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Targeted Metagenomics Reveals Hidden Chickenpox Epidemic Amid Mpox Surveillance in Uganda

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Abstract

In regions where both monkeypox virus (MPXV) and varicella zoster virus (VZV) are co-circulating, overlapping clinical manifestations can complicate clinical diagnosis. During the MPXV outbreak declared in Uganda in July, 2024, symptomatic suspected cases tested PCR negative for Mpox. To determine the cause of symptoms, we employed metagenomic sequencing with a targeted Viral Surveillance panel in 284 MPXV negative samples. VZV was identified as the predominant pathogen in 86% of MPXV-negative cases, suggesting a concurrent chickenpox surge. Using the VaricellaGen pipeline for variant calling, clade typing, and phylogeny, 118 (49%) samples that achieved ≥70% genome coverage were of clade 5 based on the single-nucleotide polymorphism (SNP) dataset. This data confirms co-circulation of VZV during the MPXV outbreak in Uganda. Our results underscore the need for laboratory confirmation of MPXV and the inclusion of VZV in the testing algorithm during the Mpox outbreak.

Keywords: Mpox, Varicella Zoster Virus (VZV), Misdiagnosis, Metagenomic Sequencing, VaricellaGen, Clade 5, Uganda

Introduction

Mpox is an infection caused by the monkeypox virus (MPXV), a member of the *Orthopoxvirus* genus [1]. Mpox presents with a wide range of clinical symptoms such as fever, lymphadenopathy, and vesiculopustular lesions, similar to other exanthematous infections, particularly chickenpox, and has been reported in previous Mpox outbreaks [2]–[5]. While MPXV can be zoonotic, VZV is predominantly human-to-human transmission with no known animal reservoir [6]. The clinical overlap between these infections makes rapid and accurate diagnosis essential for effective outbreak response and management.

VZV is a common alphaherpesvirus with a double-stranded DNA genome known to cause primarily varicella (chickenpox) in children and adolescents and herpes zoster in older and or immunocompromised persons [7], [8]. VZV is highly contagious and is airborne with a genome of approximately 125 kb containing 71 open reading frames (ORFs) [9], [10]. Currently, nine VZV clades are recognized: seven established clades (1-6 and 9) and two provisional clades (VII and VIII) [11]. In Mpox-endemic regions, especially in low- and middle-income countries with low chickenpox vaccination coverage, chickenpox remains commonly reported [2]–[5].

Conventional laboratory methods for differentiating MPXV from VZV rely on polymerase chain reaction (PCR)-based assays, which are highly specific but limited to detecting only known targets. [12]. In outbreak settings, however, an overreliance on PCR may result in undetected alternative pathogens when cases test negative for the suspected virus [13]. For instance, a study during the 2014-2015 Ebola outbreak in Conakry, Guinea, found that among 2,362 suspected Ebola cases, 1,540 (65.2%) tested negative by PCR. Notably, 98 of these PCR-negative patients died, highlighting the potential presence of other severe illnesses that were initially overlooked due to the focus on Ebola [14]. In regions where both MPXV and VZV are co-circulating, overlapping

clinical manifestations can complicate differential diagnosis, with MPXV frequently mistaken for VZV and vice versa [2], [12]. Recently, a study from Brazil deployed a multiplex PCR assay to diagnose co-infection of MPXV and VZV concurrently, but this has been limited in outbreak settings in Africa [4]. These findings underscore the need to consider alternative diagnoses and adopt comprehensive diagnostic strategies during outbreaks to avoid overlooking other clinically significant pathogens. Metagenomic sequencing offers an unbiased and powerful approach to pathogen detection, enabling the identification of viral agents beyond the initially suspected cause [15].

Between September and December 2024, only 52% cases tested positive for MPXV despite presentation with symptoms. In order to understand other causes of symptoms among MPXV negatives, we utilized metagenomic sequencing.

Methods

Ethical consideration

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. All study procedures involving human participants were performed as part of the national Mpox outbreak response. Because samples were collected for public health surveillance during an active outbreak, obtaining individual informed consent was not feasible. Consequently, a waiver of informed consent was sought and approved by the Uganda National Health Laboratory Services Research and Ethics Committee (UNHLS-2025-133).

Sample collection and PCR testing

During the MPXV outbreak declared in Uganda in July, 2024, 284 symptomatic suspected cases tested PCR negative for Mpox between

September and December 2024 were included in this study. Suspected Mpox cases were identified in accordance with the World Health Organization (WHO) outbreak case definition. Briefly, suspected cases included individuals presenting with an unexplained acute skin rash, mucosal lesions, and/or lymphadenopathy, with or without systemic symptoms such as fever, headache, myalgia, back pain, or profound weakness, or individuals with epidemiological linkage to a probable or confirmed Mpox case within 21 days prior to symptom onset. Consistent with WHO guidance, common causes of acute rash illness such as varicella (chickenpox), herpes zoster, and measles were considered during clinical assessment but did not preclude MPXV testing, given documented clinical overlap and the possibility of co-infections [16]. The samples included lesion, rash, oropharyngeal, or genital swabs in Viral Transport Media (VTM), as well as crusts collected in plain tubes - obtained as part of the public health emergency response to Mpox from affected individuals across 27 districts in the country. DNA was extracted using QIAamp DNA Kit (Qiagen, Hilden, Germany) and subsequently subjected to the Non-Variola Orthopoxvirus Real-Time PCR Primer and Probe Set (CDC, EUA) [17] in-house assay at the Central Emergency Response and Surveillance Laboratory (CERSL), Ministry of Health, Uganda. Briefly, amplification was conducted on the CFX96 Connect Real-Time Detection System (Bio-Rad Laboratories, Inc., California, USA), with denaturation at 95 °C for 8 minutes, followed by 40 cycles, each consisting of two main steps i.e., a denaturation step at 95 °C for 5 seconds and an annealing and extension step at 60 °C for 30 seconds. PCR results were analyzed using CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc., California, USA) for Ct value interpretation and quality control.

Metagenomic sequencing and taxonomic classification

High-quality DNA was extracted using the QIAamp DNA Kit (Qiagen, Hilden, Germany), normalized to a starting concentration of 50 ng, and prepared for metagenomic sequencing using a target enrichment approach with a pre-

designed Illumina Viral Surveillance Panel (VSP). Paired-end sequencing was performed on the Illumina MiSeq platform at the Genomics Core Laboratory of the Central Public Health Laboratory. Raw sequencing reads were analyzed using KrakenUniq (v1.0.3) for taxonomic classification and identification of viral pathogens [18]. Using the developed VaricellaGen pipeline, reads were checked for quality, consensus genomes generated and only samples that attained genome coverage of 70% and above were subjected to downstream analysis. The same pipeline was used to assign clades to genomes and construct a phylogenetic tree. The resulting phylogenetic tree was visualized using the Interactive Tree of Life (iTOL v7) platform for intuitive exploration and annotation of clade relationships [19].

VaricellaGen: Automated VZV genomic analysis pipeline development

To enhance the genomic characterization of VZV, we developed VaricellaGen (https://github.com/MicroBioGenoHub/VaricellaGen). The pipeline includes modules for quality control, variant calling, consensus genome generation, clade typing using a curated SNP dataset [11], and phylogenetic analysis with 1000 bootstraps and collapsing near to zero branch lengths. For the phylogenetic module, reference VZV sequences with known clade assignments were retrieved from NCBI and concatenated into a single FASTA file to serve as a background dataset [11], [20]–[32]. The sequence JN704693.1 was designated as the outgroup. Sequence identifiers were standardized and annotated in the following format: GenBank accession, three-letter country code of sample origin, year of collection, and assigned clade.

Variant calling and consensus genome assembly

The quality assessment of sequencing reads was designed to use FastQC (v0.12.1) and poor quality reads trimmed using Trim Galore (v0.6.10). The alignment of passed reads were aligned to the VZV reference genome

(NC_001348.1) using bwa (v0.7.18-r1243-dirty) and samtools (v1.21), and variants are called using freebayes (v1.3.8) and BCFtools (v1.21). [33]–[37]. Process_gvcf from ARTIC was used to generate regions to be masked during consensus genome generation. [38]. To generate consensus sequences, VaricellaGen was designed to use BCFtools, and only samples that attained genome coverage of 70% and above were meant to be subjected to downstream analysis.

Clade assignment and phylogenetic analysis

To classify the VZV strains, VaricellaGen used an integrated SNP-based clade typing module. For phylogenetic analysis, consensus genomes are concatenated and processed through VaricellaGen's phylogeny module, which performs multiple sequence alignment using MAFFT (v7.525) and constructs a maximum-likelihood phylogenetic tree with IQ-TREE2 (v2.4.0) to assess the genetic relatedness of query samples to previously reported VZV strains [39], [40]. VaricellaGen uses reference VZV sequences with known clade assignments retrieved from NCBI and concatenated into a single FASTA file to serve as a background dataset. The sequence JN704693.1 was designated as the outgroup. Sequence identifiers were standardized and annotated in the following format: GenBank accession, three-letter country code of sample origin, year of collection, and assigned clade.

Results

In this study, we analyzed 284 PCR-confirmed MPXV-negative cases from 27 districts (**Figure 1**). These included 151 (53.2%) males and 123 (43.3%) females, with a mean age of 14 years (range: 9 months - 70 years), who presented with Mpox-like symptoms, notably vesicular-pustular rash, fever, and lymphadenopathy (https://bit.ly/4iZeQDn). Gender information was unavailable for 10 (3.5%) isolates (**Table 1**).

Table 1: Demographic and clinical characteristics of 284 PCR-confirmed MPXV-negative cases presenting with Mpox-like symptoms.

Variable	Value
Total cases analyzed	284
MPXV status	PCR-confirmed MPXV-negative
Number of districts	27
Sex distribution	151 (53.2%) males and 123 (43.3%) females. Gender information unavailable for 10 cases (3.5%)
Mean age	14 years
Age range	9 months – 70 years
Clinical presentation	Vesicular-pustular rash, fever, lymphadenopathy

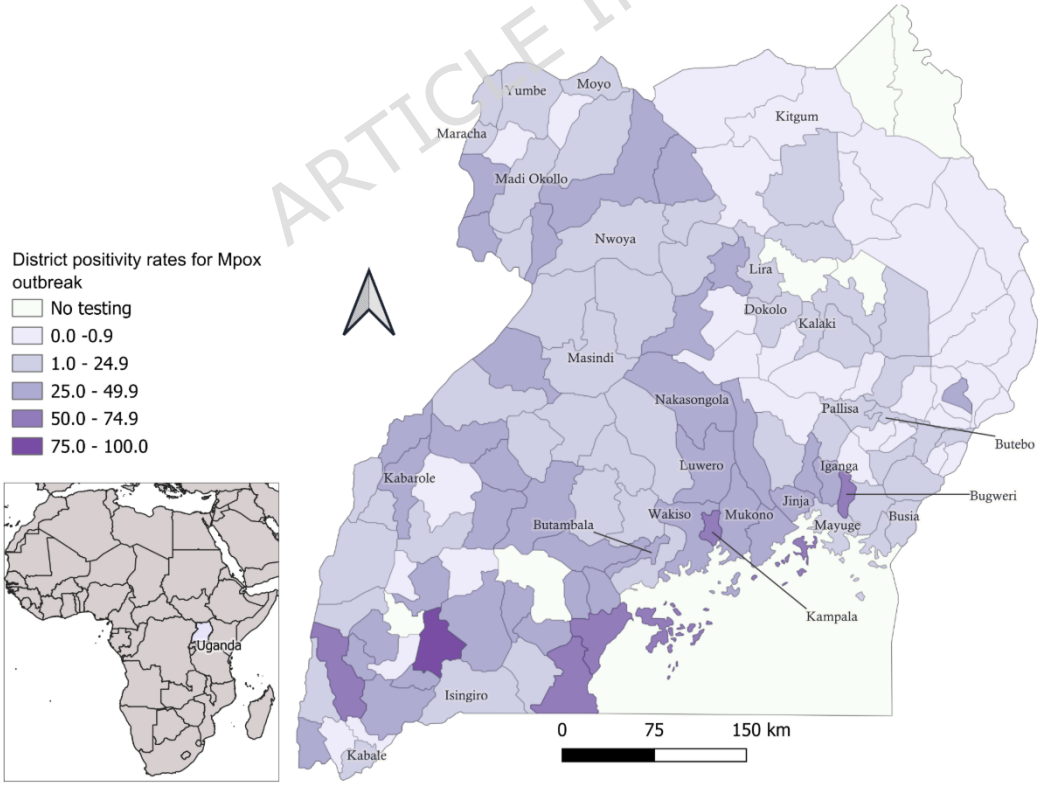


Figure 1. Geographic distribution of sample collection sites across Uganda. Map of Uganda showing district-level Mpox PCR positivity rates based on national surveillance data collected between July and December 2024, alongside the 27 districts from which MPXV-negative clinical samples were collected for metagenomic sequencing. The map was generated using QGIS (version QGIS-OSGeo4W-3.44.6-1; https://qgis.org/). Districts with no reported data or no samples tested during the study period are not labeled.

PCR screening ruled out MPXV infection in all clinically suspected cases. Metagenomic sequencing, followed by taxonomic classification using KrakenUniq, identified VZV as the predominant viral pathogen. Specifically, 86% (243/284) of the samples had at least one read aligning to VZV, while 14% (41/284) showed no VZV reads. No other clinically significant DNA viruses were consistently detected among MPXV and VZV negative samples within the limits of the DNA-based metagenomic approach used.

VaricellaGen: Automated VZV genomic analysis pipeline

To enhance the genomic characterization of VZV, we successfully developed VaricellaGen (https://github.com/MicroBioGenoHub/VaricellaGen), an automated pipeline for the genomic analysis of VZV. The pipeline integrates sequential modules for raw data quality control, variant calling, consensus genome generation, SNP-based clade typing [11], and phylogenetic reconstruction with 1000 bootstraps and collapsing near to zero branch lengths (**Figure 2**). Outputs include annotated consensus genomes, clade assignments, and a phylogenetic tree, providing a streamlined workflow from raw reads to interpretable genomic insights.

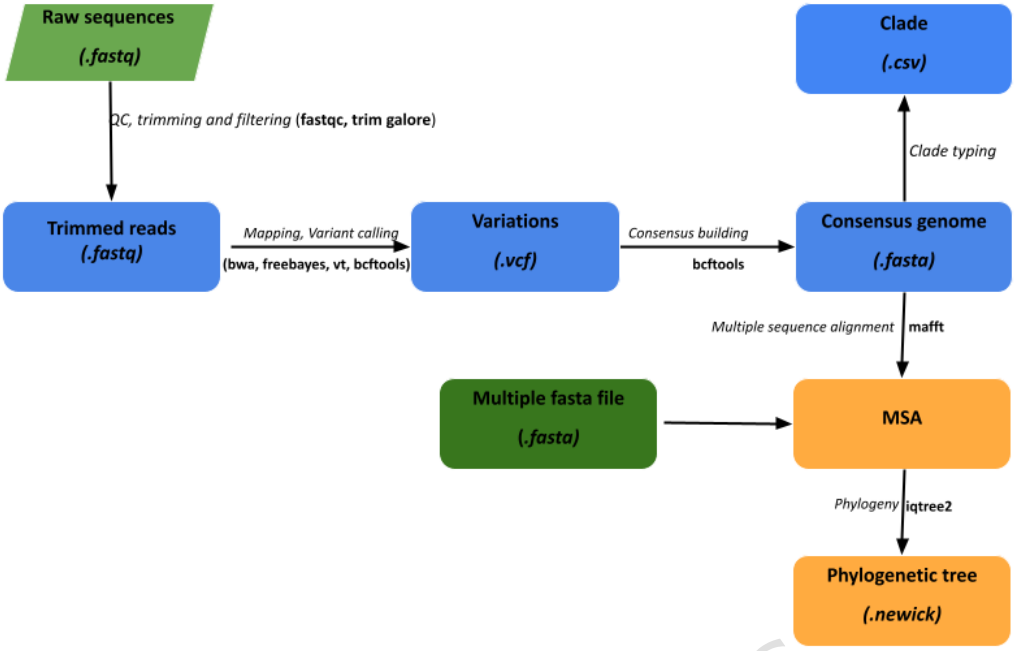
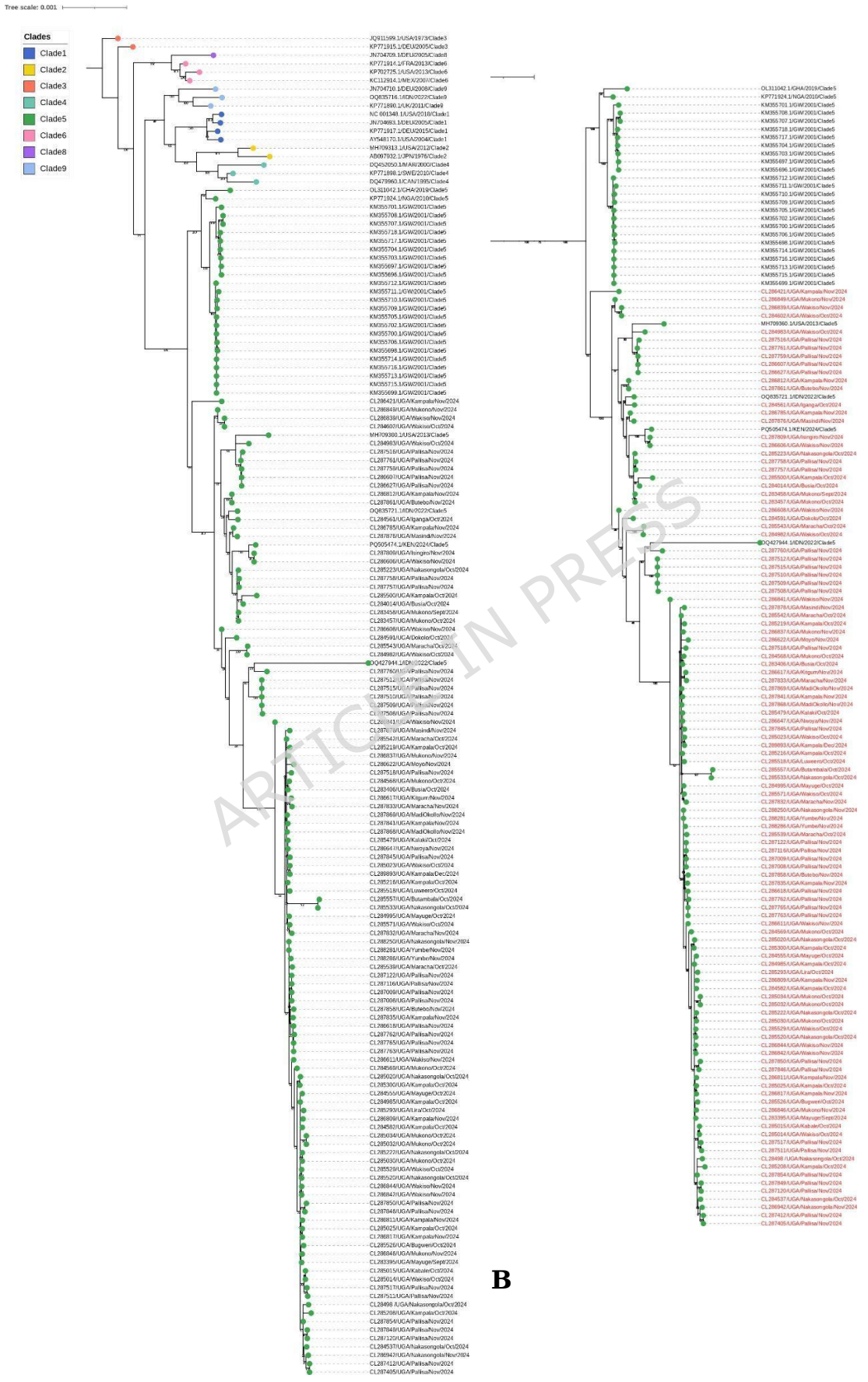


Figure 2. Flowchart of the VaricellaGen pipeline. The pipeline integrates sequential modules for raw data quality control, variant calling, consensus genome generation, clade typing (based on [11]), and phylogenetic reconstruction using a curated background dataset. Outputs include clade assignments, annotated consensus genomes, and phylogenetic trees for downstream interpretation.

Clade assignment and phylogenetic insights

Of the 243 VZV-positive samples, 49% (118/243) achieved ≥70% genome coverage using the VaricellaGen pipeline. SNP-based clade typing classified all successfully sequenced samples as VZV clade 5. This finding was corroborated by phylogenetic analysis, which revealed two distinct sub-clusters within clade 5: one cluster predominantly composed of sequences from Ugandan, India, United States, and Kenyan samples; and a second cluster comprising the remaining Ugandan sequences along with one sequence from Indian (**Figure 3**).



B

A

Figure 3. Phylogenetic analysis of Varicella Zoster Virus (VZV) genomes from Uganda. **A)** Maximum-likelihood phylogenetic tree of VZV genomes. Phylogenetic analysis of VZV-positive samples from Uganda (green) alongside reference sequences from GenBank representing known clades (Clades 1–9). **B)** All Ugandan samples (in red) clustered within Clade 5, forming two distinct sub-clusters: one grouping closely with sequences from India, the United States, and Kenya, and another clustering with only one sequence from India. The tree was constructed using IQ-TREE2 with MAFFT-based alignments; JN704693.1 (Clade 1) was used as the outgroup. Bootstrap values >30 are shown at key nodes. Tree visualization was performed using iTOL.

Discussion

Genomic pathogen surveillance is critically important, especially during outbreaks with non-specific or overlapping clinical presentations. Here, we investigated cases that presented with Mpox-like signs but had MPXV infection ruled out by PCR. Metagenomic sequencing revealed VZV as the predominant etiological agent in a substantial proportion of cases, suggesting a concurrent and previously unrecognized surge in chickenpox infections during the Mpox outbreak in Uganda.

These findings are consistent with concurrent MPXV and VZV outbreaks reported in Burundi, Kenya, and the DRC [41]–[43] and emphasize the challenge of differentiating co-circulating rash-causing viruses based on clinical signs alone [44], [45]. The similar skin manifestations of MPXV and VZV can complicate clinical diagnosis, especially in resource-limited settings with limited access to specific tests. This overlap underscores the necessity of comprehensive laboratory diagnostics, beyond single PCR assays, for

accurate identification of the causative agent and effective public health responses during outbreaks.

Using a metagenomic sequencing approach, we identified VZV in 86% of PCR-confirmed MPXV-negative cases that were clinically suspected to be Mpox. Our newly developed VaricellaGen pipeline enabled detailed genomic analysis, showing that all samples with sufficient genome coverage belonged to VZV clade 5. Phylogenetic clustering with established clade 5 reference genomes further supported this classification and suggests that this clade is prevalent in Uganda, consistent with previous findings from other African countries [32], [46].

Our phylogenetic analysis of VZV clade 5 revealed two Ugandan sub-clusters. One mainly contained sequences from Uganda, India, US, and Kenya, suggesting regional lineage mixing or historical introductions. The other primarily included the remaining Ugandan sequences, clustering with one isolate from India, indicative of multiple introductions or diverse transmission chains within Uganda. This clustering pattern also points to travel-related spread or shared networks across distant regions. These findings highlight the importance of detailed genomic surveillance for both local and international transmission tracking. The observed sub-structuring within clade 5 strongly suggests the need for a standard naming system for VZV sub-clusters to improve genomic reporting and the precision of transmission analysis for future outbreak management.

The simultaneous detection of VZV and MPXV outbreaks highlights critical gaps in current outbreak response protocols, particularly in differential diagnosis and surveillance systems. Large-scale epidemics often focus on a single high-priority pathogen, inadvertently leading to missing concurrent infections, a pattern previously seen in influenza pandemics with underrecognized bacterial co-infections despite their significant contributions to morbidity and mortality [47]. These findings emphasize the

need for integrated surveillance strategies with the ability to capture a wide spectrum of pathogens underscoring the need for multi-pathogen molecular diagnostics and genomic tools to avoid misclassification, especially in sub-Saharan Africa, where integrated disease surveillance systems have previously demonstrated the limitations of single-pathogen monitoring. [48]. A One Health approach, at the human, animal, and environment intersection further emphasizes the importance of monitoring multiple pathogens to detect emerging and re-emerging threats [49]. Our findings support the growing call for unbiased sequencing and comprehensive diagnostics in both routine surveillance and emergency responses.

Despite VZV being a DNA virus with a generally slow mutation rate [50], our analyzed sequences showed moderate genetic diversity (**Figure 3**), suggesting ongoing viral evolution or multiple introduction events. Since all sequences belonged to clade 5, the observed variation could indicate a within-clade origin reflective of probable local transmission patterns, accumulated mutations over time, or different points of origin.

Strengths of this study lie in the targeted DNA-based metagenomic sequencing, which enabled comprehensive detection and genomic characterization of DNA viruses, particularly VZV, among MPXV-negative cases, and the development of VaricellaGen, an automated pipeline for the rapid genomic characterization of VZV. By integrating quality control, variant calling, clade typing, and phylogenetics, VaricellaGen offers a scalable tool for outbreak genomic epidemiology. A key limitation of our study was that detailed genomic analyses only included samples with ≥70% genome coverage. While this threshold ensured data quality, it may have excluded informative lower-quality samples. In addition, the use of DNA-only extraction for metagenomic sequencing restricted pathogen detection to DNA viruses and excluded RNA viruses such as enteroviruses and alphaviruses (e.g., Sindbis virus), several of which are well-recognized causes of vesicular rash illness, underscoring the importance of robust sample

collection, handling, and comprehensive nucleic acid extraction strategies [51]. Future investigations incorporating total nucleic acid extraction will be required to comprehensively evaluate alternative viral etiologies of vesicular rash illness, while further research should also explore whether specific genomic features within VZV clade 5 are associated with disease severity, transmission dynamics, or geographic spread.

Overall, our findings emphasize the crucial role of integrated genomic surveillance during outbreaks, especially when clinical presentation cannot be relied on to differentiate between circulating pathogens. The identification of VZV during Uganda's Mpox outbreak demonstrates the value of metagenomic sequencing approaches for investigating alternative causes of Mpox-like illness and emphasizes the importance of strengthening diagnostic capacity during outbreaks. This study further illustrates how genomic tools can aid in differential diagnosis, guide public health response, and reduce the risks of diagnostic ambiguity. The development of VaricellaGen offers a scalable, automated tool for detailed VZV genomic surveillance, providing a valuable resource for future outbreak investigations and public health decision-making

Data availability

The source code and operation manual for VaricellaGen are available from GitHub under GNU GPL v3; (https://github.com/MicroBioGenoHub/VaricellaGen). The authors confirm that all supporting data, code, and protocols have been provided within the article. The genomic raw read files from this study are publicly available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the study BioProject ID: PRJNA1373217.

Author contributions

Conceptualization: S.K., A.A., I.S., S.N., D.J., G.M., D.P.K, A.S., S.K.T., I.S. Formal analysis: S.K., A.A., J.M.K., C.M., I.S., G.M. Funding acquisition: S.N., I.S., S.T.K., H.O., C.T., N.A., A.A., A.S., S.K, I.M, A.K. Methodology: S.K., A.A., J.M.K., H.R.O., J.S., W.T., G.T., S.W., M.M., M.N., V.Z.N., C.M., I.S., G.M., A.S, I.M, B.L, E.M.R, M.O, S.G. Validation: S.K., A.A., B.A.K., H.O., C.T., N.A., V.Z.N., N.A., G.M., A.S., S.K.T., I.S. Visualization: S.K., I.S., J.M.K. Software: S.K., J.M.K., I.S., G.M. Writing – original draft: S.K., A.A., B.A.K., V.Z.N., I.S., H.O., C.T., N.A., S.N., D.J., G.M., A.A., S.K.T., I.S. Writing – review and editing: S.K., A.A., B.A.K., V.Z.N., I.S., H.O., C.T., N.A., S.N., D.J., G.M., A.A., S.K.T., I.S, I.M, B.L, E.M.R, M.O, S.G, A.K, D.P.K.

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Competing interests

The authors declare no competing interests

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