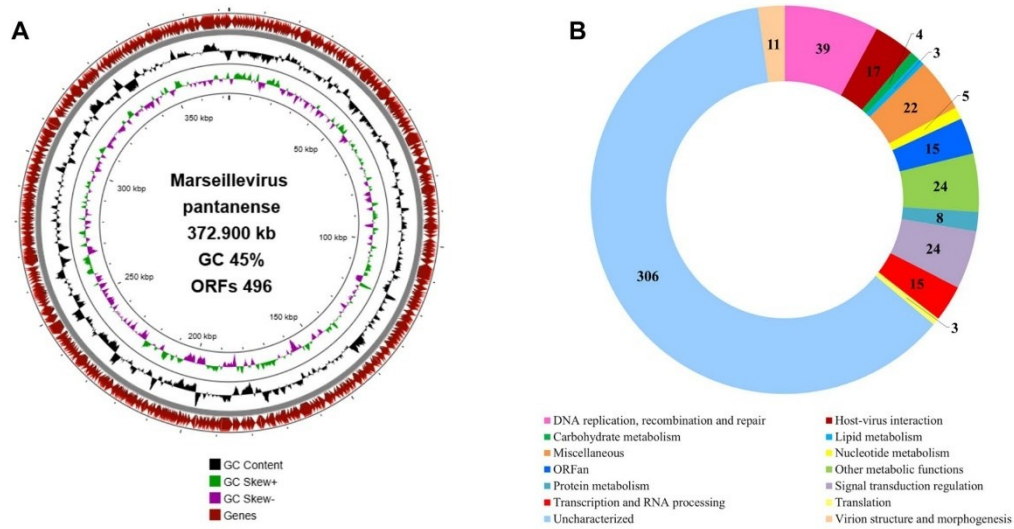


Fig. 3 Genomic analysis of Marseillevirus pantanense. **A** Circular genome map of *M. pantanense*. **B** Functional categorization of the 496 predicted proteins encoded by *M. pantanense*

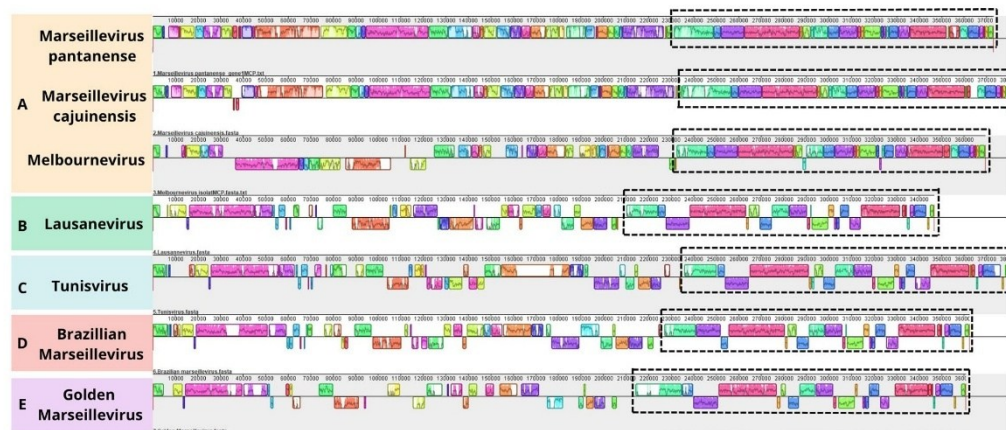


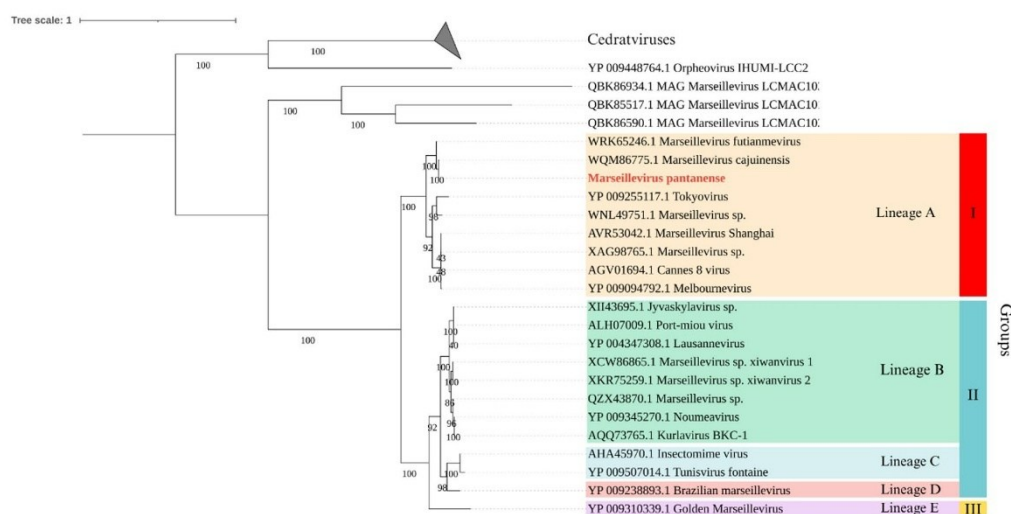
Fig. 4 Gene synteny analysis of members of the family *Marseilleviridae* representing the five described phylogenetic lineages and Marseillevirus pantanense. Each horizontal line corresponds to the genome sequence of a different virus, identified on the left. The letters A, B, C, D, and E denote the respective phylogenetic lineages of each virus. Colored blocks indicate homologous regions shared between

sequences, while uncolored areas represent unique regions exclusive to a given virus. Note: Due to their circular genomes, sequences were aligned starting from the major capsid protein (MCP) gene. Dashed rectangles indicate a conserved region previously described in Marseillevirus genomes

major capsid protein (MCP) was used as the starting point to align all sequences consistently.

The overall synteny of the gene blocks in *M. pantanense* was found to be similar to that of *M. cajunensis* and Melbournevirus (Fig. 4). Based on this similarity, we infer that

M. pantanense belongs to lineage A, group I [10], whereas Lausannevirus, Tunisvirus, Brazilian Marseillevirus, and golden Marseillevirus represent lineages B, C, D, and E, respectively [10]. This analysis also revealed a conserved



observed in the functional annotation of other *Marseillevirus* genomes, *M. pantanense* encodes restriction endonucleases (15 in total), which may have been acquired through horizontal gene transfer [2] and contribute to the genomic mosaicism of *Marseillevirus*. *M. pantanense* encodes a methylase, which we hypothesize is involved in the inhibition of host transcripts to favor the transcription of early, intermediate, and late viral factors.

Finally, genomic and phylogenetic analyses placed *M. pantanense* within lineage A, group I of the family *Marseilleviridae* [10]. This work contributes to a broader understanding of the distribution and diversity of marseilleviruses in environments with unique biotic and abiotic characteristics, such as the Brazilian Pantanal.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00705-025-06456-6>.

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Authors' contributions MFRR, NECA, JVRPC, VFQ, RALR, LHR, and JSA performed experiments and/or data analysis. TBM and LCJA collected samples. JPAJ conducted genome sequencing. MFRR, VFQ, and JSA designed the study and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Data availability The genome sequence of *Marseillevirus pantanense* will be publicly available upon the publication of the article. The raw and analyzed genome data can be accessed in the supplementary materials.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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4.2 – Artigo 2: Naiavirus: an enveloped giant virus with a pleomorphic, flexible tail

Este artigo foi publicado no periódico Nature Communications, da editora Springer Nature, e descreve o isolamento do maior vírus envelopado com cauda da virosfera conhecida, denominado de Naiavírus pantanense. Este vírus também foi isolado de amostras coletadas no bioma Pantanal, obtidas junto ao Projeto Navio, coordenado pelo Dr. Luiz Carlos Junior Alcantara, da Fiocruz. O autor da presente dissertação isolou este vírus e também atuou na caracterização da partícula e do ciclo viral utilizando técnicas de microscopia de imunofluorescência, microscopia eletrônica de transmissão, microscopia eletrônica de varredura e preparação da amostra para tomografia eletrônica. Por esta razão, durante a apresentação da dissertação somente estes resultados serão apresentados, uma vez que as demais análises foram realizadas por colaboradores do trabalho. Incentivo, no entanto, que a banca avaliadora proceda com a leitura do artigo completo, para que a importância da descoberta seja mais bem contextualizada. A descrição do Naiavírus demonstra que novos vírus gigantes permanecem por ser descobertos e destaca a importância dos estudos de prospecção viral em biomas brasileiros. Este trabalho foi desenvolvido no contexto dos projetos intitulados “Vírus gigantes de amebas: análises evolutivas e prospecção de antimicrobianos” e “Vírus gigantes de amebas nas Américas: descoberta, evolução e prospecção de antimicrobianos”, com apoio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico, Processos: 303680/2022-9 e 443344/2023-0, coordenados pelo Prof. Jonatas Abrahão. Este trabalho foi registrado no Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado, sob o número A2291C9.



Naiavirus: an enveloped giant virus with a pleomorphic, flexible tail

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Numerous studies have shown that viruses are present in a variety of environments on Earth, acting as drivers of biogeochemical cycles and powerful selective forces. Among them, giant viruses of amoebae have garnered attention from the scientific community due to their large particles and extensive genomes. Here, we describe the discovery of one of the largest tailed viruses in the known virosphere (averaging 1350 nm), named Naiavirus. This virus, isolated from a swamp biome in Brazil, has particles with a never-before-seen morphology and composition, and represents the first giant amoeba virus with an external envelope covering the capsid and extending over a flexible tail region. The Naiavirus genome, with nearly 1 million base pairs, reveals a unique set of genes, and does not resemble any other virus previously isolated so far.

Giant viruses of amoebae have garnered attention from the scientific community due to their large particles and extensive genomes, capable of encoding a significant array of unknown proteins, or proteins previously only observed in cellular organisms. Most known giant viruses infect free-living amoebae, particularly *Acanthamoeba castellanii*, which is the primary model for their study and isolation in laboratory settings¹. Among amoeba-infecting nucleocytooviruses, the first to be isolated and characterized was mimivirus, in 2003, with impressive 750 nm particles and a 1.2 Mb genome². In the following

years, several giant viruses of amoebae, all belonging to the phylum *Nucleocytooviricota*, were isolated, revealing significant structural and genomic diversity³. Despite this diversity, the known giant viruses of amoeba share some structural features, such as non-enveloped particles ranging from 0.1 to up to a micrometer or more, and the presence of an internal lipid membrane beneath the capsid, which is essential for the uncoating of genome and the proteins involved in the early stages of infection. The known giant viruses have double-stranded DNA genomes and share a series of genes encoding proteins that belong to

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the structural and replicative modules of these viruses⁴. Metagenomic studies have also shown a great abundance and diversity of giant viruses worldwide, reinforcing their presence in oceans, sediments, rivers, hot springs and permafrost samples and expanding the repertoire of genes identified in giant viruses^{3,6}.

Here we report the discovery of Naiavirus, the largest tailed virus identified to date in the known virosphere, measuring ~1350 nm (average). Isolated from a Brazilian swamp ecosystem, this virus exhibits an unprecedented particle structure and composition. It is the first giant virus infecting amoebas to feature an external envelope that encases the capsid and extends along a flexible tail. Naiavirus also possesses a genome of nearly one million base pairs, encoding a unique repertoire of genes that shows no similarity to any previously isolated virus.

Results

Naiavirus discovery, particle and cycle

We surveyed samples collected from the southern region of the Paraguay River in the state of Mato Grosso do Sul, in the Brazilian Pantanal biome. This region is widely recognized as one of the largest hotspots for biological diversity in the world. The prior isolation of the first Tupanvirus from a sample collected in this biome motivated our further exploration in the Pantanal to identify additional giant viruses⁷. To this end, water samples were collected at 44 locations along the river. In the laboratory, the samples were processed and then inoculated in *Vermamoeba vermiformis*.

After unsuccessful prospecting in 439 samples, we observed cytopathic effects in *V. vermiformis* (ATCC50237) inoculated with a water sample collected in the Porto Murtinho region. The amoebae exhibited rounding and lysis, and in the supernatant we observed regular elements, possibly giant virus particles or cells of an amoeba parasitic microorganism (Supplementary Fig. 1a, b). Once isolated, this agent was inoculated again simultaneously into *V. vermiformis*, *A. castellanii* (ATCC30010) and *A. polyphaga* (UFMG isolate), and after 48 h, we observed that all tested amoebae exhibited cytopathic effects. This lysate was subjected to crystal violet staining and then observed under an optical microscope. The cells of *V. vermiformis* were lysed, releasing a massive amount of a strongly stained entity that appeared to have a tail (Supplementary Fig. 1c). We then produced specific antibodies in mice against this entity and applied them in immunofluorescence (IF) assays. IF clearly confirmed the presence of a tail in the structure of the entity (Supplementary Fig. 1d).

Scanning electron microscopy (SEM) of the lysates revealed an unexpected and completely novel virion morphology (Fig. 1a–c). With a structure consisting of an enlarged region, here referred to as the head, and a narrowed region, here referred to as the tail, the virion was pleomorphic and asymmetric, reaching up to 1824 nm in length (mean 1350 nm) (Supplementary Fig. 2). Curiously, the entire external surface of the particles appeared to be made of the same membranous material, similar to the plasma membrane of amoebae. It is important to mention that what we refer to as a tail does not correspond to a viral tail structure *sensu strictu*, but rather to a tapered extension of the envelope structure originating from the region referred to as the head. After analyzing hundreds of particles, we identified two axes: one in the shape of a drop (head region with an average width of 540 nm) (Fig. 1a and Supplementary Fig. 2) and another in the shape of a triangle (head region with an average width of 570 nm) (Fig. 1c and Supplementary Fig. 2). At the upper end of the latter axis, at least one, and more frequently two, ostioles were present, asymmetrically arranged in an antipodal distribution. These ostioles were more clearly observed in the particle positioned along the drop-shaped axis and appeared as approximately circular protrusions on the particle surface, about 100–125 nm in size (Figs. 1d and 2a–c). In some particles, the ostioles resembled a flower of the Asteraceae family.

Transmission electron microscopy (TEM) revealed even more impressive details of this entity. In the head region, an oval structure, similar to the capsid of some giant viruses like Orpheovirus, occupied the upper region where the two ostioles were located (Fig. 1d and 2)⁸. A total of five structural layers were observed in the head region: 1) an external envelope covering the entire particle; 2) the outer layer of the oval capsid, thicker (averaging 25 nm) and electron-dense; 3) a layer below that, with internal undulations; 4) an internal membrane, as seen in nucleocytoviruses infecting amoebae; and 5) a broad electron-lucent region, where the genome is located (Fig. 2d and Supplementary Fig. 1d). In the ostiole region, there was a discontinuity in the external envelope and the layers composing the capsid wall (Fig. 2b, c). Interestingly, the internal content of the capsid is delimited in the ostiole region by the internal membrane and three additional layers of what appears to be membranous material, a structure never before described to our knowledge (Fig. 2b, c). Below the oval capsid, still within the head region, a roughly conical-shaped area (averaging 300 nm in length and 200 nm in width) was observed also covered by the external envelope (Fig. 1d). For a more detailed view of the outer membrane, new preparations were made for TEM, but using Spurr resin embedding with lower viscosity, which revealed more clearly the continuity of the envelope from the head region to the tip of the tail (Supplementary Fig. 3). In addition, longitudinal electron tomography analyses confirmed that the external envelope covers the entire viral particle from the apical region of the head to the tip of the tail (Fig. 2g, and Supplementary Movie 1). The oval-shaped capsid is clearly visible beneath the envelope, in the head region, with the aforementioned asymmetry (Fig. 2g). Particle analyses through tomography in the transverse plane revealed characteristics already described regarding the elements that make up the capsid and the tail region (Fig. 2h; and Supplementary Movies 1 and 2). At this point, we suspected that it was truly an unprecedented giant virus, with an average size of 1,350 nm, making it the largest tailed virus ever described in the virosphere and also the largest virus with an external envelope described to date. We named this entity Naiavirus, in honor of an indigenous Brazilian people, the Tupi-Guaranis. In their mythology, Naia was a beautiful indigenous woman who fell in love with and died from love for the Moon.

We then turned our attention to the tail region of Naiavirus. Unlike the head region, which showed less variation in size, the tail, with its membranous appearance, appeared quite flexible and variable in size in both SEM and TEM analyses (Fig. 3a–g and Supplementary Fig. 4). Notably, this structure appeared capable of folding, stretching, and shrinking in response to interactions with the adjacent substrate, a rare feature in the known virosphere (Fig. 3a–g). In TEM images, the tail appeared filled with an electron-lucent material and was delimited by a cylindrical membranous structure (Figs. 1d and 2c–f). Analyses of the replication cycle of Naiavirus in *V. vermiformis* and *A. castellanii* revealed that, most of the time, the first interaction between the amoebae and viral particles occurred via the tail. In several SEM images, the tail was observed attaching to the acanthopodia and pseudopodia of the amoebae, possibly stimulating their phagocytosis (Fig. 3a, b, d; Supplementary Fig. 5a and b). Once inside the phagosome, Naiavirus particles fused their internal membrane with the phagosomal membrane at the region of at least one of the ostioles, releasing the virion's internal content into the amoeba's cytoplasm (Supplementary Fig. 5c–g). TEM analyses revealed that the Naiavirus cycle occurs in the cytoplasm, with the cell's nucleus remaining intact even at late stages (Fig. 3j and Supplementary Fig. 6g and h), raising questions about whether the nucleus might play a role in the late stages of the cycle, when morphogenesis takes place. An electron-lucent viral factory occupying about 1/4 to 1/2 of the amoeba's cytoplasm was observed (Supplementary Fig. 6g and h). The morphogenesis of Naiavirus particles occurs from crescent-like structures, a feature described for several giant viruses of amoebas (Fig. 3h; and Supplementary Fig. 6a). These crescents, measuring

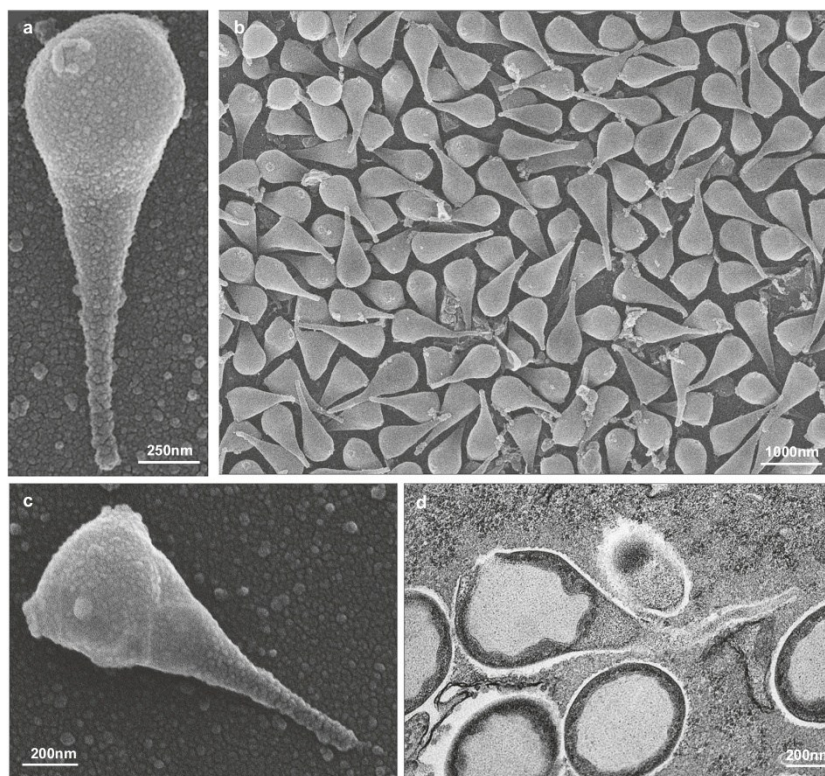


Fig. 1 | Overview of the Naiavirus particles. a–c Scanning electron microscopy (SEM) of purified Naiavirus particles. In a, the particle in its drop-like axis. In c, the particle in its triangular-like axis. **d** Transmission electron microscopy (TEM) of

Naiavirus particles within the cytoplasm of *Acanthamoeba castellanii*. SEM and TEM experiments were repeated independently three times, with similar results.

hundreds of nanometers, extend in various axes, encompassing the internal contents of the particle (Supplementary Fig. 6a–h). During their formation, at least two layers of membranous material are observed (Fig. 3h). Notably, the tail structure and the outer envelope appear to be incorporated into the particles within the viral factory (Fig. 1d; Fig. 3i, j; Supplementary Fig. 6d–h). At the viral factory's periphery, the particles seem to undergo maturation stages, with the capsid appearing thicker and its outer layer more defined (Supplementary Fig. 6e–h). No evidence of budding or exocytosis was observed. SEM and TEM analyses strongly suggest that all morphogenesis occurs within the cell's cytoplasm, and the particle release mechanism is via cell lysis induction (Fig. 3k, l; and Supplementary Fig. 7). It is important to mention that the complete replication cycle lasts ~24 h. In amoeba cultures infected synchronically, the viral factory becomes visible from 12 h; mature particles appear from 14 h; and cell lysis occurs 24 h onward.

As mentioned, Naiavirus has an external envelope that covers the entire particle. In the virosphere, envelopes are typically lipidic in nature, making them susceptible to detergents. Treatment of Naiavirus particles with Tween 20 at concentrations of 0.1% and 1% did not cause a significant reduction in viral titers. While the untreated particles induced infection with average titers of $10^{7.5}$, particles treated with 0.1% and 1% Tween induced infections with average titers of $10^{7.4}$ and $10^{7.2}$, respectively. However, when treated with a 10%

concentration, complete inactivation of the particles was observed. SEM analysis revealed damage to the viral envelope (Supplementary Fig. 8). These results suggest that the envelope of Naiavirus may have a composition different from that of regular lipids, possibly consisting of a lipid resistant to the detergent tested at lower concentrations. Although the nature of the envelope still requires further characterization, analyses using TEM, SEM, IF, and tomography indicate that the particle is completely covered by this mantle. Therefore, Naiavirus represents the largest enveloped virus in the known virosphere.

Naiavirus genome and evolutionary history

We then sequenced the Naiavirus genome using the Illumina MiSeq platform to provide insight into its evolution and genetic repertoire (see Methods). The DNA of purified virus was sequenced, and the assembly revealed a circular genome, 922 kbp in size with 33.6% G + C content and an average coverage of 791x. A total of 867 genes were predicted, ranging from 90 to 4323 bp. We performed homology searches using BLASTp against the NCBI nr database, revealing that a quarter of Naiavirus proteins had no detectable matches (i.e., ORFan genes). Furthermore, nearly 50% of the identified genes correspond to proteins with unidentified functions that were previously detected in other organisms, especially in nucleocytoovirus metagenomic sequences. To improve the annotation of Naiavirus proteins, we additionally

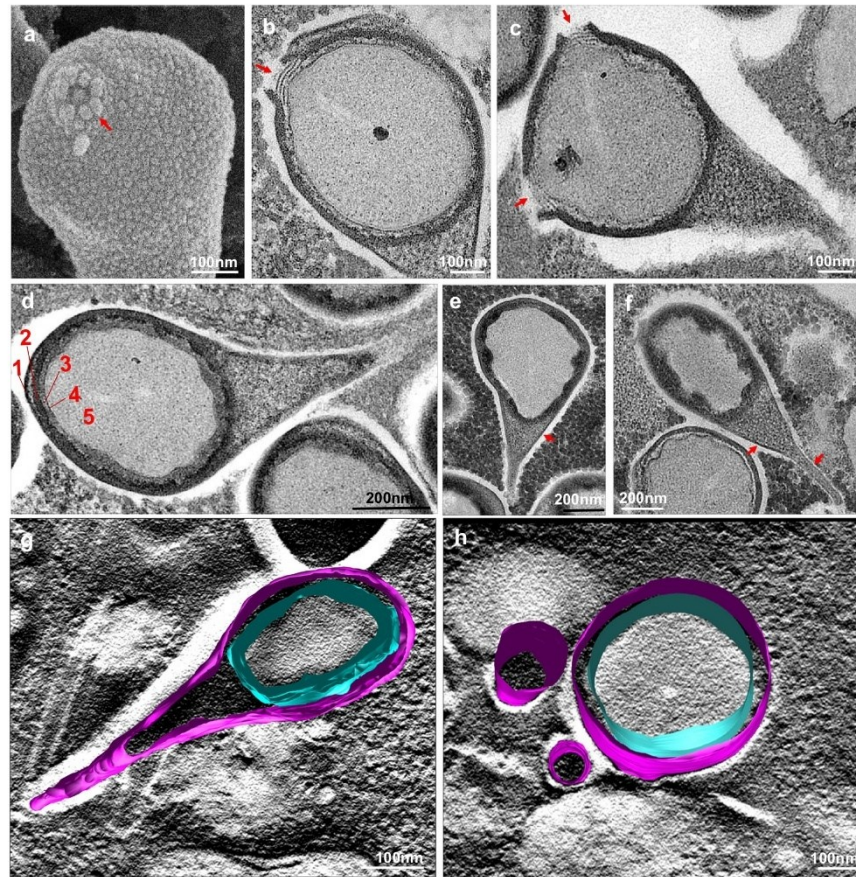


Fig. 2 | Special features of Naiavirus particles. **a–c** Ostiole of Naiavirus particles. In **a** SEM of the ostiole, highlighting the external aspect of this structure (arrow). In **b**, **c** the internal aspect of the ostiole, observed by TEM, showing that the ostiole is composed of three membranous structures (arrows), positioned over the inner membrane of the capsid. **d** Layers identified in Naiavirus particles: 1) the external envelope covering the entire particle; 2) the outer layer of the oval capsid, thicker (25 nm) and electron-dense; 3) a layer below that, with internal undulations; 4) an internal membrane, as seen in nucleocyctoviruses infecting amoeba; and 5) a broad

electron-lucent region, where the genome is located. **e**, **f** Arrows highlighting the external envelope in the tail region. **g**, **h** Electron tomography of Naiavirus particles, highlighting the external envelope (magenta) and the capsid (light blue). In **g** the particle in a longitudinal section. In **h** three Naiavirus particles in a cross-section, with the ones on the left in the tail region and the one on the right in the head region. SEM and TEM experiments were repeated independently three times, with similar results.

employed InterProScan, HHpred, and EggNOG-mapper searches^{9–12}. Nevertheless, 127 proteins remained classified as ORFans.

Analysis of these annotation results revealed that the Naiavirus genome encodes a balanced distribution of predicted functional categories. Among other functional categories widely present in the Naiavirus genome, genes associated with protein translation are notable, including 5 aminoacyl tRNA synthetases (Glycine (ID_189), Methionine (ID_222), Tryptophan (ID_457), Tyrosine (ID_657), Aspartate/Asparagine (ID_694), a peptidyl-hydrolase (ID_318) and 14 translation factors, including initiation, elongation, and termination. Additionally, 6 tRNAs were predicted in Naiavirus genome [tRNA-Ile (tat), tRNA-Ile (aat), tRNA-Leu (taa), tRNA-Leu (caa), tRNA-Cys (aca), and tRNA-Cys (gca)]. There is no clear correlation between the presence of the viral tRNA genes and the codon usage observed for Naiavirus. Nevertheless, Naiavirus encodes tRNAs associated with the most commonly used

codons for leucine and isoleucine, which may be advantageous for the viral translation process during infection (Supl. Table. 1). Genes involved in carbohydrate metabolism are also widely represented, particularly ten glycosyltransferase homologs, belonging to the families 2, 25, 45, 85 and others. Among the genes related to lipids, notable mentions include 7-dehydrocholesterol reductase (ID_7), three class-3 lipases (IDs_38, 176, 660), glycerol-3-phosphate cytidyltransferase (ID_392), fatty acid hydroxylase (ID_407), cyclopropane-fatty-acyl-phospholipid synthase (ID_444) and two patatin phospholipases (IDs_783, 533). Host-virus and signal transduction genes were also found, including several kinases, phosphatases, and an endonuclease. A total of 47 genes related to transcription and RNA processing were found, including all those necessary for transcription, such as the canonical multimeric RNA polymerase subunits (IDs_5, 393, 396, 799), RNase III (IDs_61 and 469), RNA ligase (IDs_68, 353) Cap-specific mRNA

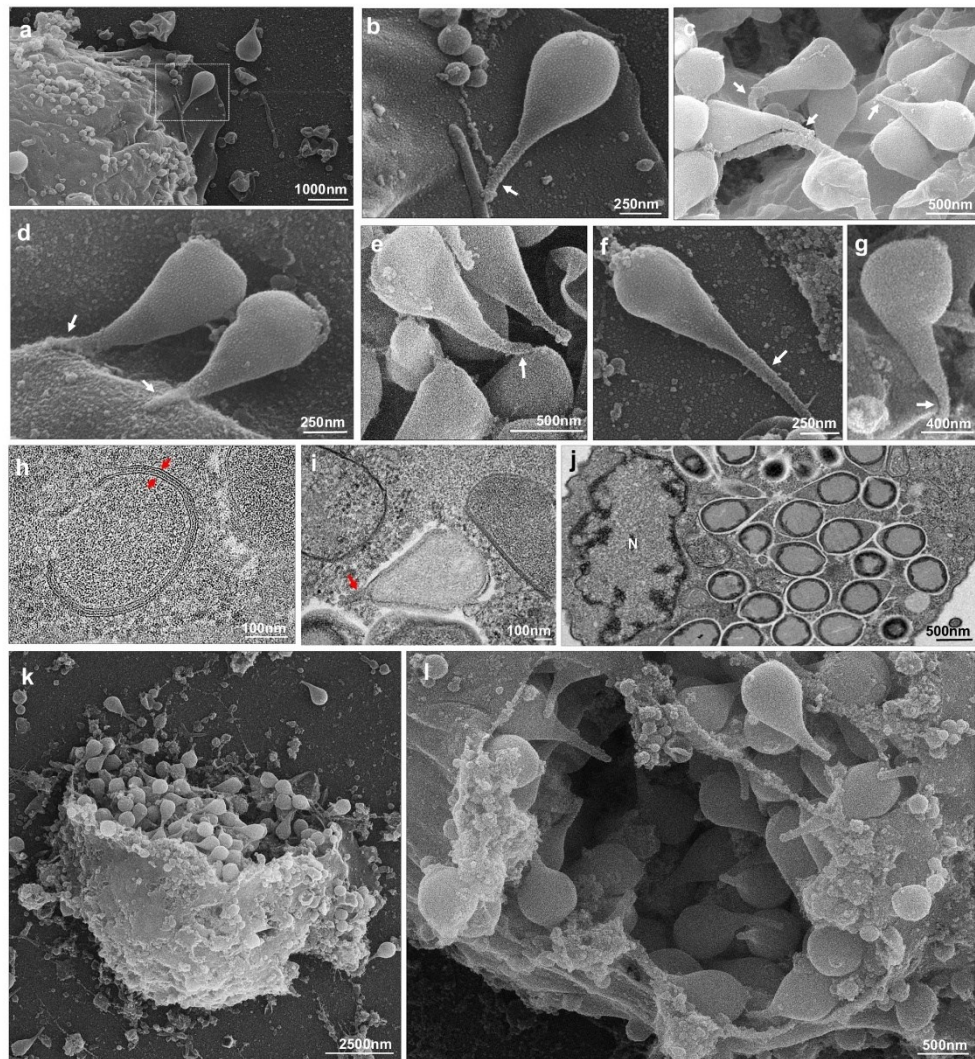


Fig. 3 | Naiavirus cycle. a–g SEM of Naiavirus particles emphasizing the flexibility and mobility of the tail region (arrows). The tail of the particles adheres to the adjacent substrate, adapting to the surface according to its topography. **h–j** Details of the cycle observed by TEM. **h** Crescents observed in the viral factory. The arrows show two juxtaposed structures at the beginning of crescent formation. **i** The tail region being formed along with the rest of the particle, in the viral factory (arrow). **j** Nuclear region (N) present at the end of the cycle. On the right, newly formed Naiavirus particle in the cytoplasm. **k, l** SEM of cells infected by Naiavirus after lysis. SEM and TEM experiments were repeated independently three times, with similar results.

(nucleoside-2-O)-methyltransferase (IDs_102 and 103) and others. An impressive number of 52 genes related to DNA replication, recombination and repair were found, including DNA mismatch repair ATPase (ID_57), AP (apurinic) endonuclease family 2 (ID_84), NAD-dependent DNA ligase (ID_92), D5 helicase-primase (IDs_120), VV D6-like helicase (ID_159), DNA primase (ID_326), DNA topoisomerase IIA (ID_398), DNA repair exonuclease (ID_430) and DNA polymerase family B (ID_582).

In addition, a long list of proteins with unexpected functions was identified in Naiavirus. A phosphoglycerate mutase (ID_648), an

enzyme involved in glycolysis and gluconeogenesis, was predicted and is essential for metabolizing glucose and 2,3-phosphoglycerate. Curiously, a staygreen protein (ID_776), typically found in plants and involved in regulating chlorophyll senescence, was also identified. Two paralogs of thaumatin family protein (IDs_365, 693) were predicted, which have also been previously identified in plants and are involved in defense functions. Bacterial-related proteins were also identified: an exosporium glycoprotein BclB-related protein (IDs_29 and 792), typically associated with the formation of the exosporium layer of bacterial

spores; and two paralogs of hemolysin III (IDs 209 and 852), a virulence factor found in some bacteria that causes hemolysis. Additionally, an aquaporin (ID_689), a mitochondrial carrier protein (ID_365), and a tumor suppressor protein, the cyclin-dependent kinase inhibitor 3 (ID_181), were predicted. Finally, HHpred predicted that ID_802 bears remote homology to a 39S ribosomal protein L38 as well as a Phosphatidylethanolamine-binding protein.

Considering that the particles of Naiavirus do not resemble any virus previously described, a central question that has intrigued us since the discovery of this entity was its phylogenetic position in the virosphere. Thus, with the genome annotated, we searched for genes commonly shared among giant viruses. Orthologs of hallmark and core genes from nucleocytoviruses were consistently found in the Naiavirus genome, including the previously mentioned DNA polymerase family B (ID_582), DNA topoisomerase IIA (ID_398), VLTf3 late transcription factor (ID_540), D5 helicase-primase (ID_120), and a major capsid protein (ID_581) with a double-jelly roll domain. A phylogenetic tree of DNA polymerase family B was then constructed, as this gene has been used in the literature as a phylogenetic marker for nucleocytoviruses (Supplementary Fig. 9). The topology of the tree suggests that Naiavirus forms a deeply-branching lineage within the known representatives of the *Pimascovirales* order. Importantly, none of the pimascoviruses discovered through viral isolation, such as Pithovirus, Cedratvirus, and Orpheovirus, cluster near Naiavirus. Interestingly, one metagenome-derived genome that exhibits phylogenetic affinity for Naiavirus is the recently described Hydrivirus, which was assembled from a Siberian permafrost sample dated to 42,000 years ago¹⁵. Since it was detected through metagenomics, the morphological characteristics of the Hydrivirus particle, as well as data related to its host and life cycle, have remained mysterious.

To examine the prevalence of other viruses with phylogenetic affinity for Naiavirus, we surveyed >15,000 publicly available metagenomes and identified several PolB sequences that fall within the new Naiavirus clade. We then performed metagenome binning to recover 11 novel metagenome-assembled genomes (MAGs) from this lineage. The other metagenomic sequences that cluster near Naiavirus were detected in various habitats, such as permafrost/cryo soil, acid mine sediment, bioreactor wastewater, water samples from lakes, etc., and several locations, including Russia, China, the USA, Canada, the Czech Republic, Sweden, Austria, and Greenland, with detections dating back to 2011 (Fig. 4c). This indicates that Naia-like viruses are present in different regions of the globe but, curiously, have never been isolated before. To assess the consistency of the results obtained from the previous phylogenetic analysis, a new tree was constructed, this time utilizing seven concatenated genes from both isolated viruses and MAGs (Fig. 4a). As previously observed, the concatenated tree revealed that Naiavirus falls within the order *Pimascovirales*, forming a distinct clade from the already known viruses in this group. Both Naiavirus, as well as Orpheovirus and Hydrivirus, are distributed among MAGs that also harbor several core genes of nucleocytoviruses. This finding indicates that this large branch of pimascoviruses remains poorly characterized, and that entirely new viruses belonging to this group are likely to be isolated in the coming years.

We also investigated the evolutionary history of Naiavirus through a global analysis of the sharing of predicted proteins among the pimascoviruses. In this analysis, not only Naiavirus but also all groups of pimascoviruses that have been isolated (cedrat-, pitho-, Orpheovirus) and metagenomic assembled genomes (MAGs) – including Hydrivirus – were considered. The result of this analysis is a network showing the protein sharing among 29 viruses (isolated or MAGs) (Fig. 4b). The largest cluster observed for Naiavirus consists of unique predicted proteins not shared with any other virus. However, several small clusters were observed between Naiavirus and various MAGs, with one large exclusive cluster between Naiavirus and MAG-Hydrivirus. This result suggests that Naiavirus has a distinct

evolutionary history, with unique predicted proteins that are not shared with other known viruses, which may indicate a significant level of genomic differentiation. The observation of small clusters between Naiavirus and various MAGs, and a large exclusive cluster between Naiavirus and MAG-Hydrivirus, suggests a possible evolutionary relationship or exchange of genetic material between these viruses and other MAGs, which may indicate processes of coevolution or interaction in specific environments. Interestingly, the analysis of the contribution of paralogs in the genomes of pimascoviruses revealed that Naiavirus and Hydrivirus have the lowest percentage of gene duplications within this group (Supplementary Table 2).

Naiavirus particles' proteome

Considering that the genome and virion organization of Naiavirus are unprecedented in the virosphere, we sought to examine the viral proteins that make up the particle. To address this, viral particles were produced by infecting 20 T175 flasks containing 10^7 *Acanthamoeba castellanii* and then purified twice, sequentially in a sucrose gradient. The ultrapurified particles were subjected to proteomics and 254 viral proteins associated with the particle (Supplementary Table 3) were identified, a number that surpasses those reported for all giant viruses, except pandoraviruses. Overall, 17 proteins involved in transcription and RNA processing were detected, containing a variety of elements that could explain the occurrence of transcription of viral genes entirely in the cytoplasm, consistent with electron microscopy findings, where no phase of the cycle was observed in the cell's nucleus. Additionally, 15 proteins involved in DNA replication, recombination, and repair were found in Naiavirus particles, suggesting that replication of its genome likely occurs in the early stages of the cycle. A homolog to a H2B histone was also found in the genome and proteomic results (ID_277), suggesting that this protein is potentially used for packaging the genome in the capsid. Finally, proteins involved in host interaction (e.g., kinases), lipid metabolism, protein metabolism, and others were also identified. Still, a total of 174 proteins were identified as ORFans or hypothetical proteins. To complement the analysis, we performed Foldseek searches on AlphaFold-predicted structures in the Naiavirus proteome (Supplementary data 1). As a result, we were able to successfully annotate only 229 proteins considering all of the approaches. A total of 19 proteins remained classified as hypothetical proteins, including the most abundant protein (ID_538), and six proteins were entirely novel, with no matches in any database (Supplementary Table 3), highlighting the vast untapped reservoir of novel proteins in giant viruses. Foldseek analysis also revealed that the viral particle packaging proteins potentially involved in host takeover, such as remote homologs to an interleukin-1 receptor-associated kinase 4 (ID_542), and a Ubx-domain-containing protein (ID_192). Notably, we identified some proteins with remote homology to phage structural proteins, including the baseplate tripod (ID_214), tail fibers (ID_206 and ID_561), and tailspike protein (ID_48). Interestingly, although the major capsid protein (MCP) is typically the most abundant protein in the capsids of various nucleocytoviruses, in Naiavirus particles, it ranks only as the 169th most abundant protein (Supplementary Table 3 and 4). Considering the completely unprecedented structure of Naiavirus particles, it is possible that the MCP's primary structural function has been modified throughout the virus's evolution, perhaps acting as a scaffolding protein. It is worth mentioning that such a phenomenon appears to have occurred in other pimascoviruses as well.

Discussion

The discovery of Naiavirus emphasizes that viral isolation studies remain crucial and complement the growing exploration of the virosphere through metagenomic methods. Unique structural elements, such as the flexible tail, the envelope, and the ostioles, demonstrate that even after more than 20 years since the description of the first

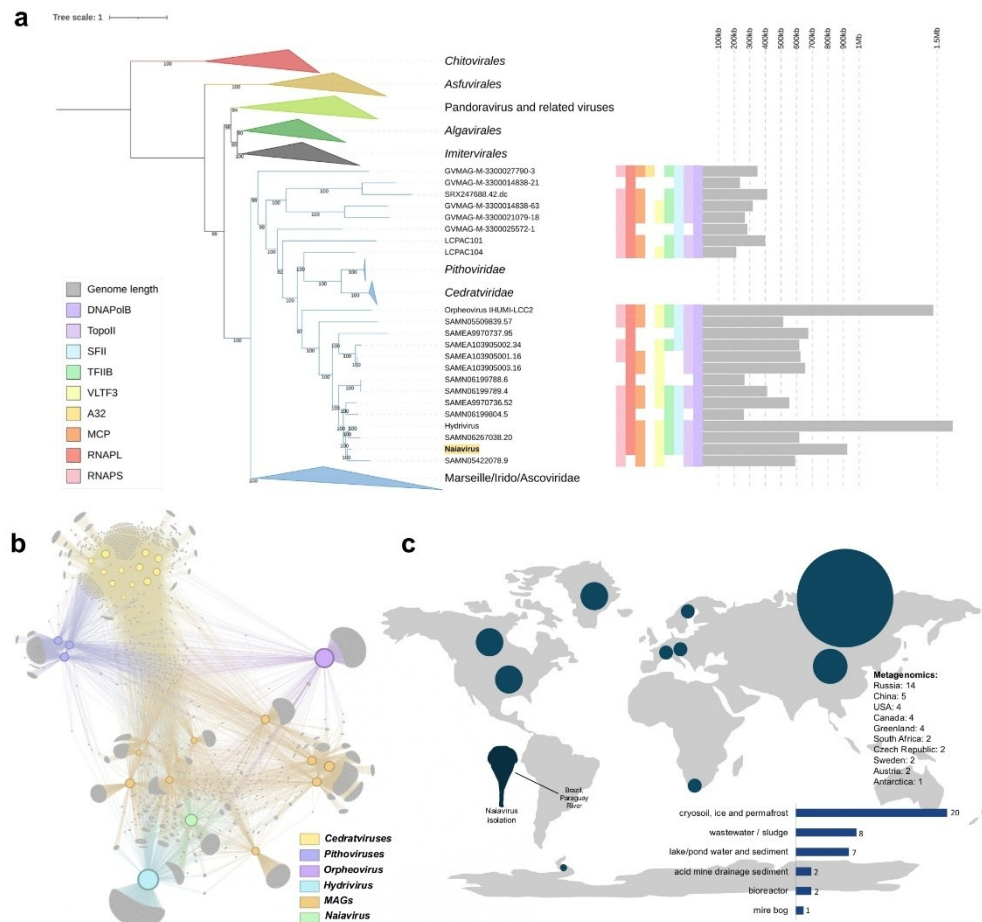


Fig. 4 | Genomics and evolution of Naiavirus. **a** Concatenated phylogenetic tree of nucleocytoviruses, showing that Naiavirus is positioned among various metagenomic sequences related to pimasoviruses, distant from any other previously isolated virus. The colored squares next to the sequences show the presence/absence of some of the core genes usually identified in giant viruses. **b** Global analysis of the sharing of predicted proteins among pimasoviruses, including

isolated viruses (cedrat-, pitho-, and Orpheovirus) and metagenomic assembled genomes (MAGs). The largest cluster observed for Naiavirus consists of unique predicted proteins not shared with any other virus. **c** Global distribution of Naiavirus detected after analysis of over 15,000 metagenomic databases. Although widely distributed around the world, Naiavirus represents the only isolated virus in this group.

amoeba viruses, giant viruses continue to surprise the scientific community with their unique structural adaptations. The fact that Naiavirus is capable of infecting different types of amoebae, a trait previously only described for Tupanviruses, suggests that the host spectrum of giant viruses is likely defined by much more complex factors than previously hypothesized, such as the presence of an almost complete set of genes associated with translation, as seen in Tupanviruses. The understanding of the evolution of giant virus genomes remains a challenge, as do the factors that determine which proteins are incorporated into their complex virions. The discovery of Naiavirus, the largest, enveloped, tailed virus ever described, symbolizes contemporary virology, where concepts and definitions regarding the boundaries of the virosphere must be frequently revisited.

Methods

Virological methods

For viral isolation, 96-well plates containing 4×10^4 cells per well of *Vermamoeba vermiformis* (ATCC 50237) in Peptone Yeast Extract Glucose (PYG) medium supplemented with ciprofloxacin (4 µg/mL), vancomycin (4 µg/mL), doxycycline (20 µg/mL), penicillin (500 U/mL), streptomycin (100 mg/mL), and amphotericin B (25 mg/mL) were inoculated with 100 µL of pure or 1:10 diluted water samples collected from 44 different points along the Paraguay River in the state of Mato Grosso do Sul, Brazil. The collections were carried out within the context of the Navio project (Expanded Navigation for Intensive and Optimized Surveillance), with support from the Brazilian Navy. For the negative control, 100 µL of PBS was used instead. The plates were

incubated at 29 °C and monitored daily under an optical microscope for cytopathic effects (CPE).

Upon CPE observation, the sample was collected, and viral stocks were produced using twenty T175 cm² cell culture flasks containing 1×10^7 *V. vermiformis* cells cultivated in PYG medium supplemented with penicillin (500 mg/mL), streptomycin (100 mg/mL), and amphotericin B (25 mg/mL). The cells were infected with Naiavirus at a multiplicity of infection (MOI) of 0.01 and incubated at 29 °C until typical CPE was observed.

For viral purification, the flask contents were collected and subjected to freezing and thawing to release viral particles that might still be trapped inside intact cells. The lysate was then centrifuged at $1200 \times g$ for 10 min to remove cell debris. The supernatant was collected, layered over a 24% sucrose gradient, and centrifuged twice at $36,000 \times g$ for 1 h. The resulting pellet was resuspended in phosphate-buffered saline (PBS), and the purified viruses were stored at -20 °C. The viral titer was determined using the endpoint method, calculated according to Reed-Muench, and expressed as the number of 50% tissue culture infective doses (TCID₅₀) per milliliter⁴⁴. Host spectrum experiments were conducted by infecting the following amoebas with the Naiavirus seed pool: *Acanthamoeba castellanii* (ATCC30010), *Acanthamoeba polyphaga* (UFMG isolate), *Vermamoeba vermiformis* (ATCC50237), and *Naegleria gruberi* (USP isolate). Considering that the virus did not replicate only in *Naegleria*, the remaining experiments were primarily conducted in *Acanthamoeba castellanii* and *Vermamoeba vermiformis*, as described in each section.

For the analysis of Naiavirus susceptibility to Tween 20, the purified viral particles were treated with this detergent at concentrations of 0.1%, 1%, and 10%. After 30 min of incubation, the particles were washed twice with PBS and then subjected to titration in *Acanthamoeba castellanii*. The detergent toxicity to the ameboid cells was previously calculated at different concentrations and dilutions, and these factors were considered in the interpretation of the results.

Microscopy

For the TEM assay, 2×10^7 cells were infected at an MOI of 0.01. Once CPE was observed, the flask contents were centrifuged for 10 min at $800 \times g$. The pellet was washed with 0.1 M sodium phosphate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for at least 1 h at room temperature. The pellet was then washed again with 0.1 M sodium phosphate buffer and resuspended in the same solution. Subsequently, the samples were fixed for 2 h in 2% osmium tetroxide, incubated overnight in 2% uranyl acetate at 2–8 °C, dehydrated in a graded ethanol series, incubated in acetone, and embedded in EPON or Spurr resin. Ultrathin sections were analyzed using a Tecnai G2-12 FEI Spirit Biotwin 120 kV electron microscope at the Microscopy Center of UFMG, Brazil.

For the SEM assay, the lysate of 4×10^6 cells or purified viral particles were added to round glass coverslips coated with poly-L-lysine and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 1 h at room temperature. The samples were then washed three times with 0.1 M cacodylate buffer and post-fixed with 1.0% osmium tetroxide for 1 h at room temperature. After this second fixation, the samples were washed three more times with 0.1 M cacodylate buffer and immersed in 0.1% tannic acid for 20 min. Next, they were washed in cacodylate buffer and dehydrated through a graded ethanol series (35–100%). Critical point drying using CO₂ was then performed, followed by mounting on stubs and coating with a 5-nm gold layer. Analyses were carried out using the ThermoFisher APREO 2 C electron microscope at the Microscopy Center of UFMG. Morphometric analyses were conducted using ImageJ software (version 1.54p).

For IF analyses, purified viral particles were stained with polyclonal anti-Naiavirus whole particle antibodies produced in mice (diluted 1:400 in PBS Tween-20 with bovine serum albumin [BSA] 3%) for 1 h at 37 °C. The samples were then incubated with anti-mouse

secondary antibodies (diluted 1:400 in PBS Tween-20 with BSA 3%) and DAPI Sigma (diluted 1:1000 in PBS) for 1 h at 37 °C. Fluorescently labeled particles were visualized (objective 100x) using an ApoTome Zeiss Axio Imager Z2 Apotome 2 microscope. Image processing was performed using Zen Lite software 2025 version (Zeiss Microscopy) at the Microscopy Center of UFMG, Brazil.

Electron tomography sample preparation and data capture

For the electron tomography preparation, *Vermamoeba vermiformis* cells were fixed in culture with 2.5% (v/v) glutaraldehyde for 2 h. Cells were then washed and resuspended in 200 mM phosphate buffer (pH 7.0). The pellet was washed, post-fixed with 1% osmium tetroxide for 2 h and stained en bloc with 2% uranyl acetate for 2 h, then dehydrated in an ethanol series and embedded in Epon resin. Sections with a nominal thickness of 250 nm were cut using an ultramicrotome and a 35 DiATOME diamond knife and collected via a water bath onto slot electron microscopy grids Slot.

Transmission electron micrographs of tilt series for single axis tomographic reconstruction were captured on a Tecnai G2-12 at 120 kV electron acceleration voltage using a 4096 × 4096 Eagle CCD (FEI). Images were captured at 1° steps from -60° to +60° using Serial EM (University of Colorado)⁴⁵ and the fiducial less tomographic reconstruction of the tilt series was performed using IMOD 5.1 suite (University of Colorado)⁴⁶. From the complete stack, 107 image slices were selected and cropped to 2183 × 2423 pixels containing the region of interest. This dataset was used for manual segmentation and modeling of each structure presented in this work.

Genome sequencing and annotation

For DNA extraction, 200 µL of purified Naiavirus was processed using the MagMAX™ CORE Nucleic Acid Purification Kit, following the manufacturer's instructions. The viral DNA was then eluted in 70 µL, and its quality and concentration were assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The genome was sequenced using an Illumina MiSeq instrument with a single-end library, prepared with the Illumina DNA Prep kit (Illumina Inc., San Diego, CA, USA). A total of 19.7 million reads were generated. The genome was assembled de novo using SPAdes software 3.12 with the parameter “-isolate”⁴⁷. Additionally, tRNA coding sequences were predicted using ARAGORN⁴⁸.

Gene prediction was performed using Prodigal v2.6.3⁴⁹. The codon usage table was generated using Jamie McGowan's Online Bioinformatics Tools (https://jamiemcgowan.ie/bioinf/codon_usage.html). All of the predicted ORFs were annotated using Diamond (Evalue of <10⁻⁵) against the NCBI nonredundant protein sequence database⁵⁰, HHpred server⁵¹, considering as valid hits those that presented probabilities greater than 80% or E values equal to or smaller than 1; eggNOG-mapper⁵² and InterProScan^{53,54}. The genomic data were deposited in the Genbank - accession: PV450503. Although the data are not yet publicly available, reviewers and editors can access them using this FigShare link: (<https://figshare.com/s/ef4eddbe9cc10b4789c4>).

Proteomic analysis

For the proteomics analysis, Naiavirus particles were produced in *Acanthamoeba castellanii* and then subjected to a two-step purification process. For protein extraction, 10⁸ viral particles were resuspended in Laemmli buffer (90 mM DTT, 2% SDS, 80 mM Tris-HCl pH 6.8, 10% glycerol, 0.1% bromophenol blue) for protein extraction and solubilization. Proteins were then separated using SDS-PAGE, and electrophoresis was halted as soon as the samples entered the resolving gel. The gel was stained with Coomassie Brilliant Blue, and the single band containing all proteins was excised for in-gel digestion. The excised gel band was destained using a solution of 50% methanol and 2.5% acetic acid, followed by protein reduction with 10 mM dithiothreitol (DTT) and alkylation with 50 mM iodoacetamide in the

dark for 30 min. The reaction was quenched by adding 10 mM DTT and incubating in the dark for 15 min. Protein digestion was performed using 1 µg of trypsin (Promega, Madison, WI, USA) at 37 °C for 16 h. The reaction was stopped by adding 5% formic acid, and the peptides extracted with 5% formic acid in 50% acetonitrile. Peptides were desalted using the StageTips method with C18 Empore disks (3M, St Paul, MN, USA). After desalting, samples were dried in a vacuum concentrator and reconstituted in 10 µL of 0.1% formic acid for further analysis.

The proteomic analysis was performed using LC-MS/MS on an Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, USA) coupled to an EASY-nLC 1200 system (Proxeon Biosystem, USA) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–40% acetonitrile gradient during 80 min, using 80% acetonitrile in 0.1% formic acid, utilizing a trap Acclaim PepMap 100 nanoViper 2PK C18 (2 cm × ID75 µm, 3 µm particle size, Thermo Scientific) in line with an analytical PepMap RSLC C18 ES 902 column (50 cm × ID75 µm, 2 µm particle size) at a flow rate of 250 nL/min. The nanoelectrospray voltage used was 1.7 kV with a source temperature of 275 °C. Full scan MS spectra (m/z 375–1500) were acquired in the Orbitrap analyzer after accumulation target value of 3e6, operating at a resolution of $r = 60,000$. The 20 most intense peptide ions, with charge states of 2 or higher, were sequentially isolated to a target value of 3e5 and fragmented using high-energy collisional dissociation (HCD) with a normalized collision energy of 27%. The signal threshold for triggering an MS/MS event was set at 1e4. Dynamic exclusion was enabled for 20 seconds, with a repeat count of 1. The maximum injection time was set to 60 ms.

For data analysis, protein identification was performed using MaxQuant version 2.4.7.0 against concatenated databases from *Acanthamoeba castellanii* and genome sequences (37,160 protein sequences, 15296385 residues) using the Andromeda search engine. Six-frame translation (6FT) was conducted using the built-in six-frame translation tool in MaxQuant. Carbamidomethylation was set as a fixed modification, while N-terminal acetylation and methionine oxidation were considered variable modifications. Peptide identification parameters included a maximum of two missed cleavages for trypsin/P, a precursor mass tolerance of 10 ppm, and a fragment ion tolerance of 0.02 Da. Protein groups were inferred automatically by the Andromeda engine following the parsimony principle. An FDR of 1% was applied to both protein and peptide identifications, calculated using reverse sequences. Protein quantification was carried out using the LFQ algorithm implemented in MaxQuant, normalizing protein abundance based on razor and unique peptide intensity values, with a minimum ratio count of one. Identifications labeled as 'Reverse,' identified only by 'site,' contaminants or present in a single sample were excluded from further analysis. LFQ intensity values were log2-transformed in Perseus version 2.0.11 for subsequent analyses.

For the additional annotation of the proteins predicted in the proteome, we ran AlphaFold v2.2 using default parameters²¹. The predicted structures were searched against PDB and Uniprot50-minimal v4 structural databases using Foldseek easy-search^{22–24}. After acquiring the raw proteomics data, a total of 274 viral proteins were detected, but 20 of them were excluded due to having only one unique peptide, low signal, and/or low coverage. The proteomics data from the particles were deposited in the ProteomeXchange platform - Project accession: PXD063493. Although the data are not yet publicly available, reviewers and editors can access them using this FigShare link - <https://figshare.com/s/39d6cf079fba30c14ce>.

Metagenome mining

To search for Naivirus relatives across a range of different habitats, we surveyed 16,801 metagenomes from the SPIRE database²⁵. We chose

the metagenomes in this set such that they represented a wide range of environments, including freshwater ponds and lakes, marine habitats, soil and sediment samples, engineered systems such as wastewater treatment plants, and others. For this analysis, we downloaded the assemblies that were already available on the SPIRE database and considered only contigs > 5 kbp in length. We predicted proteins using Prodigal v. 2.6.3 (-p meta option), and then searched all predicted proteins against the Naivirus and Hydriviruses PolB proteins using lastal v. 959 (parameter -m 100). We curated all of the best matches, in general retaining all hits with bit scores > 100. We then used these proteins for phylogenetic analysis together with select reference PolB sequences from viruses in the *Pimascovirales* order (Figure S8). Some of these proteins were placed within a clade together with Naivirus and Hydriviruses, while others were more closely related to other pimascoviruses. For metagenome-derived PolB sequences that placed within a clade with Naivirus and Hydriviruses, proceeded to bin the entire metagenomes to retrieve giant virus metagenome-assembled genomes (GVMAGs) using methods similar to those previously described⁵. We first downloaded 10 million reads associated with each metagenome and mapped them against the assembled contigs using coverm v0.4.0 (parameters “-m metabat -min-covered-fraction 0”) and then used the coverage file to bin the contigs using MetaBat2 v2.12.1 (parameter “-s 2000000”) ²⁶. We then identified bins that contained the contigs on which we found Naivirus-like PolBs and curated these GVMAGs with ViralRecall v. 2.0 (parameter “-c”) ²⁷ removing contigs with scores < 0.

Orthologous groups analysis

For the analysis of orthologous groups, the genomes of 17 pimascovirus representatives available at the National Center for Biotechnology Information (NCBI) database, along with 11 curated GVMAGs, were used to predict proteins using prodigal v2.6.3¹⁹. Orthologous groups were generated from the protein predictions from a set of 29 genomes, including Naivirus, using OrthoFinder 2.5.5 (parameter “-og”) ²⁸. A bipartite network was built from the orthofinder output using Gephi 10.1, followed by manual customization of node sizes²⁹.

Phylogeny analysis

For phylogenetic analysis, proteins of different nucleocytoviruses were predicted using prodigal v2.6.3 with default parameters¹⁹. The nclsv_markersearch tool was used to predict marker genes, create the dataset and perform the alignments (https://github.com/faylward/nclsv_markersearch.git). A set of seven marker genes that were previously benchmarked was used⁴. Alignments of individual or concatenated datasets were trimmed with trimAl v1.5. (parameters “-gt 0.1” or “-gt 0.5”, respectively) ³⁰. Phylogenetic analysis was performed using Iqtree2 2.3.6 (parameters “-m LG + F + R10 -bb 1000 -safe”) ³¹. The resulting phylogenetic trees were visualized and edited using iTOL³².

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Ethics

We confirm that our research complies with all relevant ethical regulations. The production of anti-Naivirus antibodies in mice was conducted with approval from the Animal Experimentation Ethics Committee of Universidade Federal de Minas Gerais (235/2023). This research was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge - SISGEN (number A2291C9).

Data availability

The raw and analyzed proteome data generated in this study have been deposited in the FigShare (<https://figshare.com/s/>)

39d6cf079fbaf30c14ce). The Naiavirus proteome generated in this study have been deposited in the ProteomeXchange database under accession code PXD063493. The raw and analyzed genome data generated in this study have been deposited in the FigShare (<https://figshare.com/s/ef4eddbe9cc10b4789c4>). The Naiavirus genome generated in this study have been deposited in the Genbank database under accession code PV450503. Sequences and protein predictions from the metagenome-assembled genomes generated in this study can be found on Zenodo (<https://zenodo.org/records/16950814>).

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brother, who passed away this year and is deeply missed, and to all those committed to individual and collective mental health.

Author contributions

Sample collections: T.M., C.B.C.S. and L.C.J.A. Virus isolation: M.R. Vir-
ological experiments: M.R., B.N., N.A., E.R. and J.S.A. Microscopical
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J.R.C., V.Q., A.J., J.S.A., F.O.A. Study design and coordination: J.S.A.
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Competing interests

The authors declare no competing interests.

Additional information

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5 – DISCUSSÃO

A descrição do *Acanthamoeba polyphaga* mimivírus (La Scola et al. 2003) foi precursora para a prospecção de várias amostras que resultaram no isolamento de diversos vírus gigantes de ameba. Tais isolados com grandes partículas, simetrias diversas e genomas com informações nunca antes relacionadas aos vírus proporcionou um cenário de grandes mudanças no entendimento da virosfera. O isolamento desses vírus junto a sua detecção por metagenômica em diversos tipos de amostras advindas de várias regiões do planeta é um demonstrativo de sua ubiquidade. Isso levanta diversas questões relacionadas a sua complexidade evolutiva, interações com seu hospedeiro e sua diversidade estrutural (Abrahão, 2025).

A partir dessa mudança do entendimento da virosfera é que podemos perceber o quanto importante são os estudos prospectivos virais em amostras ambientais, uma vez que permitem isolar mais cepas ou até mesmo novas entidades virais com características morfológicas e/ou genômicas únicas. O que permite a caracterização biológica e genômica, e o fomento de novos questionamentos frente à existência, evolução e relações intra e interespecíficas que os vírus gigantes de ameba possuem, como sinergia, facilitação, antagonismo, competição e mutualismo (Andreani et al. 2025).

Considerando esse contexto e o amplo território brasileiro que possui biomas com características únicas com uma rica e abundante biodiversidade (Alho, 2008), é que o Brasil ganha destaque mundial para o isolamento de vírus gigantes. Até o momento, centenas de nucleocitovírus associados a amebas, pertencentes a diferentes famílias virais, já foram isolados em diversas regiões do território nacional (Boratto et al. 2022; Machado et al. 2024). Visando enfatizar a relevância desse processo prospectivo realizado no Brasil, é que podemos destacar o isolamento até então somente no Brasil do Tupanvírus com uma simetria peculiar (Abrahão et al. 2018) e Yaravírus com um genoma nunca antes registrado em bancos de dados genômicos (Boratto et al. 2020).

Na relevância de novos isolamentos no território brasileiro é que neste estudo apresentamos a descoberta de dois vírus gigantes de amebas, sendo um deles uma nova cepa de Marseillevírus e o outro um novo vírus nunca antes descrito na virosfera, denominado de Naiavírus. Ambos foram isolados em amostras coletadas no Pantanal brasileiro, um bioma que possui múltiplos ecossistemas devido ao fenômeno das inundações sazonais que cria diversos habitats com relações ecológicas complexas (Alho, 2007).

O *Marseillevirus* isolado foi denominado de *Marseillevirus pantanense* e apresenta diversas características biológicas e genômicas que condizem com outros membros da família *Marseilleviridae*. Ele foi encontrado em amostra de água doce, semelhante ao descrito para outros isolados de *marseillevirus* (Boyer et al. 2009; Thomas et al. 2011; Aherfi et al. 2013; Doutre et al. 2014; Dornas et al. 2016) o que possivelmente está relacionado à presença de seu hospedeiro nesse ambiente. O *M. pantanense* também possui partículas icosaédricas com 190 nm de diâmetro, o que se enquadra na faixa de 180 a 250 nm observada nos demais *marseillevirus* isolados (Boyer et al. 2009; Sahmi-Bounsiar et al. 2021).

Apesar de seu ciclo de multiplicação e suas estruturas serem condizentes com a literatura (como as fábricas virais eletrolucuentes) foi visualizado durante ensaios de imunofluorescência, a localização ou migração de proteínas virais para a periferia celular no tempo de 3 hpi. Isto nos possibilita deduzir uma produção de componentes estruturais virais compartimentalizada na periferia da célula, que poderia otimizar o processo de morfogênese (Fabre et al. 2017; Sahmi-Bounsiar et al. 2021). Todavia, este resultado necessita de confirmação por outros grupos de pesquisa e também deve ser avaliado em experimentos em que o inóculo inicial é removido.

O genoma de *M. pantanense* possui um tamanho de 372,900 kbp com 496 genes preditos e um conteúdo de GC de 45%, o que também entra em concordância com outras cepas de *marseillevirus* já isoladas, com genomas variando de 348 a 404 kbp com 386 a 515 genes preditos e um conteúdo GC de 42,9% a 45,2% (De Azevedo et al. 2024; Sahmi-Bounsiar et al. 2021). Apesar da necessidade de experimentos de transcriptômica para confirmação, os tRNA-Glu e tRNA-Gln foram detectados em *M. pantanense*. O que condiz com a anotação gênica de para outro *marseillevirus* (De Azevedo et al. 2024).

Assim como observado em grande parte dos nucleocitovírus que infectam amebas, grande parte dos genes anotados são proteínas não caracterizadas (Raoult & Boyer, 2010). Em *M. pantanense*, dos 496 genes preditos 212 se enquadram nessa categoria. Já nas análises filogenéticas, *M. pantanense* está posicionado dentro da linhagem A, grupo I da família *Marseilleviridae* (De Azevedo et al. 2024). Logo, apesar de ser uma cepa com características biológicas e genômicas próximas de outras cepas da família *Marseilleviridae* podemos compreender a partir deste estudo a distribuição e diversidade de *marseillevirus* em ambientes como o Pantanal brasileiro.

Já o Naiavírus é um vírus nunca antes descrito e sua caracterização biológica e genômica trazem uma nova percepção de que há muitos vírus complexos a serem descobertos. As partículas de Naiavírus possuem uma morfologia viral pleiomórfica e assimétrica, com uma região denominada de cabeça e outra de cauda. Suas partículas podem atingir até 1824 nm de comprimento com média de 1350 nm, perdendo somente para as super partículas do Tupanvírus com até 2300 nm (Abrahão et al., 2018).

Considerando a região de cabeça é que podemos visualizar internamente a presença do capsídeo com um formato oval, semelhante a outros vírus gigante, como o Orpheovírus (Andreani et al., 2018). Esse capsídeo é envolvido por um manto aparentemente de natureza lipídica que se afunila formando uma cauda cilíndrica e pegajosa que se ajusta dependendo do substrato no qual está inserido. Hipotetizamos que tal característica possa se relacionar com seu processo de ser fagocitado pelo hospedeiro, uma vez que a cauda pegajosa possa se aderir aos pseudópodes da ameba.

As características morfológicas apresentadas a partir de diversas técnicas de microscopia já permitem inferir que o Naiavírus é o maior vírus envelopado com cauda já descrito até momento. Essa descoberta enfatiza a grande importância nos estudos de prospecção viral e principalmente no Brasil considerando sua megadiversidade de espécies. Elementos estruturais únicos, como a cauda flexível, o envelope e os ostíolos, demonstram que, mesmo após mais de duas décadas da descrição dos primeiros vírus de ameba, os vírus gigantes continuam surpreendendo os virologistas.

Os resultados deste estudo ressaltam mais uma vez a grande importância do isolamento de mais vírus gigantes de amebas para ampliar a compreensão da abundância e distribuição dessas entidades nos biomas brasileiros. As descobertas do Marseillevírus pantanense e do Naiavírus fomentam novas discussões referentes a compreensão das interações que essas entidades possuem entre si, com o seu hospedeiro junto a aspectos relacionados aos processos virais, evolutivos, e o seu papel na manutenção das comunidades microbianas.

6 - CONCLUSÕES

- Foram isolados dois vírus gigantes do bioma Pantanal.
- O isolado Marseillevírus pantanense pertence ao Grupo I e linhagem A dos marseillevírus.
- Características biológicas únicas como a migração e/ou localização de proteínas de M. pantanense foram observadas 3hpi na periferia da célula. Embora ensaios comprobatórios ainda sejam necessários, as análises aqui realizadas sugerem que proteínas virais parecem migrar para a periferia das células 3hpi, fato antes não descrito para outros marseillevírus.
- Foi isolado o maior vírus envelopado com cauda da virosfera conhecida, denominado Naiavírus.
- O Naiavírus possui genoma e estruturas únicas, nunca antes descritas.
- O presente trabalho demonstra a grande importância dos estudos de prospecção viral em amostras de biomas brasileiros.

7 – REFERÊNCIAS BIBLIOGRÁFICAS

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8 – PRODUÇÕES, EVENTOS CIENTÍFICOS E APRESENTAÇÕES

- Foi cursada a carga horária (30 créditos) exigida pelo Programa de Pós-Graduação em Microbiologia para o período do Mestrado.

8.1 - Participações em eventos científicos

- Avaliador: 12ª Feira Brasileira de Trabalhos de Iniciação Científica na Educação Básica e Técnica. Centro Pedagógico da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 01 de novembro de 2024.
- Organização de evento: Workshop: A academia e o marco legal da biodiversidade. Auditório 1 da Faculdade de Ciências Econômicas, campus Pampulha. Belo Horizonte, Minas Gerais/ Brasil, 11 de dezembro de 2024.
- Participação: XI Simpósio de Microbiologia da UFMG. Centro de Atividades Didáticas 1 - CAD 1 da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 2024, 17 a 18 de dezembro de 2024.
- Trabalho apresentado: **Rodrigues, M. F. R.**, Arias, N. E. C., Neiva, B. N., Machado, T. B., Queiroz, V. F., Junior, J. P. A., Alcântara, L. C. J., & Abrahão, J. S. ANÁLISE GENÔMICA E FILOGENÉTICA DE UM MARSEILLEVÍRUS ISOLADO DE UMA REGIÃO REMOTA NO PANTANAL. Centro de Atividades Didáticas 1 - CAD 1, Belo Horizonte, Minas Gerais / Brasil, 17 de dezembro de 2024.
- Organização de evento: I Simpósio Brasileiro de Vírus de Microrganismos. Auditório Nobre do Centro de Atividades Didáticas 1 - CAD 1, Belo Horizonte, Minas Gerais / Brasil, 01 de agosto de 2025.
- Palestrante: Ciclo de Palestras da LAM-UFMG: Desenvolvimento e publicação de artigos científicos. Instituto de Ciências Biológicas da UFMG, 09 de outubro de 2025.
- Avaliador: 13ª Feira Brasileira de Trabalhos de Iniciação Científica na Educação Básica e Técnica. Centro Pedagógico da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 30 de outubro de 2025.
- Participação: 36º Congresso Brasileiro de Virologia & 20º Encontro de Virologia do Mercosul. Centro de Atividades Didáticas 1 - CAD 1 da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 20 a 23 de novembro de 2025.

- Trabalho apresentado: **Rodrigues, M. F. R.**, Queiroz, V. F., Arantes, T. S., Limborço, H., Neiva, B. N., Arias, N. E. C., Machado, T. B., Barcelos, M. G., Cortines, J. R., Thiemann, O. H., Marques, R. E., Melo-Hanchuk, T. D., Celis, E. L. H., Junior, J. P. A., Reis, E., Alcântara, L. C., Santos, C. B. C., Jivaji, A. M., Rodrigues, R. A. L., Aylward, F. O., & Abrahão, J. S. Naiavirus: the largest tailed virus of the known virosphere, Centro de Atividades Didáticas 1 - CAD 1 da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 22 de novembro de 2025.
- Participação: 2ª Mostra Acadêmico-Científica de Estudantes Negres da UFMG. Centro de Atividades Didáticas 2 - CAD 2 da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 28 de novembro de 2025.
- Trabalho apresentado: **Rodrigues, M. F. R.**, Queiroz, V. F., Arantes, T. S., Limborço, H., Neiva, B. N., Arias, N. E. C., Machado, T. B., Barcelos, M. G., Cortines, J. R., Thiemann, O. H., Marques, R. E., Melo-Hanchuk, T. D., Celis, E. L. H., Junior, J. P. A., Reis, E., Alcântara, L. C., Santos, C. B. C., Jivaji, A. M., Rodrigues, R. A. L., Aylward, F. O., & Abrahão, J. S. Naiavírus: O Maior Vírus Envelopado da Virosfera. Centro de Atividades Didáticas 2 - CAD 2, Belo Horizonte, Minas Gerais / Brasil, 28 de novembro de 2025.
- Participação: XII Simpósio de Microbiologia da UFMG. Centro de Atividades Didáticas 1 - CAD 1 da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 03 a 05 de dezembro de 2025.
- Trabalho apresentado: **Rodrigues, M. F. R.**, Queiroz, V. F., Arantes, T. S., Limborço, H., Neiva, B. N., Arias, N. E. C., Machado, T. B., Barcelos, M. G., Cortines, J. R., Thiemann, O. H., Marques, R. E., Melo-Hanchuk, T. D., Celis, E. L. H., Junior, J. P. A., Reis, E., Alcântara, L. C., Santos, C. B. C., Jivaji, A. M., Rodrigues, R. A. L., Aylward, F. O., & Abrahão, J. S. NAIAVÍRUS: O MAIOR VÍRUS ENVELOPADO DA VIROSFERA CONHECIDA. Centro de Atividades Didáticas 1 - CAD 1, Belo Horizonte, Minas Gerais / Brasil, 05 de dezembro de 2025.

8.2 - Formação complementar

- I Treinamento em Microscopia Eletrônica de Transmissão Aplicada a Ciências da Vida. Centro de Microscopia da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 12 a 27 de agosto de 2024.
- I Treinamento em Microscopia Eletrônica de Varredura Aplicada a Ciências da Vida. Centro de Microscopia da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 26 de agosto de 2025.

8.3 - Atividades de extensão

- Curso de Inglês Iniciante (A1.1). Centro de Extensão da Faculdade de Letras da UFMG. Faculdade de Letras da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais / Brasil, 01 de abril a 02 de julho de 2024.

8.4 – Outros artigos científicos publicados durante o Mestrado

- De Aquino, I. L. M., Azevedo, B. L., Arias, N. E. C. **Rodrigues, M. F. R.**, & Abrahão, J. S. (2025). The final cut: how giant viruses of protists are released from their hosts' cells. *Arch Virol*, 170(77), 1-9.
- Arias, N. E. C., **Rodrigues, M. F. R.**, Neiva, B. N., Tatara, J. M., Almeida, G. M. F., & Abrahão, J. S. (2025). An alternative procedure for the storage and preservation of *Vermamoeba vermiformis* and other amoebae in the laboratory. *Current Protocols*, 5, e70164.