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229E- and NL63-like coronaviruses in phyllostomid bats, Belize

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ABSTRACT

Coronaviruses (CoVs) are a diverse group of RNA viruses that infect a broad range of hosts, including many bat species. The emergence of several CoVs, specifically α-CoVs and β-coVs causing significant human and domestic animal diseases, has been linked to bats. Characterizing the evolutionary relationships and host associations of bat-borne CoVs is crucial for understanding and mitigating zoonotic risks. While most bat CoV research has focused on the Eastern Hemisphere, the diversity of CoVs in Neotropical bats remains relatively understudied. Here, we report novel and previously identified α-CoV diversity in three phyllostomid bats (*Desmodus rotundus*, *Carollia sowelli*, and *Sturnira parvidens*) in Belize. Our analysis targeting the partial RNA-dependent RNA polymerase gene revealed varying prevalence (22.22 - 36.36 %) across these species. Our phylogenetic analysis suggests strong similarity between two Neotropical bat α-CoV lineages and human CoVs (229E and NL63), illustrating new evolutionary relationships compared to prior bat α-CoVs.

1. Introduction

Coronaviruses (CoVs), paramyxoviruses, and lyssaviruses are among the most epidemiologically relevant zoonotic viruses of bats [1]. These viruses have caused outbreaks, epidemics, and even pandemics in humans and domestic animals, emphasising the importance of understanding their diversity and prevalence in wild bats [1]. However, most CoV and paramyxovirus studies have focused on bats from the Eastern Hemisphere, especially in the tropics [2,3]. The Neotropics are undersampled for both bat viruses, despite hosting the world's most diverse bat communities [2] and showing evidence for zoonotic lineages [4]. While substantial work has focused on rabies virus, studies are largely biased towards *Desmodus rotundus* (the common vampire bat) [5].

The Neotropics host the most diverse bat communities globally, with the family Phyllostomidae representing a unique radiation of over 200 species spanning divergent ecologies [6]. This diversity offers unique scenarios for viral diversification and specialisation [7]. Earlier studies have sampled Neotropical bats from Brazil, Costa Rica, and Mexico, identifying rabies virus, α-CoVs and β-CoVs, and rubulaviruses and morbilliviruses (*Paramyxoviridae*) [8]. However, the diversity, geographic range, and host range of such viruses, and their clinical implications, remain mostly poorly understood across the Neotropics. Our study contributes to this effort by investigating the presence and diversity of these viral taxa (CoVs, paramyxoviruses, and lyssaviruses) and improving sampling efforts for this disproportionately underrepresented region.

2. Materials and methods

2.1. Wild bat sampling

In April 2019, we conducted preliminary viral surveillance among three phyllostomid bat species in Belize: *Desmodus rotundus* ($n = 27$), *Carollia sowelli* (Sowell's short-tailed bat; $n = 10$), and *Sturnira parvidens* (little yellow-shouldered bat; $n = 11$). *D. rotundus* is broadly distributed across the Neotropics, whereas the latter species are restricted to Central America (Fig. 1A). We focused on these species based on prior reports of all three viral taxa in *D. rotundus* and from the genera *Carollia* and *Sturnira* [8]. Over 12 nights (6–10 PM), we captured bats in the Lamanai Archeological Reserve, Orange Walk District. We used sterile miniature

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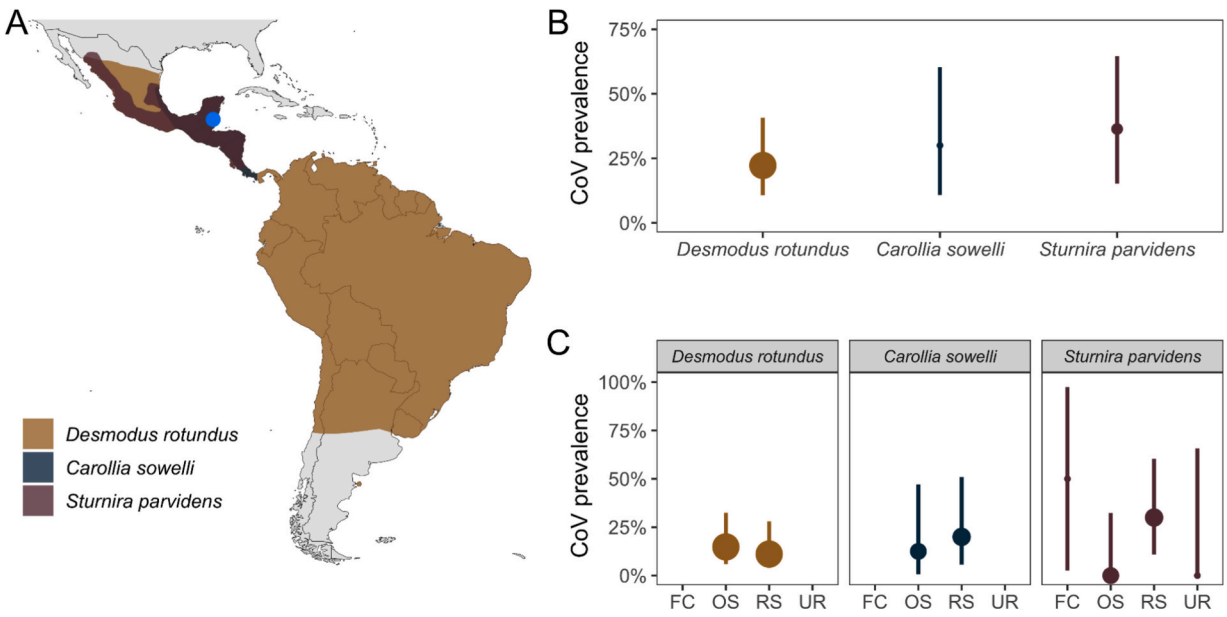

Fig. 1. A) Map showing the sampling location in Belize (blue point) with the distribution of *Desmodus rotundus*, *Sturnira parvidens*, and *Carollia sowelli.* The dark reddish-brown areas indicate regions where all three species distributions overlap B) Species-level CoV prevalence based on positivity in at least one sample type tested per individual. C) CoV prevalence by sample type: feces (FC), oral swabs (OS), rectal swabs (RS), and urine (UR). FC and UR were only collected from *S. parvidens*. Point estimates of CoV prevalence are scaled by sample size. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Sample size across the analyzed three bat species and four sample types.

	Saliva (OS)	Rectal swab (RS)	Feces (FC)	Urine (UR)
Desmodus rotundus ($n = 27$)	27	27	–	–
Carollia sowelli ($n = 10$)	8	10	–	–
Sturnira parvidens ($n = 11$)	8	10	2	2
Total ($n = 48$)	43	47	2	2

rayon swabs (1.98 mm, Puritan) and sterile cotton swabs (4.78 mm, Puritan) to collect saliva (OS) and rectal (RS) samples in 1 mL DNA/RNA Shield (Zymo Inc) (Table 1). We also collected paired swabs in viral transport medium (VTM; Rocky Mountain Biologicals) to attempt viral isolation. We opportunistically collected feces and urine in 1 mL DNA/RNA Shield. Samples were preserved in a cryoshipper prior to storage at −80 °C. All bats were released after processing. Sampling was approved by the Institutional Animal Care and Use Committee of the American Museum of Natural History (AMNHIACUC-20190129) and Belize Forest Department (FD/WL/1/19(06), FD/WL/1/19(09)).

2.2. Viral RNA detection

We extracted RNA from samples in DNA/RNA Shield using the *Quick*-RNA Viral Kit (Zymo Research). RNA was converted into cDNA using an iScript Synthesis Kit (BioRad), used as the template in three semi-nested PCRs for viral diagnostics. For CoVs, we targeted a 216-bp fragment of the RNA-dependent RNA polymerase (RdRp) gene [9]. We targeted a 200–500 bp fragment of the L gene [10] and a 164-bp fragment of the N gene [11] for paramyxoviruses and lyssaviruses, respectively. All PCRs included negative controls. Amplicons were Sanger sequenced and aligned using Geneious 2025.1.2, followed by analysis using NCBI BLAST and deposition in GenBank (PV197952–61). A subset ($n = 4$) of *D. rotundus* CoV sequences were published previously (OM240577–80) [12].

2.3. Viral isolation

For PCR-positive samples, we attempted viral isolation by inoculating eluates from paired VTM samples onto monolayers of Vero E6 cells (ATCC CRL-1586) and primary kidney, brain, lung, liver, and spleen primary cell cultures derived from *Carollia perspicillata* [13]. Cultures were maintained in DMEM (Gibco) supplemented with 2 % fetal bovine serum (Gibco), 1× sodium pyruvate (Gibco), and 1× antibiotic-antimycotic (Gibco) at 37 °C with 5 % CO_2. Cells were examined microscopically every 2–3 days for 10 days to detect viral infection–induced cytopathic effects. After 10 days, cell culture media were passaged to fresh cell monolayers and incubated for another 10 days. Media from each culture were then analyzed by PCR using the above protocols for CoVs. Viral culture was performed under BSL-3 conditions at Indiana University (Institutional Biosafety Committee protocol IN-1348).

2.4. Statistical and phylogenetic analyses

We fit a generalized linear model with binary response and small sample size bias correction in R with the *brglm2* package to assess the effects of species, sample type (OS or RS) and their interaction on the probability of infection. We then used a likelihood ratio test to evaluate the significance of each predictor and their interaction term.

We aligned our CoV RdRp sequences, along with other bat and human CoVs, using MUSCLE in Geneious 2025.1.2, and constructed a maximum-likelihood phylogeny using PhyML in NGPhylogeny.fr [14].

3. Results and discussion

We detected CoVs in all three species with varying prevalence (Fig. 1B): *D. rotundus* (22.22 %, 95 % confidence interval

[CI]:10.6–40.75 %), *C. sowelli* (30 %, 95 % CI: 10.77–60.32 %) and *S. parvidens* (36.36 %, 95 % CI: 15.16–64.61 %). We also observed variable prevalence between sample types within species (Fig. 1C). In *D. rotundus,* OS had higher prevalence (14.81 %, 95 % CI: 5.91–32.47 %) compared to RS (11.11 %, 95 % CI: 3.85–28.05 %). In *C. sowelli,* RS had higher prevalence (20 %, 95 % CI: 5.66–50.98 %) compared to OS (12.5 %, 95 % CI: 0.64–47.08 %). For *S. parvidens*, we had urine and feces as additional samples, and feces showed higher prevalence (50 %, 95 % CI: 2.56–97.43 %) than RS (30 %, 95 % CI: 10.77–60.32 %); no OS ($n = 8$) or urine ($n = 2$) samples were positive. However, the likelihood ratio test revealed no significant effect of species ($\chi^2 = 0.20$, $P = 0.90$), sample type ($\chi^2 = 2.36$, $P = 0.49$), or their interaction ($\chi^2 = 1.96$, $P = 0.37$) on CoV infection status.

We did not detect viral infection–induced cytopathic effects or CoV RdRp amplicons in any of the cultured samples during a 20-day period. Similarly, no samples were positive for paramyxoviruses or lyssaviruses (and hence no culture was attempted), which may be attributed to the very low prevalence of these viruses among phyllostomid bats (e.g., [15]) and the limited sample sizes we had, particularly for *C. sowelli* and *S. parvidens*. Moreover, our data are based on a single sampling event at one site, which likely further limited our ability to detect these viruses. These considerations highlight the importance of a sampling strategy with both temporal and spatial replication to improve viral detection and characterization [3].

Our analysis revealed three genetically distinct α-CoV lineages circulating in phyllostomid bats in Belize (Fig. 2). The first lineage appears to be novel and consists of ten closely related monophyletic sequences from *D. rotundus*, *C. sowelli*, and *S. parvidens*, suggesting strong local-scale circulation and host switching. These sequences form a sister group to α-CoV sequences previously reported from other phyllostomid bats in Brazil (e.g., OM265179–80), indicating that this viral lineage is unique to the region but shares deeper evolutionary history with related viruses elsewhere in the Neotropics [16]. Notably, the other two lineages show close phylogenetic similarity to NL63 and 229E, two human coronaviruses (HCoV) associated with respiratory tract infections [17]. Specifically, the second lineage, previously identified and consisting of a single sequence from *D. rotundus* (OM240577) [12], is phylogenetically similar to multiple NL63 sequences (e.g., MT118694), with a NL63-like bat CoV from Kenya forming a basal clade. The third lineage included sequences previously identified from two *D. rotundus* (i.e., OM240580,

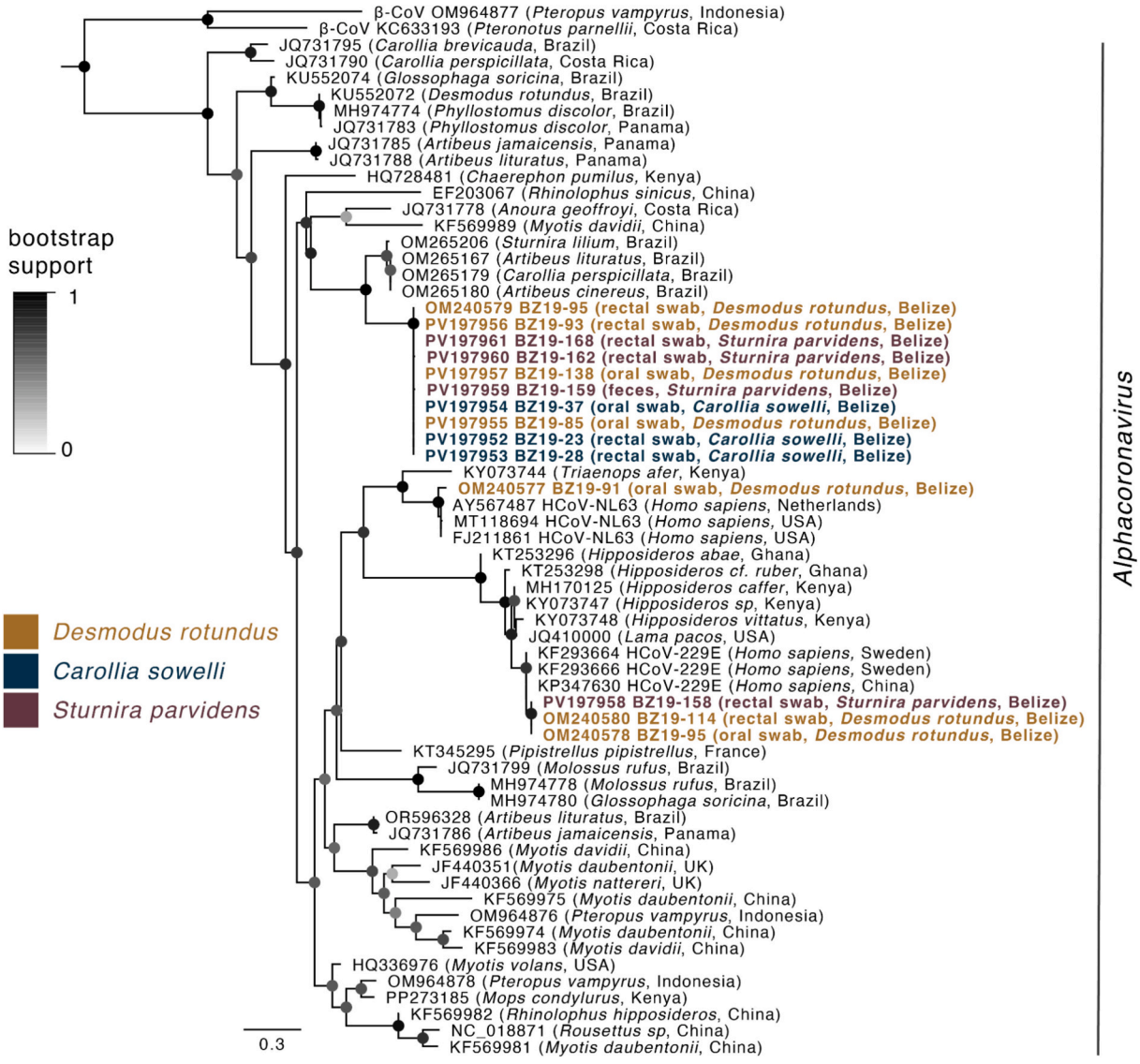

Fig. 2. Maximum likelihood phylogeny displaying the evolutionary relationships among bat α-CoVs, HCoVs 229E and NL63, and the alpaca α-CoV using partial RdRp sequences. GTR + G nucleotide substitution model with 1000 bootstrap replicates was used to infer tree topology and assess node support. The phylogeny was rooted with two bat β-CoV sequences. Sequences generated from Belize bats here are highlighted. Nodes are colored by bootstrap support (values <50 % are not shown).

OM240578) [12] and one newly identified from *S. parvidens* (PV197958), both exhibiting close phylogenetic similarity to 229E (e.g., KP347630). All other 229E-like bat CoVs formed a basal clade to this group, corroborating the known evolutionary ancestry of 229E to Afrotropical insectivorous bats [18]. To provide additional evolutionary context, we included the alpaca α-CoV (JQ410000) in our analysis, as it is known to be closely related to 229E [19]. This virus is clustered with bat CoV sequences from the Afrotropics but not with the Neotropical bat 229E-like CoVs we identified.

Our inferences, based on partial RdRp sequences, do not confirm the evolutionary origin and pathogenicity of these viruses. Serological surveys targeting sympatric species, including humans, are necessary to determine both prior exposure and cross-species transmission of these viruses. However, existing evidence of CoV evolution through host switching among both sympatric bats and other animals raises concern and highlights the need for continued surveillance [18,20], including whole-genome sequencing and receptor characterization, to better assess their spillover potential.

4. Conclusions

Our study provides significant insights into the diversity and circulation of CoVs in the Neotropics, identifying three distinct α-CoV lineages in phyllostomid bats from Belize. The detection of these lineages across multiple species suggests a broader host range and ecological generalism of these viruses and their propensity to be found in other phyllostomid bats in the region. Notably, the similarity of these bat α-CoV sequences to HCoVs compared to Afrotropical bat sequences poses several questions, including the directionality of spillover (bats to humans or humans to bats), pathogenicity of these viruses, and the risk to both bats and humans. Addressing these questions will require additional surveillance efforts targeting a broad range of hosts across the Neotropics to better understand viral evolution and the ecological context of spillover events. Our work and such future directions align with a more general need for One Health approaches to better understand the dynamics and risks of bat viruses [21].

CRediT authorship contribution statement

B.R. Ansil: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Guang-Sheng Lei:** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Aaron J. Santos:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Michael Letko:** Methodology, Resources, Writing – review & editing. **Stephanie N. Seifert:** Methodology, Resources, Writing – review & editing. **M. Brock Fenton:** Methodology, Investigation, Project administration, Writing – review & editing. **Nancy B. Simmons:** Methodology, Investigation, Project administration, Writing – review & editing. **Ryan F. Relich:** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Daniel J. Becker:** Conceptualization, Methodology, Investigation, Supervision, Project administration, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors report no competing interests.

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Data availability

All RdRp sequence data are available on GenBank under accessions OM240577–80 and PV197952–61.

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