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Molecular characterization and phylogenetic relationships of a novel *Pipistrellus ceylonicus*-derived picobirnavirus

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Abstract

The *Picobirnaviridae* family comprises small, non-enveloped viruses with a bi-segmented, double-stranded RNA genome, exhibiting broad host range and high genetic diversity. Despite over 7,000 entries in public databases, only 53 originate from bats, and merely one contains a near-complete capsid protein sequence, highlighting a significant knowledge gap. This study reports the detection and genomic characterization of a novel picobirnavirus derived from *Pipistrellus ceylonicus* bat from the Kon Chu Rang Nature Reserve, Gia Lai province, Vietnam. Using metagenomic sequencing, we obtained genomic sequences for both segments (dsRNA1: 1,902 bp; dsRNA2: 1,626 bp), encoding the capsid protein and RNA-dependent RNA polymerase (RdRp), respectively. Phylogenetic analysis of RdRp amino acid sequences robustly placed the virus within the International Committee on Taxonomy of Viruses (ICTV)-recognized genogroup 2 and the proposed genus *Alphapicobirnavirus*. The obtained capsid protein showed the highest sequence similarity to picobirnaviruses isolated from a human and a macaque (genetic distances of 0.59 and 0.62), while exhibiting a distant relationship (divergence of 0.79) to the only known near-complete bat picobirnavirus capsid sequence. This significant genetic distance, particularly from the sole chiropteran reference, supports the identification of a novel viral species within the *Picobirnaviridae* family. The characterized virus expands the known diversity of bat-related picobirnaviruses and provides crucial genomic data, contributing to a more accurate understanding of the family's evolution and host range.

Keywords

Bats; Metagenomics; Phylogeny; RNA Viruses; Vietnam; Viral Genomics; Virus Classification.

Introduction

The *Picobirnaviridae* family comprises small, non-enveloped viruses with a double-stranded RNA (dsRNA) genome of 4.1–4.6 kbp in length, consisting of two segments (with the larger being 2.4–2.7 kbp and smaller - 1.7–1.9 kbp), though a rare single, fused segment has also been documented (Delmas *et al.*, 2019). Segment 1 contains two or three open reading frames (ORFs). The function of the first ORF, which is non-obligatory, remains unknown, as does that of the second ORF-encoded peptide. The third ORF encodes the precursor of the nucleocapsid protein. Segment 2 contains a single ORF encoding the RNA-dependent RNA polymerase (RdRP).

A single genus, *Orthopicobirnavirus*, is currently recognized within the family. This genus is subdivided into five distinct genogroups, which exhibit substantial genetic diversity among themselves (Giannitti *et al.*, 2015). Genogroups I and II are predominantly associated with vertebrate hosts, while genogroup III is primarily linked to

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invertebrates (Shi *et al*., 2016). Limited information is currently available for the proposed genogroups IV and V (Knox *et al*., 2018). A proposed alternative taxonomic classification subdivides the family into 7 genera based on RdRP amino acid sequence similarity (Sadiq *et al*., 2024).

Genomic sequences related to *Picobirnaviridae* have been identified in a wide range of vertebrates, including mammals, fish, birds (such as chickens and rheas), and humans. Invertebrate-associated picobirnaviruses, in contrast, were discovered through extensive screening of invertebrate transcriptomic datasets (Shi *et al*., 2016).

Of the known picobirnaviruses, those associated with mammals, including humans, constitute the most extensively characterized group. Potential non-human mammalian hosts include horses (Giannitti *et al*., 2015), pigs (Ganesh *et al*., 2012), deer (Huaman *et al*., 2021), rodents (including marmots) (Peng *et al*., 2025), bats (Yan *et al*., 2019) and others. While these viruses are most frequently detected in fecal samples, they have also been identified in the sputum of patients with acute respiratory disease (Berg *et al*., 2021). There is some evidence linking picobirnaviruses to gastrointestinal illness in humans, particularly among immunocompromised individuals (Giordano *et al*., 1998; Legoff *et al*., 2017). However, a definitive causal relationship has never been established, supporting the hypothesis that they are opportunistic agents (Malik *et al*., 2014). This pattern extends to other mammals as well, with picobirnaviruses detected in both symptomatic and clinically healthy animals.

The inability to cultivate picobirnaviruses in animal cell culture, combined with the lack of a clear virus-disease relationship, raises fundamental questions about their true host range *(Kashnikov et al., 2023)*. Several genetic features of picobirnaviruses suggest they might infect bacteria or fungi rather than animal cells. Supporting evidence includes the presence of Shine-Dalgarno sequences upstream of open reading frames as a hallmark commonly found in bacteriophage genomes (Krishnamurthy & Wang, 2018). Furthermore, some picobirnaviruses use a non-standard genetic code, such as the fungal/invertebrate mitochondrial code (Yinda *et al*., 2018), and the vast majority of detections occur in bacteria-rich environments like the gut. Nevertheless, the definitive host of *Picobirnaviridae* remains unresolved. Confirmation will likely require either the development of a functional cultivation system complicated by the fact that many gut microbes thought to be the actual hosts are themselves unculturable (Kim *et al*., 2011), or further structural and functional studies of the viral capsid to identify potential bacterium-specific entry mechanisms.

Chiroptera are recognized as a major reservoir for diverse viruses, and due to their capacity for long-distance movement and occupation of varied biomes, their virome diversity is considerable, even within a single country (Lapshina *et al*., 2025). While numerous studies have identified diverse viruses in Vietnamese bats (Arai *et al*., 2019; Inagaki *et al*., 2020; Latinne *et al*., 2023), picobirnaviruses had remained undetected prior to this research. Of the over 7,000 *Picobirnaviridae* records currently available in public genomic databases, however, only 53 originate from bats with only one containing a capsid protein sequence longer than 400 amino acids. In this study, we report the detection of a potentially novel picobirnavirus derived from a *Pipistrellus ceylonicus* bat captured in the Kon Chu Rang Nature Reserve, Gia Lai province, Vietnam. We present the genomic sequences for both viral segments and the corresponding near-complete amino acid sequences for the RNA-dependent RNA polymerase (RdRP) and capsid protein. These sequences, identified through metagenomic sequencing, were used for subsequent molecular and phylogenetic analysis.

Materials And Methods

Sample collection and animal handling

A subadult female *P. ceylonicus* was captured on June 3, 2025, in the Kon Chu Rang Nature Reserve, Gia Lai province, Vietnam, using a mist net. Taxonomic identification was conducted following standard taxonomic guidelines (Kruskop, 2013). All animal handling

procedures were performed by trained personnel in compliance with ethical standards for wildlife research, under a protocol approved by the local ethics committee of the Joint Vietnamese-Russian Scientific Research and Technology Center (Protocol No. 1824 dated 21.05.2025-approval number 1837/CN-HDDDDV dated 21.05.2025). The fecal collection was performed by placing the bat in a sterile bag and waiting for natural excretion. The sample was harvested from the bag using sterile instruments, omitting the surface layer of the produced biosample. Immediately after collection, the fecal sample was placed in a 2.0 ml cryovial, flash-frozen in liquid nitrogen in the field and maintained under these conditions until transfer to the laboratory, where it was stored at -80 °C prior to the study.

Nucleic acids extraction

The fecal sample was homogenized by vortexing until a uniform suspension was achieved. The homogenate was then centrifuged at 17,761g, and the resulting supernatant was subjected to a second round of centrifugation to remove any remaining particulate debris. Total nucleic acids were extracted from 100 µl of the clarified supernatant using the RIBO-prep kit (AmpliTest, Russia) according to the manufacturer's instructions.

Library preparation and sequencing

The nucleic acid extract was treated with DNAse I (NEB) and the remaining RNA was purified using MagPure A4 XP magnetic beads (Magen, China) with 1.2:1 beads:sample ratio. The first strand cDNA synthesis was performed using NEBNext Ultra II RNA First Strand Synthesis Module (New England Biolabs, USA) with denaturation and random priming performed at 65 °C for 5 minutes. The second strand cDNA synthesis was performed with NEBNext Ultra II RNA Second Strand Synthesis Module (New England Biolabs, USA); the end repair was performed using NEBNext Ultra II End Repair/dA-Tailing Module. The adapter from the PCR Barcoding Expansion 1-96 (Oxford Nanopore Technologies, UK) was ligated using NEBNext Ultra II Ligation Module (New England Biolabs, USA); the sample was then purified with MagPure A4 beads with 0.3:1 beads:sample volume ratio. Barcoding PCR amplification was performed using PCR barcode primers from PCR Barcoding Expansion 1-96 (Oxford Nanopore Technologies, UK) and BioMaster LR HS-PCR 2x mix (Biolabmix, Russia) with 5 minutes elongation time. The final third generation sequencing library was afterwards prepared using Ligation Sequencing Kit V14 (Oxford Nanopore Technologies, UK) and sequenced on the R10.4.1 MINIon flow cell (Oxford Nanopore Technologies, UK) with Nanoporus sequencer (Nanoporus, Russia).

Sequencing data analysis and viral genome assembling

Basecalling was performed using Dorado v.7.2.13 in super-accurate mode, yielding approximately 2.25 million reads (1.07 gigabases) for the sample. Adapter trimming and chimera resolution were conducted with Porechop v.0.2.4 (Wick & Volkening, 2017). Reads were assembled *de novo* into contigs using Canu v.2.2 (Koren *et al.*, 2017), followed by removal of non-target sequences with Kraken2 v2.1.3 (Wood *et al.*, 2019). Taxonomic classification was optimized using a combined nucleotide and protein approach with BLASTn v2.16.0+ (Altschul *et al.*, 1990) and DIAMOND v2.1.16 (Buchfink *et al.*, 2021) to identify *Picobirnaviridae* contigs. Raw reads were then mapped back to the identified contigs, and the assemblies were polished with Pilon v1.24 (Walker *et al.*, 2014) to correct insertion, deletion, and mismatch errors.

Genome annotation and phylogenetic analysis

Genome annotation, protein translation, identification, and visualization were performed manually using a combination of SnapGene Viewer (Tello *et al.*, 2022) and the protein BLAST (pBLAST) tool *(McGinnis & Madden, 2004)*. A maximum likelihood phylogenetic tree was constructed based on an alignment of RNA-dependent RNA polymerase (RdRP) protein sequences from viruses belonging to the family *Picobirnaviridae* following an approach used in other *Picobirnaviridae* taxonomy studies (Sadiq *et al.*, 2024). The analysis was performed using RAxML-NG version 1.2.2 (Kozlov *et al.*, 2019) under the LG+I+G substitution model with 1000

bootstrap replicates. Reference sequences included formally classified taxa from the ICTV *Picobirnaviridae* report as well as proposed genera suggested by recent taxonomic evaluations (Delmas *et al.*, 2019).

Results

A subadult female of *P. ceylonicus* species was captured on June 3, 2025, in the tropical forest of Kon Chu Rang Nature Reserve, Gia Lai province, Vietnam (Figure 1).

Figure 1. **(A)** *P. ceylonicus* captured in the Kon Chu Rang Nature Reserve, Gia Lai province, Vietnam (Photo: Yuzefovich A.P.). **(B)** Undisturbed forest habitat in Kon Chu Rang Nature Reserve, Gia Lai province, Vietnam, where the *P. ceylonicus* was captured (Photo: Yuzefovich A.P.). **(C)** Geographic location of the capture site (14.474; 108.542).

Genome sequencing and assembly yielded sequences for two segments: dsRNA1 (1,902 bp, GenBank accession PZ196361) and dsRNA2 (1,626 bp, GenBank accession PZ196362). The dsRNA1 segment includes an open reading frame (ORF), encoding a protein homological to capsid proteins of other members of *Picobirnaviridae* family. The incompleteness of the obtained assembly due to the high presence of host and bacterial nucleic acids prevented the recovery of the other two ORFs (ORF1 and ORF2, both of unknown function), which are typically found in the dsRNA1 segment of *Picobirnaviridae*. The dsRNA2 segment was found to contain a single ORF encoding an RNA-dependent RNA polymerase (RdRP).

BLASTx analysis against the non-redundant protein database revealed that the predicted capsid protein is most closely related to that of the picobirnavirus isolate human/BEL/HPBV1112/2010, detected in diarrheic feces from an immunosuppressed patient with ulcerative colitis, with a pairwise identity of 39.05%. The predicted RdRP protein shows the highest similarity to the RdRP of picobirnavirus isolate Svole211_picobirna21, identified in *Neodon fuscus* feces. Detailed annotations for both proteins are provided in Table 1.

Table 1. Characteristics of identified *Pipistrellus ceylonicus*-derived picobirnavirus genomic sequences.

Protein	Obtained nucleotide sequence length	Length of the obtained protein sequence	Weight	Nucleotide accession	The closest amino acid homologue, pairwise identity
Capsid	1,902 bp	569 a.a.	62.9 kDa	PZ196361	AOW41964.1 Picobirnavirus sp. (human/BEL/HPBV1112/2010), 39.05%
RdRP	1,626 bp	515 a.a.	59.2 kDa	PZ196362	XNT21246.1 Picobirnaviridae sp. (rodent metagenome), 66.41%

Phylogenetic analysis was performed on the amino acid sequences of the RdRp. The maximum-likelihood tree was constructed using representatives of the entire *Picobirnaviridae* family (Figure 2). The maximum-likelihood tree illustrates the evolutionary relationships among representative picobirnaviruses based on their RdRP amino acid sequences. Colored backgrounds demarcate clusters corresponding to proposed genera (*Alphapicobirnavirus, Betapicobirnavirus*, etc.), with labels also indicating the officially recognized genogroups (Genogroup 1, 2, or 3), the proposed genus name, the host or isolation source, and the formal ICTV species name where applicable. The *Pipistrellus ceylonicus*-derived virus identified in this study is highlighted and its placement within Genogroup 2 and the proposed *Alphapicobirnavirus* genus is indicated. Bootstrap support values (based on 1000 replicates) are shown at major nodes.

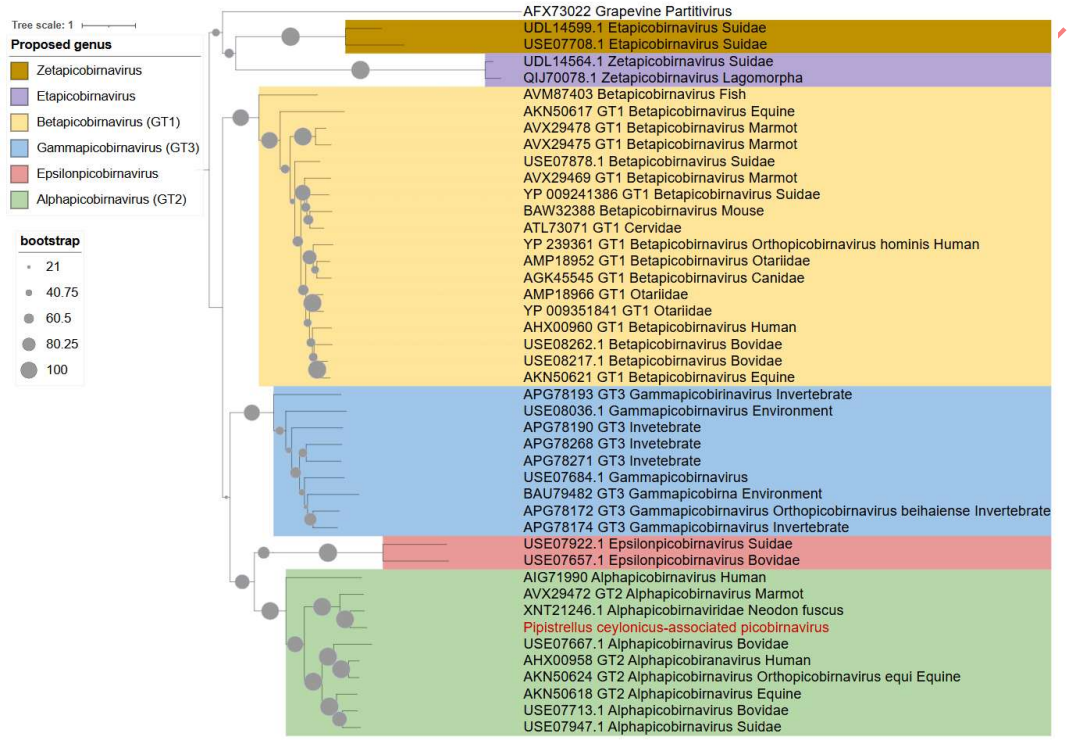

Figure 2. Phylogenetic analysis of RdRP amino acid sequences of *Pipistrellus ceylonicus*-derived picobirnavirus across the *Picobirnaviridae* family. The maximum likelihood analysis based on 803 aa alignment was performed using under the LG+I+G substitution model with 1000 bootstrap replicates Colored ranges indicate proposed genera; the sequence titles indicate proposed genus, ICTV-recognized genotype and the isolation source. The newly identified virus is highlighted in red.

The *Pipistrellus ceylonicus*-derived picobirnavirus robustly clusters within ICTV-recognized genogroup 2 and the proposed genus *Alphapicobirnavirus*. The neighboring clade include sequences identified in samples obtained from human and rodents (*Marmota himalayana* and *Neodon fuscus*). The fact that newly observed virus fits into one of the proposed genera additionly supports this proposed division.

The pairwise genetic distances (p-distance) for the capsid protein amino acid sequences are visualized in a distance matrix, which includes the amino acid sequences of the capsid proteins of representative members of *Picobirnaviridae* family, for which the capsid protein sequence is available in the public databases (Figure 3). The distances for *Pipistrellus ceylonicus*-derived picobirnavirus are shown in the first column; the titles include the recognized genotypes, the proposed genera titles (for sequences where the corresponding RdRp sequence used for genotyping was available) and the isolation sources of corresponding viruses.

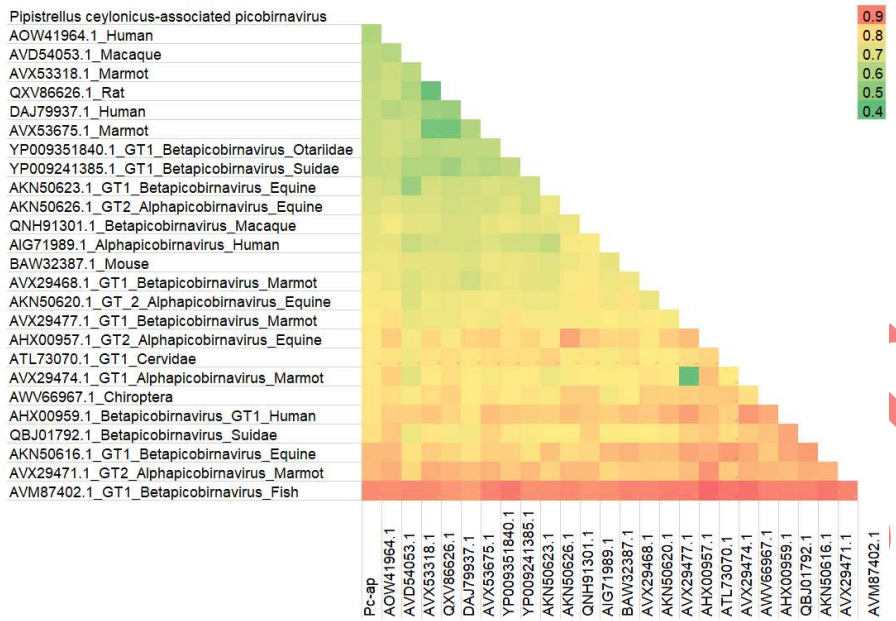

Figure 3. The pairwise distance matrix of the amino acid sequences of the capsid proteins of representative members of *Picobirnaviridae* family.

Notably, sequence diversity among capsid proteins in the *Picobirnaviridae* family is in general high and shows no correlation with either the RdRP-defined genotype or the isolation host. The *Pipistrellus ceylonicus*-derived picobirnavirus demonstrates the greatest, yet still low, capsid protein similarity to viruses isolated from a human and a macaque (genetic distances of 0.59 and 0.62, respectively). In contrast, its divergence from AWV66967, the only previously known near-complete picobirnavirus sequence from a chiropteran host, is 0.79, indicating a distant phylogenetic relationship.

Discussion

Significant gaps remain in our knowledge of the *Picobirnaviridae* family. The absence of a reliable picobirnavirus culture system restricts their detection and characterization to metagenomic sequencing (Kashnikov *et al.*, 2023). As this method has become more accessible, it has facilitated the recent discovery of novel picobirnaviruses in a diverse range of potential hosts, including wild and domestic birds (Pankovics *et al.*, 2018; Ullah *et al.*, 2022), cattle (Huaman *et al.*, 2021; Woo *et al.*, 2019), primates (Woo *et al.*, 2019), marsupials (Chong *et al.*, 2019), pigs (Chauhan *et al.*, 2022), deer (Huaman *et al.*, 2021a, 2021b), bats (Yinda *et al.*, 2018) and various other mammalian species. However, fundamental questions persist regarding its true host range, the relationship between host species and viral genetics, and its potential role in pathogenesis (Kashnikov *et al.*, 2023). The characterization of this novel picobirnavirus contributes to addressing these questions by expanding the known diversity of the family and providing new insights into both its potential host breadth and the composition of the Vietnamese bat virome.

Currently, the *Picobirnaviridae* family contains only a single recognized genus (Delmas *et al.*, 2019). However, the extreme genetic diversity within this genus (Ghosh *et al.*, 2009) and the broad range of host species from which these viruses have been isolated suggest that the current taxonomy does not accurately reflect their evolutionary relationships. A proposed classification system based on RdRP amino acid sequences defines several distinct genera (Sadiq *et al.*, 2024), which aligns well with the observed clustering of known picobirnavirus sequences. The novel virus described in this study is consistent with this proposed classification system, as it robustly clusters within the proposed *Alphapicobirnavirus* genus.

Current ICTV species demarcation criteria for

Picobirnaviridae are based primarily on host specificity (Delmas *et al.*, 2019). However, the official report notes that, given the substantial genetic distances observed, each picobirnavirus with a complete genome may eventually be classified as a distinct species. The *P. ceylonicus*-derived picobirnavirus is genetically distant from known bat-associated picobirnaviruses, while its closest relatives were isolated from different hosts (human and macaque), supporting its designation as a novel species within the family. Furthermore, its highest capsid protein similarity is to a human-derived virus rather than to other bat-associated picobirnaviruses.

While a broad correlation exists between genomic lineage and host range for some *Picobirnaviridae* representatives, such as members of the proposed *Gammapicobirnavirus* genus (Genotype 3), which are primarily found in environmental and invertebrate samples (Shi *et al.*, 2016), this link is virtually absent among more closely related vertebrate hosts. For example, betapicobirnaviruses have been detected in hosts ranging from fish to humans (Sadiq *et al.*, 2024). The genetic distances between capsid protein sequences, which are theoretically linked to host specificity, show an even weaker association with the isolation source, underscoring the complex and poorly constrained host-virus relationships within this family.

This study has several limitations, partly due to the limited knowledge available regarding the *Picobirnaviridae* family. Owing to the absence of a culture system, definitive host identification of the newly detected virus is not possible, and in-depth virological characterization is nearly unattainable. Consequently, as in most studies on *Picobirnaviridae*, the characterization of novel viral species is based solely on sequences obtained from metagenomic experiments. As previously noted, it remains possible that the virus is associated with the gut bacterial microbiota rather than the bat host itself, or alternatively, that it originates from the bat's diet (e.g., insects). However, the latter scenario appears unlikely, given that the newly identified virus is genetically related to viruses isolated from mammalian feces and distant from known invertebrate-associated picobirnaviruses. Likewise, the fecal sample collection protocol cannot completely rule out the possibility that the virus originated from the exterior surface of the animal rather than the intestinal tract. Nevertheless, these limitations are typical for animal-derived viruses that lack established cultivation systems.

Conclusion

While surveillance studies often focus on viral families with established zoonotic and pandemic potential, such as *Coronaviridae*, *Orthomyxoviridae* and *Flaviviridae*, the broader bat virome holds critical insights into the diversity of other understudied viral groups. The *Picobirnaviridae* family exemplifies this, characterized by a broad host range, substantial genetic diversity, and numerous unresolved questions regarding its ecology and evolution. Answering these questions requires expanded data on both its genetic spectrum and true host associations. The identification of a novel picobirnavirus in *P. ceylonicus* contributes to this goal by expanding the known host associations and enriching the documented viral diversity within Vietnamese bats.

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Statement on the Use of Generative AI (GenAI)*: No AI Used*

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