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Opportunistic untargeted sequencing can complement targeted viral surveillance workflows for unresolved respiratory illness in Uganda

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Abstract

Routine respiratory surveillance in low- and middle-income countries relies heavily on targeted molecular diagnostics for influenza and SARS-CoV-2, leaving a substantial proportion of influenza-like illness (ILI) cases etiologically unresolved. To address this diagnostic blind spot, we evaluated the utility of opportunistic, untargeted shotgun next-generation sequencing of viral RNA on a small cluster of four unresolved clinical samples collected in Uganda in December 2025 that had tested negative via routine RT-qPCR. This opportunistic sequencing approach successfully identified clinically relevant viral pathogens in two of the four cases. From one sample, a near-complete genome of Human Metapneumovirus was recovered (13,273 bp, 45.6× coverage). This genome belonged to the globally circulating B2 lineage and revealed greater sequence divergence in the attachment G gene relative to the rest of the genome. The second sample yielded a 368 bp partial fragment of Human Respirovirus 1. These findings demonstrate that the opportunistic application of untargeted sequencing to diagnostically unresolved ILI cases can effectively complement targeted viral workflows, offering a robust, periodic tool to uncover missed pathogens in respiratory viral surveillance.

1. Introduction

Acute upper respiratory tract infections (URTIs) are the most common infectious disease cause for clinical presentations worldwide[1] affecting productivity, driving unnecessary antibiotic prescriptions and causing a huge economic burden[2]. A substantial proportion of URTI cases go without identification of the etiological agent after routine diagnostic testing[3–5].In many clinical and surveillance settings, diagnostic algorithms for Influenza-like illness (ILI) prioritize a narrow set of pathogens—most commonly influenza viruses and, more recently, SARS-CoV-2[6–8]. While such targeted testing is efficient and cost-effective, it inherently limits pathogen detection and leaves a residual burden of unexplained respiratory disease.

As a result, respiratory viruses with established clinical relevance may remain undetected, especially when case management or surveillance activities rely exclusively on targeted molecular assays. Untargeted metagenomic next-generation sequencing (mNGS) offers a complementary approach by enabling the detection of a broad spectrum of viral genomes directly from clinical samples without prior assumptions regarding pathogen identity[9,10]. Although not intended for routine diagnostics, periodic or opportunistic application of mNGS has been shown to uncover viral agents missed by conventional testing and to provide genomic data from infections that would otherwise remain uncharacterized[11,12].

In low- and middle-income countries, including those in sub-Saharan Africa, the diagnostic challenges are amplified by limited access to expanded diagnostic panels[13]. In Uganda, respiratory virus surveillance has largely been embedded within influenza sentinel systems, with broader respiratory pathogen testing performed mainly in research settings[8,14]. Such research has reported that a notable

fraction of ILI cases is attributable to non-influenza viruses, highlighting persistent diagnostic blind spots in routine respiratory surveillance[3,15].

However, untargeted genomic sequencing is rarely applied outside outbreak investigations or structured research studies. Here, we report the results of an opportunistic metagenomic analysis of nasopharyngeal samples collected from four (4) individuals presenting with flu-like symptoms who tested negative for influenza and SARS-CoV-2. This work was not designed as a systematic surveillance study but rather an exploratory application of untargeted sequencing, intended to demonstrate the value of periodically applying such approaches to unresolved respiratory infections on a cluster of individuals with flu-like illness who tested negative for influenza and SARS-CoV-2 (figure F1).

2. Results

Four (4) participants were swabbed and tested for Influenza A and SARS-CoV-2 by real-time reverse transcription polymerase chain reaction (RT-qPCR). They presented with symptoms described in Table T1 below. All four were residents of Wakiso District in Uganda and adults aged between 31 and 53 years, with three (3) males and one (1) female.

All four samples tested negative for influenza and SARS-CoV-2 by RT-qPCR, but two of them yielded respiratory viral sequence reads by shotgun metagenomic sequencing. Of these two samples, 1 revealed human metapneumovirus (hMPv) and 1 revealed human Respirovirus 1 (Table 1). Beyond these viral signals, all four samples yielded bacterial reads consistent with typical upper respiratory tract microbiota, predominantly Prevotella melaninogenica and Corynebacterium propinquum, alongside low-abundance environmental taxa common in metagenomic datasets (such as Phyllobacterium and Methylobacterium). No reads corresponding to those taxa were detected in the negative controls.

Table T1
Clinical characteristics and metagenomic findings of the participants

1	**Age | Sex**	**Clinical presentation**	**Influenza / SARS-CoV2 RT-qPCR**	**Shotgun mNGS (Relative abundance after host filtering | no. of reads)**
	36 | M	**Fever, headache, sore throat, cough, chest pain, rhinorrhea, dyspnea, vomiting, reduced appetite, weakness**	**Negative**	**Human Metapneumovirus (1.08% | 717)**
2	53 | M	Sore throat, cough, rhinorrhea, headache, chest pain, weakness	Negative	No significant respiratory viral signal
3	31 | F	Fever, headache, sore throat, cough, chest pain, rhinorrhea, myalgia/arthralgia, weakness	Negative	Human Respirovirus 1 (2.7% | 18)
4	50 | M	Fever, headache, sore throat, dry cough, chills, myalgia/arthralgia, reduced appetite, weakness	Negative	No significant respiratory viral signal

Note: *No chronic medical illness was reported by any participant.*

2.1. The human Metapneumovirus (hMPv) case

The hMPv case presented with the broadest range of reported symptoms and yielded the highest number of sequence reads on metagenomics sequencing. De novo assembly yielded a non-fragmented and unambiguous dominant contig corresponding to the near-complete hMPv genome (13,273 bp) with a coverage of 45.6× (supplementary figure S1). This has been deposited in the NCBI GenBank database under the accession number PZ228342.

Phylogenetic analysis based on complete whole genome sequences placed the hMPv genome within the B2 lineage where it clustered with previously reported B2 strains from other geographic regions and showed no atypical divergence (figure F2). A BLASTn analysis of the sequence against the NCBI nucleotide database returned closely related hMPV B2 genomes, including sequences from China and the USA, with nucleotide identities exceeding 99%, query covers of 100% and e-values of zero (0).

The G gene exhibited slightly lower sequence conservation relative to the full genome. When compared to both the RefSeq sequence (NC_039199.1), the nucleotide identity was 65.1% for the G gene vs 81.1% for the entire genome while comparison with its closest GenBank sequence, (PV218031.1), showed a nucleotide identity of 96.7% for the gene vs the 99.3% seen genome-wide. This reduced even further to 56.5% for the gene and 92.5% for entire genome, when comparing amino acid sequences. All coding regions of the recovered hMPv genome encoded full-length open reading frames without premature stop codons or frameshifts, and the predicted protein lengths were comparable to closely related B2 reference strains.

2.2. The Human respirovirus 1 / Human Parainfluenza Virus 1 (HPIV1) case

The HPIV1 case, had a limited number of reads assigned to it. De novo assembly recovered a 368 bp contig representing approximately 2% of the genome, at low assembly coverage of 0.87×. BLASTn showed a 98.64% nucleotide identity to a GenBank HPIV1 sequence (MH828705.1) with 100% query cover and zero (0) e-value.

To further assess the taxonomic assignment, reads mapping to the HPIV1 RefSeq genome (NC_003461.1) were examined individually, and their closest BLASTn matches consistently corresponded to HPIV1 sequences. The recovered 368 bp fragment mapped to the F gene of the virus and has been deposited in the NCBI GenBank database under the accession number PZ210030. However, the limited read count, low coverage and short fragment length prevented recovery of a complete genome and limited further genomic characterization.

3. Discussion

Acute respiratory infections remain a leading cause of morbidity globally, but a substantial proportion of cases remain etiologically unresolved following routine diagnostic testing, especially since most diagnostic panels are restricted to a limited number of pathogens, commonly Influenza and SARS-CoV-2[28–30]. This diagnostic gap continues to be reported in both high and low resource settings, where so many ILI cases cannot be attributed to commonly tested viruses[15,31,32]. In this work, the opportunistic application of shotgun metagenomic sequencing to Influenza and SARS-CoV2 negative samples enabled the identification of other clinically relevant respiratory viruses, highlighting the importance of untargeted approaches in resolving unexplained respiratory infections.

No respiratory virus was found in two (2) of the four (4) samples, but this does not necessarily indicate absence of infection. Rather, this may have reflected a non-viral respiratory pathogen or low viral loads for example during early or resolving stages of infection, which can fall below the detection threshold of shotgun metagenomics, a method which has a lower sensitivity compared to targeted molecular assays[33,34]. This limitation is inherent in untargeted sequencing approaches and should be noted when interpreting a negative result.

Human metapneumovirus (hMPv) was found in one (1) of the four (4) samples and a near-complete genome of the virus was recovered. hMPv is a globally distributed respiratory pathogen first described in 2001 and is now recognized as a significant cause of upper and lower respiratory tract disease[35,36]. Despite its clinical relevance, it is not routinely included in many diagnostic workflows, especially in low and middle income countries during routine surveillance[37]. Moreover, to the best of our knowledge, this is only the second near-complete hMPV genome from Uganda available in a public database, following a sequence reported by our laboratory in 2024[15] (GenBank accession: PQ226465.1). The hMPv genome in

this work exhibited high coverage and was assembled as a single dominant contig which is evidence of a well-supported viral signal[34].

Phylogenetic analysis placed the hMPv sequence within the B2 lineage, a major lineage circulating globally. This is in line with previous reports of widespread circulation of genetically similar strains across diverse regions[38,39]. The integrity of the recovered genome was further supported by the observation that all coding regions retained intact open reading frames, and predicted protein lengths were comparable to reference sequences. The G gene exhibited reduced sequence conservation relative to the rest of the genome, a feature well documented for paramyxoviruses since their surface glycoproteins are highly variabile[40,41].

In contrast, the detection of human respirovirus 1 (HPIV1) was supported by a limited number of reads and a short genomic assembly. HPIV1 also known as human parainfluenza virus 1 is also a recognized cause of respiratory disease globally but is not part of routine molecular diagnostic panels in surveillance[42,43]. While the recovered fragment showed high nucleotide identity to reference HPIV1 sequences and similar taxonomic assignments at both contig and read levels, the limited coverage and short fragment length prevented comprehensive genomic characterization. This limited recovery likely stems from a low viral load or RNA degradation and highlights a limitation of untargeted sequencing in detecting low-titer infections[34,44].

Despite using a viral RNA extraction protocol, some bacterial taxa such as Prevotella melaninogenica and Corynebacterium propinquum, consistent with known upper respiratory tract microbiota[45], were detected across the samples. The presence of these low-abundance taxa also reflects background signals common in metagenomic datasets, including potential contributions from laboratory environments[46]. This reinforces the importance of distinguishing clinically relevant pathogens from background microbial signals in untargeted sequencing.

This work demonstrates that even limited and exploratory metagenomic analyses performed opportunistically can yield meaningful insights into respiratory virus diversity in routine clinical samples especially in regions where the full spectrum of circulating respiratory viruses remains incompletely characterized[3,47]. This work however, had some limitations including a small sample size and an opportunistic design that prevented inference as regards prevalence and epidemiological trends. In addition, the absence of virus-specific RT-PCR validation limited confirmation of low-abundance HPIV1 detection.

4. Conclusion

This work attests that opportunistic application of shotgun metagenomic sequencing to diagnostically unresolved flu-like illness cases can reveal clinically relevant respiratory viruses that may not be detected by routine testing. The recovery and genomic characterization of a near-complete human metapneumovirus genome highlights the robustness of this approach, while the detection of a low-

abundance human respirovirus 1 signal speaks to both its sensitivity and its limitations in low-titer infections. These findings underscore the potential value of periodic untargeted sequencing as a complementary tool to targeted diagnostics, especially in settings where the full spectrum of circulating respiratory pathogens is not completely characterized.

5. Methods

5.1. Study Population and Sample Collection

Nasopharyngeal swabs were collected from individuals presenting with flu-like symptoms in December 2025. Swabs were placed in Universal Transport Medium (UTM) and stored at − 80°C until processing. Baseline demographic and clinical characteristics of the enrolled participants were documented at the time of collection.

5.2. Nucleic Acid Extraction, RT-qPCR, Library Preparation and Sequencing

Viral RNA extraction using QIAamp Mini Viral RNA Extraction Kit from Qiagen (Halde man, Germany) and RT-qPCR testing using CDC's Influenza and SARS-CoV-2 Multiplex Assay were performed following the CDC protocol as previously described[8]. The RNA was then used to prepare DNA libraries for shotgun metagenomics sequencing with the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Inc., USA) following the manufacturer's protocol. The libraries were quantified and pooled for sequencing on the Illumina MiSeq platform using the v3 300-cycle kit, generating 2×150 bp paired-end reads. Negative controls were included during nucleic acid extraction and were processed alongside the clinical samples through the library preparation, sequencing and bioinformatic analysis.

5.3. Bioinformatics Analysis

Raw sequencing reads were quality trimmed (Q30) using trim galore v0.6.10[16] and the quality metrics were assessed using FastQC v0.12.1[17] and MultiQC v1.33[18]. Host-derived sequences were removed by aligning all reads to the human reference genome (hg38) using bowtie2 v2.5.4[19], and retaining only non-aligned reads. Taxonomic classification of filtered reads was performed using Kraken2 v2.1.3[20] against a standard reference database (https://benlangmead.github.io/aws-indexes/k2 accessed on 5 January 2026) to identify microbial taxa present in each sample.

For viruses detected at notable read abundances (> 5 reads), pathogen confirmation was done using reference-based read mapping against their respective RefSeq reference genomes with bwa-mem2 v2.2.1[21]. From the alignment files, the number of aligned reads plus depth were assessed with samtools v1.18 [22] and the consensus genomes were called using sam2consensus v3 (https://github.com/vbsreenu/Sam2Consensus) - min. depth of 5.

In parallel, de novo assembly of non-human reads was performed using SPAdes v3.15[23] and the resulting contigs were queried against the NCBI nucleotide database using blastn v2.14.1[24] to confirm taxonomic assignments and evaluate sequence similarity. Multiple Sequence Alignment was performed for the longest viral contig with other closely related GenBank and RefSeq sequences using Mafft v7.526[25] (1000 iterations). Maximum likelihood phylogenetic tree construction was done using IQ-TREE v3.0.1[26] (1000 bootstraps) with the best fit substitution model chosen based on the Bayesian Information Criterion. The tree was then edited in itol v7[27]

5.4. Ethical Considerations

The data was collected and reported through the Health Management Information System for routine influenza surveillance as a public health activity for early detection and reporting under the Uganda Ministry of Health and therefore did not require formal ethical review. Data was anonymized upon collection, and authors did not have access to identifying information.

Declarations

Grant Information

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Competing Interests:

The authors declare that they have no competing interests.

Author Contribution

Conceptualization, F.Y.; writing – original draft preparation, F.Y, J.N and J.T.K.; methodology and investigation, F.Y, CAN, J.N, N.J, B.N, E.A and J.T.K.; data analysis, F.Y.; data curation, N.O.; supervision, project administration, and funding acquisition, J.N, J.K, J.T.K and J.J.L. All authors read and agreed to this final version of the manuscript.

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Data Availability

The sequence data generated by this work is available in GenBank

Supplementary Materials

Supplementary figure S1: De novo assembly graph showing recovery of the hMPV genome.

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Figures

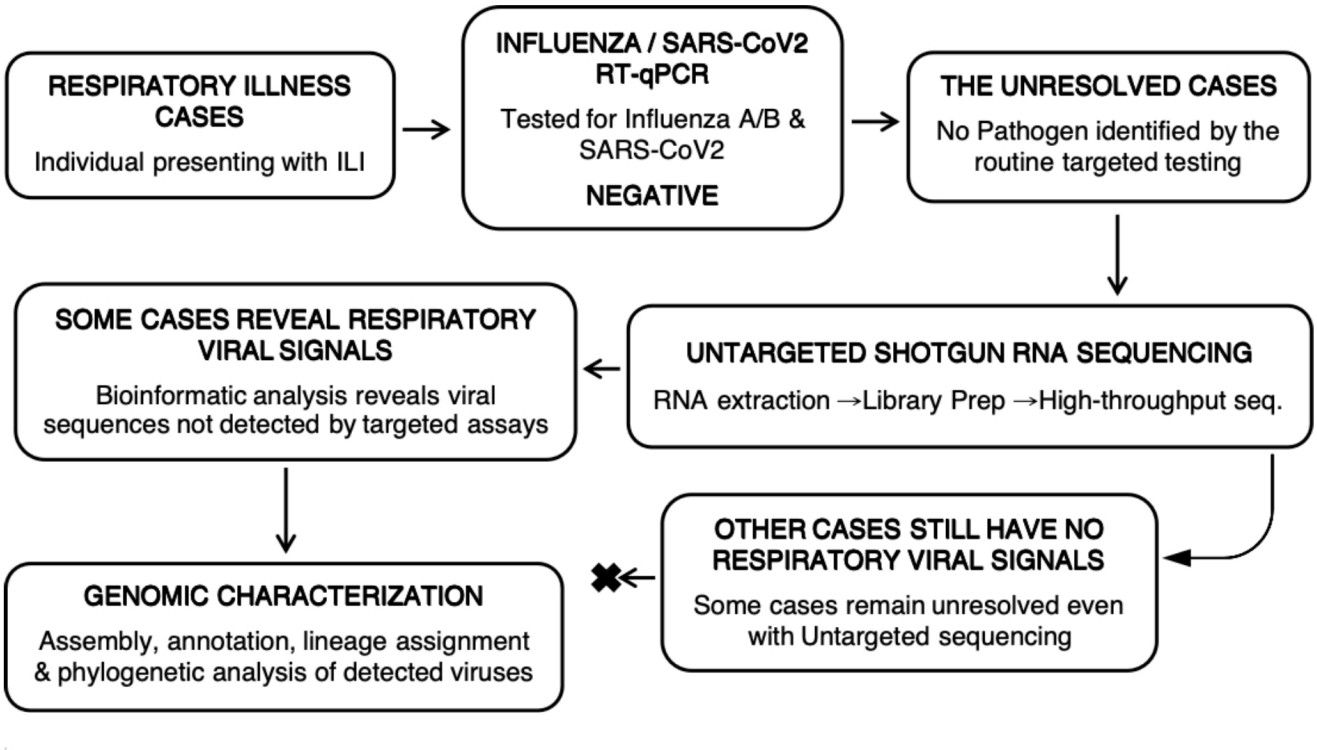


Figure 1

Conceptual framework of applying untargeted shotgun RNA sequencing to investigate unresolved ILI cases. Respiratory illness cases that test negative for influenza and SARS-CoV-2 by routine RT-qPCR remain diagnostically unresolved. Untargeted shotgun RNA sequencing can be applied to such cases to identify respiratory viral signals that may not be captured by targeted assays, followed by genomic characterization of the detected viruses.

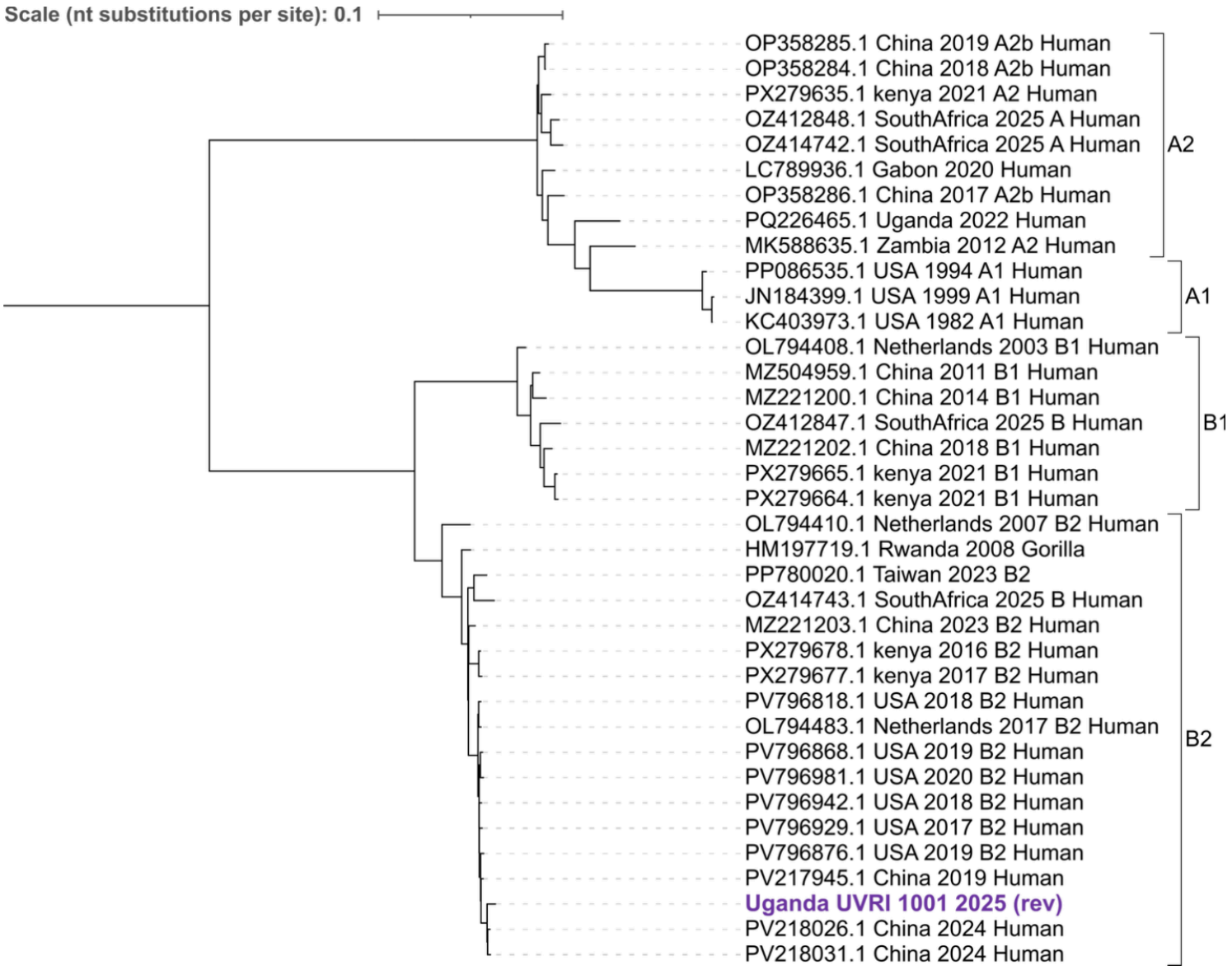


Figure 2

Phylogenetic placement of the recovered hMPV genome. Maximum-likelihood phylogenetic tree of representative hMPV genomes constructed from full-genome sequences and rooted at the midpoint. The genome generated in this study (Uganda UVRI 1001 2025; highlighted in purple) clusters within the hMPV B2 lineage. Reference sequences were selected to represent major hMPV lineages and diverse geographic regions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryfigureS1.pdf