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Article

Detection and Genomic Characterization of a Bat Orthohepadnavirus in Urban Areas of Brazil: Implications for Zoonotic Surveillance

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Simple Summary

Bats host a wide diversity of viruses, including some that may affect animal and human health. Monitoring viruses circulating in bat populations is therefore important for understanding viral diversity and identifying potential emerging pathogens. In this study, we analyzed bat samples originally collected for routine rabies surveillance using a metagenomic sequencing approach. This strategy allowed the detection and genomic characterization of a hepatitis B–like virus in the bat species *Molossus rufus*. The recovered viral genome showed the typical organization of viruses belonging to the Hepadnaviridae family but also displayed genetic differences compared with previously described bat viruses. These findings expand current knowledge about the diversity of hepatitis B–related viruses in bats and highlight the value of integrating metagenomic approaches into existing public health surveillance systems for the discovery of previously unknown viruses.

Abstract

Bats are recognized reservoirs for a vast array of viral diversity, including members of the Hepadnaviridae family. Within a One Health framework, genomic surveillance of these animals is fundamental to understanding viral diversity and the potential risks of zoonotic spillover in high-density human population areas. This study describes the detection of a bat hepadnavirus through agnostic viral metagenomics in samples from passive surveillance collected in urban and peri-urban areas in Brazil. Sequencing was performed using the Oxford Nanopore Technologies (MinION) platform, and the bioinformatics pipeline involved *de novo* assembly and taxonomic identification against viral databases. We identified several contigs with similarity to the Tent-making bat hepatitis B virus (TBHBV) in a single liver sample. The largest contig (3,182 bp) represents the complete genome, exhibiting a nucleotide identity of 80.93% with the original reference isolate. Our findings document the circulation of this viral lineage in a new epidemiological setting (the Brazilian urban interface), underscoring the importance of continuous surveillance to monitor the evolution and geographic distribution of bat orthohepadnaviruses and their relevance to public health.

Keywords: hepadnavirus; bats; metagenomic

1. Introduction

Bats are recognized as important reservoirs of viral diversity [1]. Due to their broad ecological range, wide geographic distribution, and social behavior, these animals play a significant role in the maintenance and transmission of several viral families, including viruses with zoonotic potential [2]. Consequently, surveillance programs targeting bat populations, especially in urban settings, have become increasingly important for the early detection and characterization of such viruses [3,4].

In recent years, several hepadnaviruses have been described in bat species from different geographic regions, expanding the known host range and genetic diversity of these viruses [5–8]. Members of the

family Hepadnaviridae are small enveloped DNA viruses with partially double-stranded circular genomes of 3.0 to 3.4 kb [9]. Viruses within the genus Orthohepadnavirus infect not only bats, but a wide range of mammalian hosts, including humans, primates, rodents, and cats [10–13].

In this regard, virus discovery efforts have been greatly facilitated by advances in high-throughput sequencing [14]. One strategy is the metagenomic approaches which allow the unbiased detection of viral genomes directly from biological samples and have become valuable tools for pathogen surveillance and viral discovery [15]. In the context of public health surveillance, samples collected for diagnostic purposes can also be explored using metagenomics, enabling the identification of previously unknown viruses [16]. As part of routine zoonotic surveillance activities, the Laboratory of Zoonoses and Vector-Borne Diseases of the Zoonoses Surveillance Division of the municipality of São Paulo (LabZoo/DVZ/COVISA/SEABEVS/SMS) employs agnostic viral metagenomic sequencing to investigate viral diversity in wildlife specimens submitted for diagnostic purposes within a One Health framework. In this study, we report the identification and genomic characterization of a divergent hepadnavirus detected in a bat specimen collected in an urban setting in São Paulo, Brazil, and submitted for rabies diagnosis through passive surveillance. Using genome reconstruction, we obtained the complete viral genome and performed comparative and phylogenetic analyses to determine its relationship with previously described members of the genus Orthohepadnavirus.

2. Materials and Methods

2.1. Sample Origin and Surveillance Context

As part of routine surveillance activities, our laboratory employs metagenomic sequencing approaches to explore viral diversity present in wildlife samples submitted for diagnostic purposes. Therefore, 24 carcasses of bats obtained through passive surveillance and submitted to LabZoo for rabies diagnosis in the year of 2025 were subsequently repurposed for research aimed at investigating viruses of zoonotic interest.

This study was performed in compliance with the guidelines established by the Brazilian Ministry of Health's Ordinance No. 1138 regarding the use of these specimens. According to article 3, Zoonoses Surveillance Units are authorized to: receive animal carcasses (item XI), perform laboratory diagnostics for zoonoses of public health relevance (item IV), and collect biological samples for diagnostic purposes beyond rabies (item XIV).

2.2. Nucleic Acid Extraction and Metagenomic Sequencing

Because the objective of this study was unbiased viral discovery, liver tissue was used for shotgun metagenomic analysis due to its potential to harbor viruses circulating systemically in the host. Approximately 10 mg of liver tissue from each bat was individually homogenized using L-Beater 24 (Loccus, Cotia, SP, Brazil) and subsequently filtered through a 0.22 µm syringe filter. The filtrate was treated with TURBO DNase (2 U/µL; Invitrogen, Carlsbad, CA, USA), and total nucleic acids were extracted using TRIzol® Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions.

Metagenomic sequencing was performed using the SMART-9N protocol as described elsewhere [17]. The resulting amplicons were purified with AMPure XP beads (Beckman Coulter, UK) at a 1:1 ratio, quantified via Qubit 3.0 fluorometry (Life Technologies, Carlsbad, CA, USA), and normalized to 65 ng per sample. Libraries were prepared using the NEBNext Ultra II End Repair/dA-tailing and Ligation modules (NEB, Ipswich, MA, USA), with multiplexing handled by the Native Barcoding Kit 24 V14 (ONT, Oxford, UK). Sequencing was conducted on a MinION Mk1C platform (ONT) using R10.4.1 flow cells loaded with 33 ng of the final library and operated for 72 hours to maximize depth of coverage.

2.3. Genome Assembly and Consensus Reconstruction

Raw signal processing, including high-accuracy (HAC) basecalling and demultiplexing, was performed using Guppy (ONT). Following adapter, primer removal, and quality filtering via Cutadapt v4.8 and Porechop v0.3.2 (ONT), genomes were reconstructed through *de novo* assembly with MEGAHIT v1.2.9 [18], followed by polishing with Medaka v1.11.3 (ONT) to generate high-fidelity consensus sequences.

Sequence alignment was performed against the NCBI RefSeq viral protein database (updated February 10, 2026) via DIAMOND v2.1.9 [19] (blastx mode, more-sensitive setting). To ensure the accuracy of the viral identification and filter out spurious hits, all potential viral sequences underwent a secondary screening against the NCBI non-redundant (nr) protein database.

To refine the viral genome, sequencing reads were mapped back to the assembled contig and a consensus genome sequence was generated. Variant calling and consensus reconstruction were performed using BCFtools v1.17 [20], and the resulting sequence was further repolished using Medaka v1.11.3 (ONT) to correct residual errors associated with long-read sequencing data.

The resulting repolished consensus sequence was visually inspected by mapping the filtered reads back to the genome using Minimap2 2.28-r1209 [21] and examining the alignment in Tablet 1.17.08.17 [22] to verify read support across the genome and to confirm the integrity of predicted open reading frames.

2.4. Molecular Identification of the Host Species

Host species identification was performed using mitochondrial sequences recovered from the metagenomic dataset. Nanopore reads were mapped against a dataset of bat mitochondrial gene sequences retrieved from GenBank using Minimap2 2.28-r1209 [21]. The resulting alignments were processed with SAMtools v1.18 [23], and variant calling was performed using BCFtools v1.17 [20]. A consensus mitochondrial sequence was generated from the mapped reads. The resulting consensus sequence was compared with sequences available in the NCBI nucleotide database using BLASTn to determine the host species identity.

2.5. Genome Annotation

Open reading frames (ORFs) were predicted and annotated based on similarity to previously described members of the genus Orthohepadnavirus through BLASTn searches. The presence and organization of the canonical hepadnavirus genes, including polymerase, surface, precore/core, and X proteins, were confirmed through sequence comparison and translation analysis.

2.6. Sequence Similarity and Phylogenetic Analysis

The viral genome sequence and predicted proteins were compared to sequences available in public databases using similarity searches as well representative sequences of hepadnavirus available at International Committee on Taxonomy of Viruses (ICTV) homepage. Multiple sequence alignments were performed using MAFFT v7.505 [24]. Phylogenetic relationships between the divergent identified virus and representative members of the family Hepadnaviridae were inferred using maximum-likelihood methods implemented in IQ-TREE v2.0.7 [25]. Statistical support for tree topology was assessed using bootstrap resampling. The tree was visualized using Interactive Tree of Life (iTOL) v7.4.2 [26].

2.7. Genome Organization and Sequencing Depth Visualization

The genomic organization of the recovered hepadnavirus sequence was visualized using the software Circos v0.69-8 [27]. ORFs were identified based on annotation of the assembled genome and included the polymerase (Pol), surface (S), precore/core (preC/C), and X genes. Sequencing reads were mapped to the assembled viral genome to determine coverage depth across genomic positions. The depth of coverage was calculated using SAMtools v1.18 [23], and the resulting coverage data were used to generate a circular genome plot.

2.8. Sliding Window Analysis of Nucleotide Identity

Nucleotide identity between the recovered viral genome and the closest related sequence, Tent-making bat hepatitis B virus (TBHBV - Accession Number: KC790381.1), was evaluated using a sliding window approach implemented in R v4.4.0 within the RStudio environment (v2025.05.1). The aligned nucleotide sequences were imported from a FASTA alignment file produced after MAFFT v7.505 [24]. Sequence identity was calculated across the genome using a window size of 200 nucleotides and a step size of 20 nucleotides. For each window, the percentage of identical nucleotides between the two sequences was computed. The resulting identity values were plotted against genomic position using the ggplot2 package v4.0.2 to generate a line graph representing sequence similarity across the genome. Annotated ORF positions were overlaid on the plot to indicate the genomic locations of the polymerase, surface, precore/core, and X genes.

2.9. Pairwise Nucleotide Identity Analysis

Pairwise nucleotide identities among complete viral genomes were calculated using global pairwise alignments implemented in Python with the BioPython library (pairwise2 module). For each pair of sequences, nucleotide identity was calculated as the proportion of identical positions in the alignment relative to the alignment length. Identity values were compiled into a similarity matrix and visualized as a heatmap using the Python libraries *pandas* v2.2.3, *seaborn* v0.13.2, and *matplotlib* v3.10.0.

3. Results

3.1. General Results

A metagenomic library was generated from 24 bat samples submitted for rabies diagnosis through passive surveillance. All specimens tested negative for Rabies virus detection using the Direct Fluorescence Antibody Test (DFA), the gold standard for post-mortem laboratory diagnosis of rabies [28].

Metagenomic sequencing generated 404,056 raw reads, of which 380,600 were retained after primer and adapter trimming as well as quality filtering. The filtered reads had a mean length of 417 bp and an N50 length of 450 bp (Supplementary Table S1).

Viral sequences related to the family Hepadnaviridae were identified in one sample derived from the liver of a bat originated from an urban area of Sao Paulo State, Brazil (Sample ID 3848/25 - Supplementary Table S2).

3.2. Molecular Identification of the Host Species

A mitochondrial 12S rRNA fragment was successfully reconstructed from the metagenomic reads. The consensus sequence showed complete coverage across the reference fragment and, when compared with the NCBI nucleotide database using BLASTn, exhibited 100% nucleotide identity and 100% query coverage with sequences of *Molossus rufus*.

3.3. Genome Characterization of the Bat Hepadnavirus

The assembled viral genome, named *Molossus rufus* bat hepadnavirus (MRBHV), was 3,182 nucleotides (nt) in length with a GC content of 46.86%. Mapping of sequencing reads to the reconstructed genome identified 16,300 reads, corresponding to approximately 4.3% of the dataset. These reads provided 100% genome coverage with an average sequencing depth of 3,135x, supporting the reconstruction of a complete viral genome (Supplementary Table S1).

Sequencing reads were mapped across the entire genome, with a minimum depth of 29x and a maximum depth of 7,138x, indicating complete genome recovery without uncovered regions (Supplementary Figure S1).

Genome annotation revealed the typical hepadnavirus genomic organization, including the polymerase (Pol), surface (S), precore/core (preC/C), and X ORFs. The polymerase ORF spanned nucleotides 2132–3182 and 1–1466 and encoded a predicted protein of 838 amino acids (aa). The

surface gene was located between nucleotides 1–672 and encoded a protein of 223 aa. The core ORF was located between nucleotides 1714–2280 and encoded a predicted capsid protein of 188 aa. An upstream region preceding the core gene (nt 1618–1713) was identified and likely represents a precore region encoding a 32 aa peptide. The X gene spanned nucleotides 1211–1618 and encoded a predicted protein of 135 aa (Figure 1A).

Comparative analysis using BLASTn revealed that the genome shared the highest similarity with TBHBV, showing 80.93% nucleotide identity with 99% query coverage and an E-value of 0.0 (Supplementary Table S3).

3.4. Genomic Similarity Analysis

Sliding window analysis of nucleotide identity between the MRBHV genome and the TBHBV revealed identity values ranging from approximately 55% to 95% across the genome. The identity plot revealed heterogeneous similarity, with higher nucleotide identity observed in the surface gene and parts of the polymerase region. The lowest similarity was detected toward the terminal portion of the polymerase gene, indicating increased divergence in this region (Figure 1B).

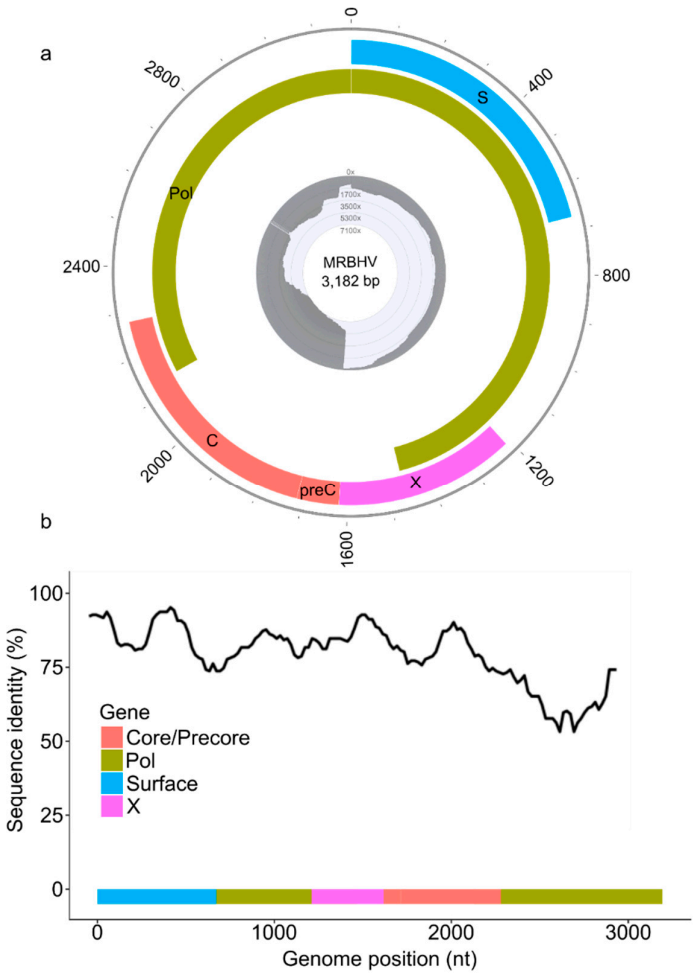


Figure 1. Genome organization and sequencing coverage of the bat hepadnavirus identified in this study (MRBHV). (a) Genome organization of the divergent bat hepadnavirus MRBHV showing the four overlapping open reading frames encoding the polymerase (Pol), surface (S), precore/core (preC/C), and X proteins. Grey histogram indicates sequencing coverage depth obtained from metagenomic reads. Complete genome of the bat

hepadnavirus 3,182 kb. (b) Sliding window analysis of nucleotide identity between MRBHV genome and the closest related virus (TBHBV - Accession Number: KC790381.1). Colors indicate the genomic regions corresponding to each gene.

3.5. Phylogenetic Analysis

Phylogenetic reconstruction based on the amino acid sequence of the polymerase protein was performed using the maximum likelihood method under the Q.pfam+F+R5 evolutionary model (Supplementary Table S4; Supplementary Data S1). The resulting tree showed that the MRBHV clustered within the genus Orthohepadnavirus and within the clade of TBHBV sequences, forming a well-supported clade with an ultrafast bootstrap value of 100. These results indicate that the virus identified in this study forms a distinct lineage within the bat-associated hepadnaviruses, with strong statistical support (Figure 2).

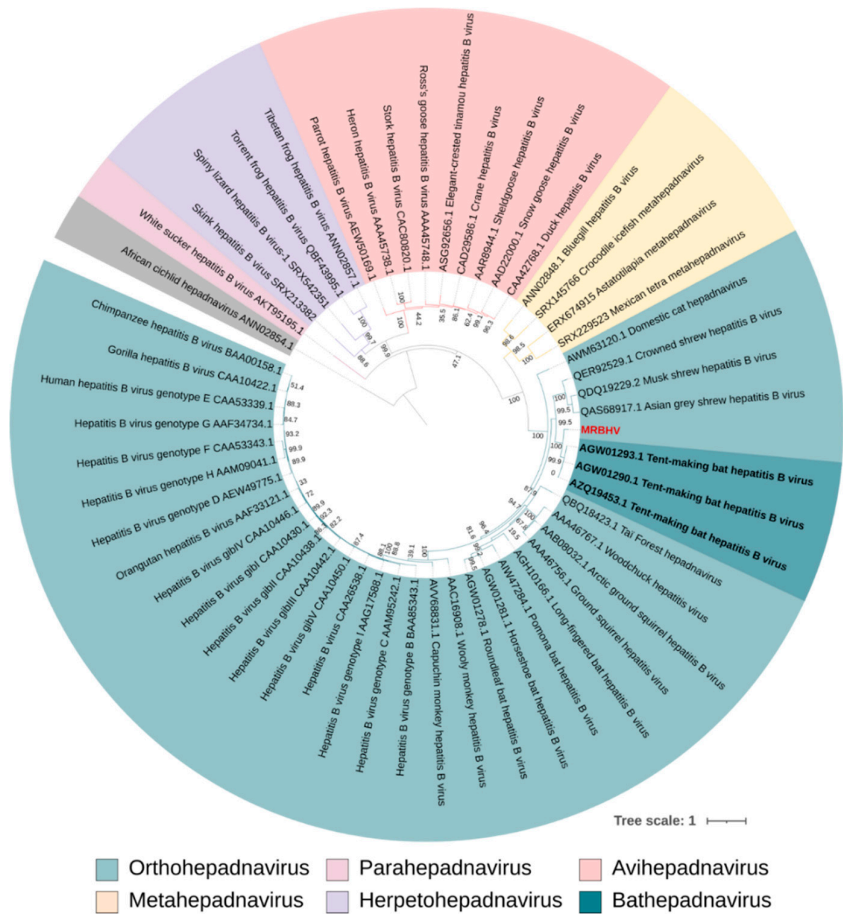


Figure 2. Phylogenetic analysis of the bat hepadnavirus identified in this study (MRBHV) based on polymerase amino acid sequences. The phylogenetic tree was reconstructed using representative polymerase amino acid sequences of the Hepadnaviridae family. The sequence identified in this study (MRBHV) is highlighted in red. Bat hepadnavirus are highlighted in bold. Node values represent bootstrap support percentages. The tree was rooted using sequence African cichlid hepadnavirus ANN02854.1. The scale bar indicates the number of nucleotide substitutions per site.

3.6. Pairwise Nucleotide Identity Analysis

Pairwise nucleotide identity analysis based on complete genome sequences showed that the virus identified in this study shares the highest similarity with previously reported bat hepadnaviruses (TBHBV - GenBank Accession Numbers KC790380.1 and KC790381.1) while lower

identity values were observed when compared with more distantly related hepadnaviruses. This pattern is consistent with the phylogenetic results and further supports that the detected virus represents a distinct lineage within bat-associated hepadnaviruses (Figure 3).

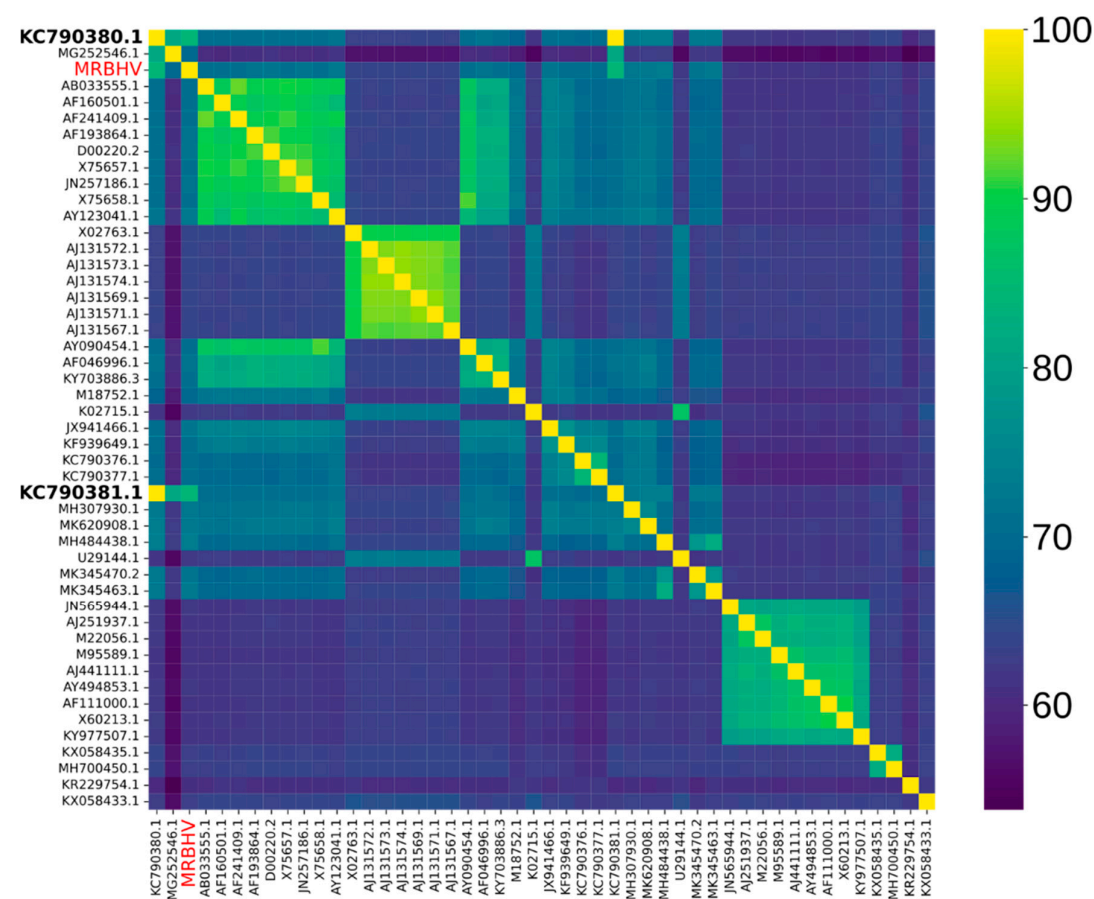


Figure 3. Pairwise nucleotide identity among complete genomes of representative hepadnaviruses. Heatmap showing pairwise nucleotide sequence identity calculated from complete genome sequences of representative members of the family Hepadnaviridae. The virus identified in this study (MRBHV) is highlighted in red on both axes. Sequences showing the highest similarity are in bold. Colors represent the percentage of nucleotide identity, ranging from low identity (purple/blue) to high identity (yellow). Clusters of closely related viruses are visible as blocks of higher similarity along the diagonal.

4. Discussion

In this study, we report the detection and genomic characterization of a hepadnavirus identified in liver tissue from a *Molossus rufus* bat. The recovered genome displayed the canonical genomic organization typical of members of the family Hepadnaviridae, including the polymerase, surface, precore/core, and X open reading frames arranged in an overlapping configuration [9]. The genome length (3,182 nt) is consistent with those reported for other bat-associated hepadnaviruses [29].

Comparative sequence analysis revealed that the virus detected in this study is most closely related to Tent-making bat hepatitis B virus, sharing approximately 80.9% nucleotide identity across the genome and clustering with this lineage in phylogenetic analyses based on the polymerase protein. The high bootstrap support observed for this clade indicates a robust evolutionary relationship between these viruses. Nevertheless, sliding window analysis demonstrated heterogeneous levels of nucleotide identity across the genome, with values ranging from approximately 55% to 95%, indicating the presence of both conserved and more divergent genomic regions. This pattern is consistent with the evolutionary dynamics commonly observed in

hepadnaviruses, whose compact genomes and extensive gene overlap impose distinct selective pressures across coding regions [30]. Thus, the virus identified in this study likely represents a distinct evolutionary lineage within bat-associated hepadnaviruses and may represent a previously unrecognized bat-associated hepadnavirus closely related to Tent-making bat hepatitis B virus.

Genome annotation also revealed a region upstream of the core gene consistent with a predicted precore sequence. In orthohepadnaviruses, the precore region encodes the precursor of the secreted hepatitis B e antigen (HBeAg), a protein involved in modulation of host immune responses [31]. Although functional characterization was not performed in the present study, the presence of this genomic feature further supports the structural similarity between the virus identified here and other members of the Hepadnaviridae.

Although the virus identified in this study clustered with Tent-making bat hepatitis B virus in phylogenetic analyses, the observed nucleotide identity of approximately 80.9% across the genome indicates a considerable level of genetic divergence. Similar levels of divergence have been reported among distinct lineages of bat-associated hepadnaviruses, reflecting the substantial evolutionary diversity of this viral group. The genomic differences observed here may represent host-specific evolutionary adaptation or long-term diversification of hepadnaviruses circulating in bat populations [8,32].

Regarding the host, the detection of a divergent hepadnavirus in a *Molossus rufus* bat expands the known host range of bat-associated hepadnaviruses. Given that bats harbor a diverse range of viruses within the family Hepadnaviridae, this finding further contributes to the understanding of the diversity and evolutionary history of this viral group [29,32].

The genus *Molossus* comprises insectivorous bats widely distributed throughout the Americas [33]. *Molossus rufus*, in particular, has been reported to inhabit urban environments in Brazil [34]. Noteworthy, the bats analyzed in this study came from urban areas where they frequently occupy buildings and other human-modified structures. This ecological context places these animals as synanthropic fauna as they are in close proximity to human populations and domestic animals, increasing opportunities for viral detection at the human–animal interface [35].

Nevertheless, the zoonotic potential of bat-associated hepadnaviruses remains incompletely understood. Previous studies have demonstrated that certain bat hepadnaviruses are antigenically related to human hepatitis B virus and may share functional similarities with members of the genus Orthohepadnavirus [29]. However, there is currently no evidence that bat hepadnaviruses are capable of infecting humans under natural conditions. The discovery of genetically diverse hepadnaviruses in bats nonetheless highlights the importance of continued surveillance, particularly in synanthropic species that frequently occur in close proximity to human populations. Monitoring the diversity and evolution of these viruses as well as others may contribute to a better understanding of their host range and potential for cross-species transmission [5,29] especially in urban settings [36,37].

Importantly, the virus described in this study was identified through a metagenomic surveillance strategy applied to samples obtained from passive surveillance activities. The analyzed bats were originally submitted to the public health diagnostic network for rabies testing and subsequently repurposed for viral discovery using shotgun metagenomics. This approach highlights the value of integrating research-oriented viral surveillance with existing public health diagnostic workflows [38]. Passive surveillance programs, particularly those focused on rabies diagnostics, routinely receive wildlife specimens and therefore represent an important and wasted resource for the detection of additional viral agents circulating in animal populations. In this context, the application of shotgun metagenomics within public health surveillance frameworks represents a powerful tool for the detection of potentially zoonotic viruses before spillover events occur [39,40].

Although this study provides important insights, it has several limitations that should be considered when interpreting the results. First, the detection of the virus was based solely on metagenomic sequencing, and independent confirmation by targeted PCR or Sanger sequencing was not performed. Although the viral genome was recovered with substantial read support, additional

molecular confirmation would further strengthen the findings. Second, the virus was detected in a single bat specimen, which precludes any inference regarding its prevalence or distribution in bat populations. Furthermore, only liver tissue was analyzed, preventing evaluation of viral tissue tropism or systemic infection. Finally, the use of random amplification during metagenomic library preparation may introduce biases in genome representation. Future studies including targeted screening of additional bat samples and molecular confirmation approaches will be important to better understand the distribution, host range, and evolutionary diversity of this virus.

Taken together, the identification and genomic characterization of the *Molossus rufus* bat hepadnavirus underscores the importance of integrating wildlife surveillance, diagnostic infrastructure, and metagenomic technologies to improve our understanding of viral diversity and to strengthen preparedness for emerging zoonotic threats.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Sequencing depth across the complete viral genome of MRBHV and distribution of coverage values; Table S1: Sequencing statistics; Table S2: Samples analyzed; Table S3: BLASTn similarity results; Table S4: Polymerase amino acid sequences used for phylogenetic analysis; Data S1: Polymerase amino acid sequences used in phylogenetic analysis.

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Institutional Review Board Statement: Ethical review and approval were waived for this study due to Brazilian legislation (Law No. 11.794/2008 and CONCEA regulations), ethical approval for animal experimentation is only required when animals are handled specifically for research or educational purposes. In this study: no animals were captured, handled, or euthanized for the purpose of this research project. The study utilized post-mortem samples (sample from passive surveillance, i.e., found dead on the ground by citizens) from animals that were already part of the official rabies diagnostic routine of the municipality. The research is a secondary use of biological material obtained during primary public health surveillance actions.

Informed Consent Statement: Not applicable.

Data Availability Statement: The complete viral genome sequence generated in this study has been deposited in GenBank under accession number PZ158833. Raw sequencing reads have been deposited in the Sequence Read Archive under BioProject PRJNA14366300; BioSamples from SAMN56479928 to SAMN56479951. The scripts used for the pairwise identity analysis and figures generation are available at GitHub (<https://github.com/julianaamorim-arch/bat-hepadnavirus-genome-analysis>).

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Conflicts of Interest: The authors declare no conflicts of interest.

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