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Use of Metagenomic Next-Generation Sequencing to Investigate Respiratory Pathogens in Influenza-Negative Nasopharyngeal Swabs From Cameroon's National Influenza Surveillance

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ABSTRACT

Background: In Cameroon, national influenza surveillance identifies influenza-positive cases, but the causes of non-influenza ARIs remain poorly characterized. We applied metagenomic next-generation sequencing (mNGS) to investigate respiratory pathogens in influenza-negative nasopharyngeal swabs collected through national influenza surveillance.

Methods: We analyzed 199 influenza-negative nasopharyngeal swabs collected between September and December 2023 from patients with influenza-like illness or severe acute respiratory infection. Mixed DNA/RNA libraries were prepared and sequenced on an Illumina NovaSeq platform. Taxonomic classification was performed using METAMIC (v2.0) and SPAdes (V3.15.5) bioinformatic pipelines.

Results: Viral sequences were detected in 120/199 samples (60.3%, 95% CI: 53.5%, 67.1%), representing 15 viral species. The most frequent viruses were rhinovirus (43/131), respiratory syncytial virus (33/131), influenza virus, parainfluenza virus and enterovirus. Two non-respiratory viruses (Rotavirus and Papillomavirus) were also detected. Sequencing generated an average of 26.1 million reads per sample, with a mean depth of 870.3 per sample and an average genomic coverage of 82%. Eleven viral co-infections were identified. Several bacterial and fungal genera were also detected.

Conclusion: Our study shows that mNGS provides a powerful, unbiased approach for characterizing respiratory pathogens in Cameroon, revealing substantial viral diversity among influenza-negative ARI cases. However, cost and bioinformatics capacity remain major barriers to routine implementation. Longitudinal studies and clinical correlation are needed to strengthen interpretation and guide integration of mNGS into national surveillance.

1 | Introduction

Acute respiratory infections (ARIs) are the leading cause of infectious disease-related morbidity and mortality worldwide [1]. ARIs range from mild, self-limiting illnesses such as the

common cold to severe, life-threatening conditions including pneumonia and acute respiratory distress syndrome. ARIs can be caused by various infectious agents, including viruses, bacteria, fungi and parasites. Approximately 24% of patients with ARIs in Cameroon require hospitalization [2]. In low- and

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middle-income countries (LMICs), significant gaps remain in understanding the causes of ARI, reflecting the limited access to reliable molecular diagnostic tools [1].

Current diagnostic methods to detect ARIs in LMICs are mainly based on PCRs, sequence-dependent molecular amplification techniques. These conventional viral diagnostic techniques generally rely on prior knowledge of the virus, which limits their usefulness in identifying unexpected or emerging pathogens. These limitations highlight a critical need for improved diagnostic strategies. Metagenomic next-generation sequencing (mNGS), an unbiased and high-throughput approach capable of detecting a wide spectrum of viruses, has emerged as a promising tool for the identification of respiratory pathogens [3–6].

Influenza is an infectious viral disease and the major cause of acute respiratory disease in humans. Influenza surveillance has been implemented across multiple countries to monitor circulating strains in diverse geographical regions. Influenza surveillance began in 2007 in Cameroon and Centre Pasteur du Cameroun (CPC) is the national influenza centre [7, 8]. Each year, approximately 1000 samples are collected from patients with respiratory illness in sentinel sites which are part of the influenza surveillance system in Cameroon [7, 8]. A 10-year study shows an annual positivity rate ranging from 24% to 29% [8]. COVID-19 disrupted influenza seasonality and reduced detection rates [9].

After several years of influenza surveillance in Cameroon, information on the causes of non-influenza ARIs remains limited [10–12], highlighting a significant gap in the national system for the surveillance and management of infectious diseases. Previous studies have relied primarily on targeted PCR assays; consequently, emerging, re-emerging, rare, or highly specific pathogens—if present—may remain undetected. Here, we applied mNGS to perform a comprehensive investigation of respiratory pathogens in nasopharyngeal swabs collected from patients who had tested negative during influenza surveillance in Cameroon.

2 | Material and Methods

2.1 | Sample Collection

We conducted a cross-sectional study analyzing nasopharyngeal swabs from national influenza surveillance in Cameroon. We included nasopharyngeal swabs collected between September and December 2023 from patients with influenza-like illness (ILI) or with severe acute respiratory infection (SARI) based on the WHO case definition as previously described [13]. We conveniently selected the first 203 samples with sufficient volumes (> 200 µL). These samples were obtained from seven of Cameroon's 10 administrative regions: West, Northwest, Southwest, East, South, Littoral and Centre. Nasopharyngeal swabs were collected in 2 mL cryovials containing viral transport medium in influenza sentinel surveillance sites in Cameroon. Within Cameroon's surveillance system, two principal categories of sites are distinguished: ILI sites, which collect data from outpatients and SARI sites, which collect data exclusively from hospitalized patients.

The criteria for selecting sentinel sites encompassed the willingness of hospital administrative and clinical staff to participate, the feasibility of transporting samples to the reference laboratory, the size of the hospital's catchment area and the geographical distribution of the sites. Samples collected were stored in the refrigerator at 4°C for a maximum of 48 h and transported to the CPC for analysis. We included in our study nasopharyngeal swabs tested negative for influenza virus. Patient information—including age, sex, region and district of collection and date of symptom onset—was extracted from the influenza surveillance database.

2.2 | Sample Processing and Metagenomic Sequencing

2.2.1 | Wet-Lab Procedures

Shotgun metagenomics represents a semi-quantitative, pan-microbial next-generation sequencing (NGS) approach that can identify any pathogen present in a sample, independent of prior genetic sequence knowledge, offering a transformative tool for diagnostics and surveillance [3]. The protocol employed in this study has undergone independent evaluation of both wet- and dry-lab procedures [14, 15] and its performance has been benchmarked against other molecular approaches across diverse contexts [3, 5]. Briefly, nasopharyngeal swabs were first subjected to mechanical disruption, enzymatic digestion and chemical lysis treatments. Total nucleic acids were then extracted using the QIAAsymphony system with the QIAAsymphony DSP DNA Midi Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Subsequently, DNA and RNA libraries were prepared with the Illumina DNA Prep Kit and the Illumina Stranded Total RNA Prep with Ribo-Zero Plus Kit (Illumina, San Diego, CA, USA), respectively. Sequencing was performed on a NovaSeq 6000 platform (Illumina) using a NovaSeq 6000 S4 Prepared Pool v1.5.

2.2.2 | Dry-Lab Procedures

Data analysis was conducted using MetaMIC v2.0 software (available on GitHub: <https://github.com/mndebe/MetaMIC>) [3], as previously reported [5, 14]. The workflow began with demultiplexing of forward (R1) and reverse (R2) sequence files. DNA and RNA datasets were processed separately for each patient. Raw paired-end reads (2×150 bp) were trimmed with an in-house script (ReadChainsaw) to remove adapter sequences, low-quality reads (< 50 nt or Phred score < 22/sliding window of 3 nt) and low-complexity sequences. Human reads were aligned to the GRCh38 reference genome (NCBI RefSeq assembly GCF_000001405.40) using SNAP [16]. Unaligned sequences were subsequently compared against a curated 'non-pathogenic' database of non-parasitic, non-fungal eukaryotes to exclude sequences corresponding to plants, insects, or mammals. Remaining non-human, non-pathogenic reads were extracted with Samtools [17] and aligned using SNAP [16] to an in-house database derived from a filtered and validated version of the NCBI NT database (release June 2018). Species-level annotation was performed using NCBI TaxID nomenclature with an in-house script.

Only nasopharyngeal swabs with a cumulative DNA and RNA read count exceeding 10 million were included, in line with current guidelines [18, 19], which recommend a threshold of 10 million reads per sample. According to our standard operating procedures, a pathogen read count-to-total read count ratio of $1E-5$ was applied to identify viral, bacterial, fungal, or parasitic species. Background noise was controlled using an environmental sample. A quantitative quality control (ZymoBIOMICS Microbial Community Standard II—Log Distribution) was incorporated in triplicate to validate the entire workflow (extraction, library preparation and sequencing). This control comprises eight bacterial species and two fungal species mixed to produce a log-distributed abundance. The overall methodology, including both wet- and dry-lab procedures, is accredited under ISO 15189 standards.

2.2.3 | Further Analysis for Viral Populations

For samples that did not align properly to the selected references but in which a viral species was identified by MetaMIC, de novo assembly was performed using SPAdes v3.15.5. The resulting contigs were then used as custom references to realign the original reads, enabling reconstruction of genetic variability within each sample. Consensus genomes were subsequently generated with SamTools/BCFTools v1.19, applying a minimum base frequency threshold of 50%. Genotyping followed a tiered approach: NextClade and Genome Detective were used when species-specific databases were available, whereas unresolved cases were analyzed through BLAST homology searches against the NCBI database. Phylogenetic trees were constructed using reference sequences retrieved from NCBI viruses.

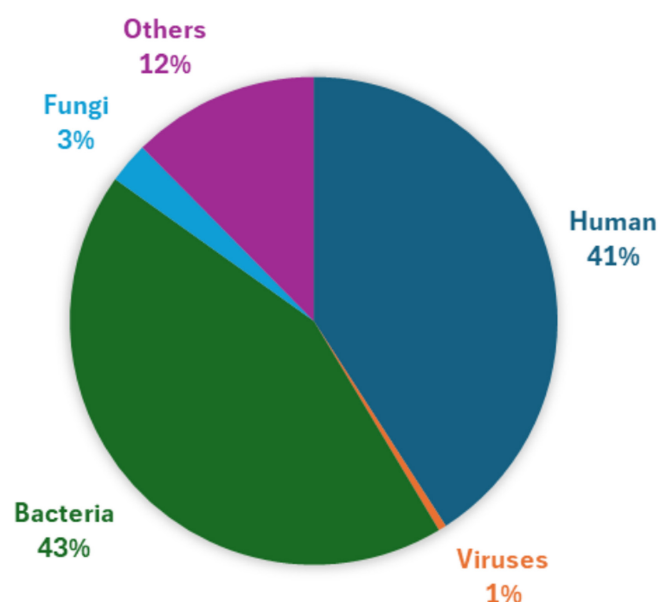


FIGURE 1 | Taxonomic distribution of metagenomic sequencing reads from all nasopharyngeal swabs. Data presented are based on average values of reads.

3 | Results

3.1 | General Characteristics of the Studied Population

mNGS was successfully performed on a total of 199 nasopharyngeal swab samples, including 150 samples from patients with ILI and 49 samples from patients with SARI. They were collected between September and December 2023 from 7 of 10 regions of Cameroon (Centre, Littoral, West, East, South-West, North-West and South). The study population consisted of 104 females and 95 males, aged between 0 and 80, with an average age of 14.1 years. Four samples failed to reach the 10M threshold, were classified as uninterpretable and consequently excluded from the analyses.

3.2 | Viral Detection and Genetic Diversity

Metagenomic sequencing of all nasopharyngeal swabs generated an average of 26.1 million reads, after quality trimming and filtering. On average, 41% of the reads were derived from human genome, 43% from bacteria, 3% from fungi and 1% from viruses. The rest of the reads (12%) were derived from other viruses that did not have any significant match in the known databases and non-pathogenic taxa (Figure 1). Viral reads, with a mean sequencing depth of 870.3 per sample, were identified in 120 of the 199 nasopharyngeal swabs analyzed (60.3%; 95% CI: 53.5%, 67.1%). Following genome assembly, the identified viral genomes showed an average coverage of 82%, ranging from 5% to 100%. In total, we detected 11 cases of viral co-infection and recovered 131 viral sequences. These sequences belonged to Rhinovirus (43/131), Respiratory Syncytial Virus (33/131), Influenza virus (14/131), Parainfluenza virus (13/131), Enterovirus (11/131), Adenovirus (4/131), Cytomegalovirus (3/131), Metapneumovirus (2/131), Polyomavirus (2/131), SARS-CoV-2 (1/131), Torque teno virus (1/131), Human Coronavirus HKU1 (1/131) and Epstein-Barr virus (1/131). Two non-respiratory viruses—Rotavirus and Papillomavirus—were also detected in two patients. Overall, 92.4% (121/131) of the identified viral strains belonged to five major respiratory viruses: Rhinovirus, Respiratory Syncytial Virus, Influenza virus, Parainfluenza virus and Enterovirus.

Phylogenetic analyses revealed substantial genetic variability among rhinovirus, with 23 distinct subtypes identified across the species *Enterovirus alpharhino* (RV-A), *Enterovirus betarhino* (RV-B) and *Enterovirus cerhino* (RV-C) (These refer to the different rhinovirus species). The enterovirus strains were grouped into three serotypes corresponding to *Enterovirus alphacoxsackie* (EV-A), *Enterovirus betacoxsackie* (EV-B) and *Enterovirus coxsackiepol* (EV-C). The influenza strains included 5 *Influenza A virus*, 4 *Influenza B virus* and 5 *Influenza C virus*. Respiratory syncytial virus sequences clustered into two types (RSV-A and B), with type A predominating. The parainfluenza virus detected corresponded to the serotypes *Human orthorubulavirus 4* and *Human respirovirus 1* (Figures 2 and 3). The remaining viruses exhibited lower diversity, with sequences grouping within a single type (Figure 2).

3.3 | Bacterial and Fungal Detections

On an average, 43% and 3% of the reads were derived from bacteria and fungi, respectively (Figure 1). Bacteria and fungi were detected in 61 and 21 of the 199 nasopharyngeal swabs analyzed, respectively. The bacteria identified belong to: *Chlamydia pneumoniae*, *Corynebacterium* (*propinquum*, *efficiens*, *urealyticum*, *yudongzhengii*, *pseudodiphtheriticum*, *doosanense*, etc.), *Enterobacter* (*hormaechei*), *Haemophilus* (*influenzae*, *aegyptius*, *haemolyticus*, *seminalis*, *caudoviricetes* sp., *haemolyticus*), *Klebsiella pneumoniae*, *Moraxella* (*catarrhalis*, *nonliquefaciens*, *osloensis*), *Staphylococcus* (*aureus*, *epidermidis*), *Streptococcus* (*oralis*, *pneumoniae*, *pyogenes*, *mitis*), *Actinomyces* (*oris*, *naeslundii*). The fungi identified belong to *Candida* (*parapsilosis*, *metapsilosis*, *orthopsilosis*, *auris*).

4 | Discussion

ARIs present with nonspecific symptoms, making etiologic diagnosis difficult, particularly in resource-limited settings where testing relies on targeted PCR assays [20]. These assays detect only known pathogens and often leave underlying causes untyped. This gap has driven interest in unbiased virome approaches capable of characterizing both well-known and less-characterized viral communities [21].

This study represents the first application of mNGS to investigate respiratory pathogens in Cameroon, providing a comprehensive view of viral diversity among influenza-negative ARI cases. Despite viral reads representing only 1% of total sequencing data, mNGS detected viral sequences in 60.3%

of samples. Our viral detection rate of 60.3% is higher than 30.5% (58/190) from Kenya [4] and lower than 97.6% (82/84) from South Africa [22], possibly due to differences in sample processing or sequencing depth. The mNGS approach offers major advantages for pathogen detection, but its use for routine diagnostics in resource-limited settings such as Cameroon remains challenging. Although the clinical surveillance increased during and after the COVID-19 pandemic, the high cost of reagents and the limited availability of bioinformatics expertise represent significant barriers. Reducing reagent costs and expanding access to bioinformatics training would be key steps towards enabling the full potential of NGS technologies to improve health outcomes in such settings. We detected 11 cases of viral co-infections, which is a significant finding as co-infections are often underestimated by targeted-PCRs. To strengthen influenza surveillance, we recommend adopting multiplex respiratory panels—already commercially available—that cover nearly all viruses, or alternatively, multiplex respiratory panels integrating the viruses which were frequently detected in this study.

The samples analyzed in this study were those that had tested negative for *influenza virus* within Cameroon's national surveillance programme [7, 8]. Using mNGS, influenza viruses were detected in 14 nasopharyngeal swabs. The detection of influenza C virus is not unexpected, as this type is not included in the routine respiratory testing panels used in Cameroon's influenza surveillance programme. In contrast, the identification of influenza A and B viruses is more concerning, given that our study specifically included samples that had previously tested negative for both types [7, 8]. Several studies comparing mNGS with routine PCR-based surveillance have reported discrepancies

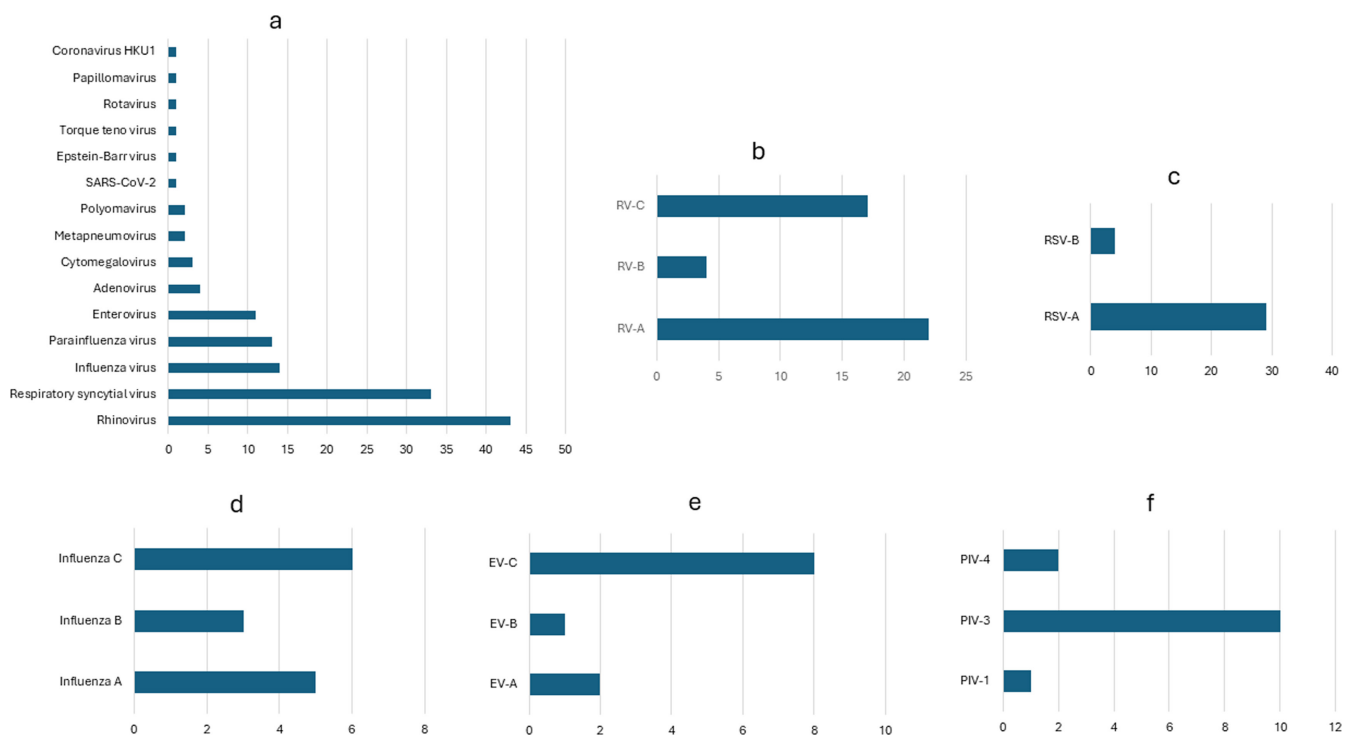


FIGURE 2 | Number of viral sequences for the 15 identified viruses (a) and the number of sequences by type for rhinovirus (b), respiratory syncytial virus (c), influenza (d), enterovirus (e) and parainfluenza (f). Rhinovirus was the most frequently detected virus (43/131 sequences), followed by RSV (33/131).

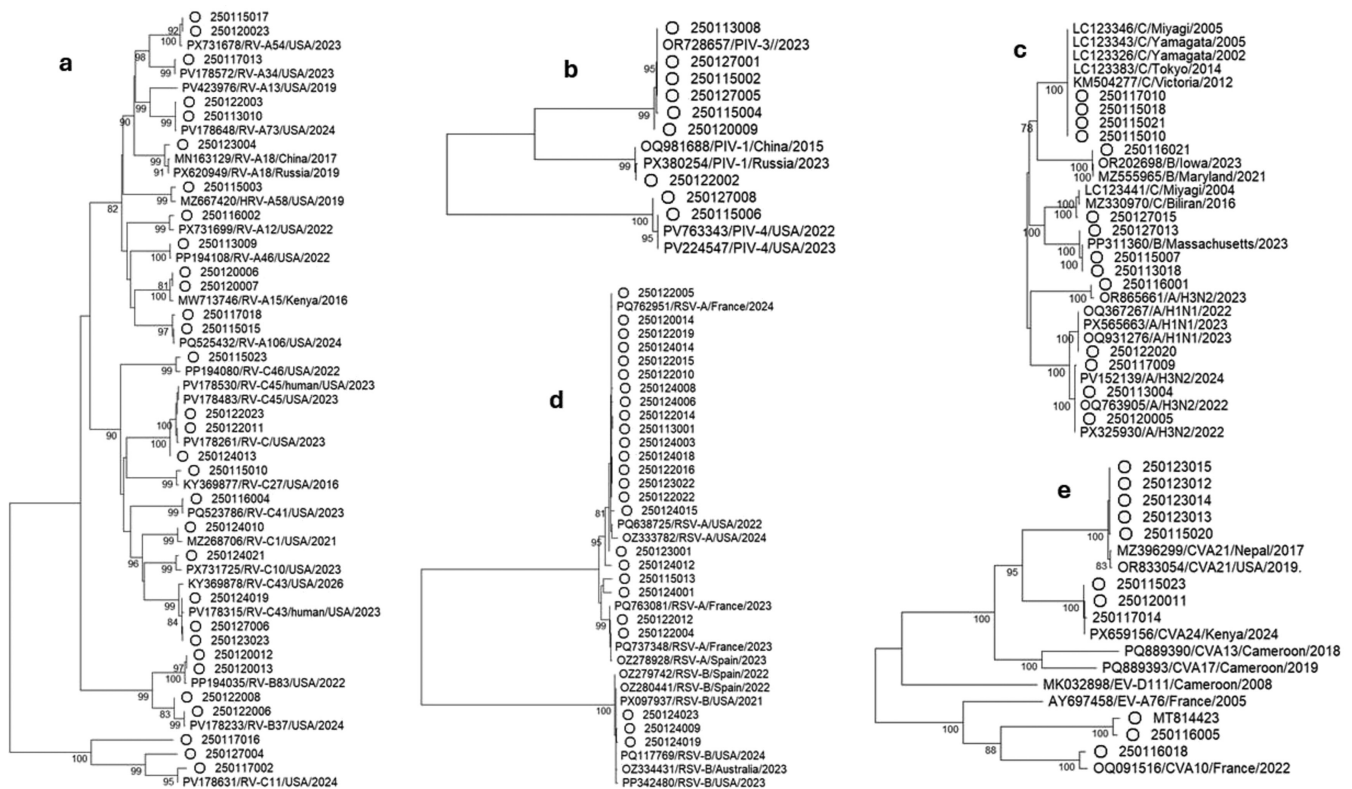


FIGURE 3 | Phylogenetic trees generated using GenBank reference sequences for rhinovirus (a), parainfluenza (b), influenza (c), respiratory syncytial virus (d) and enterovirus (e). Phylogenetic trees were constructed using the neighbour-joining method with MEGA 12 software. Genetic distances were calculated using the Kimura two-parameter model as a correction factor and 1000 replicates. Branch lengths represent genetic distance (Kimura 2-parameter model). Only bootstrap values > 70% are presented. The accession number, genotype/subtype, country of origin and host are shown for each GenBank HEV strain used in the phylogenetic analysis. Each strain identified in our study is indicated by (○) followed by lab identifier.

between the two methods, without demonstrating that mNGS offers superior sensitivity [6, 23, 24]. These studies concluded that integrating mNGS with routine PCR testing can provide a more accurate characterization of respiratory infections in clinical samples from resource-limited settings.

We unexpectedly detected rotavirus and papillomavirus in nasopharyngeal swabs. This is the first report of these viruses being detected in respiratory samples in Cameroon. Although studies conducted elsewhere have documented the presence of rotavirus [25, 26] and papillomavirus in nasopharyngeal secretions [27, 28], the association between their detection and clinical symptoms (ILI observed in rotavirus-positive patient and SARI observed in papillomavirus-positive patient) remains unclear. In line with these previous studies, we believe that further investigations are needed to confirm whether these viruses are true respiratory pathogens or contaminants.

We detected 3% fungal reads and 43% bacterial reads. These percentages are high for nasopharyngeal samples and could be explained by contamination (e.g., environmental DNA) or by asymptomatic colonisation (e.g., *Streptococcus pneumoniae* in the nasopharynx). Certain viruses (polyomavirus, CMV), bacteria (*Actinomyces oris*, *Moraxella catarrhalis*, *Corynebacterium* spp. and *Haemophilus influenzae*) and fungi (*Candida parapsilosis* and *C. pseudohemulonius*) should not be considered true respiratory pathogens, as they are typically

part of the normal oropharyngeal microbiota. They can be pathogenic but are not typical causes of ILI characteristic of the large number of patients in whom they have been identified. Interpreting bacterial results in mNGS is complex, as it is difficult to distinguish actual pathogens from contaminants or members of the normal microbiota. The main challenges include the high sensitivity of the method, the risk of false positives and the lack of standardised thresholds for defining clinical relevance [29, 30]. The detection of EBV is intriguing, given previous reports describing the detection of EBV DNA in nasopharyngeal samples and its association with nasopharyngeal carcinoma (NPC) [31, 32]. NPC is a highly invasive and metastatic cancer that is endemic in southern China, Southeast Asia and northern Africa [31, 32]. Although Cameroon is located in a non-endemic region the situation is unknown and further investigation remains necessary.

This study provides valuable insights but is limited by restricted regional representation (samples from Cameroon's three northern regions—North, Far North and Adamawa—remain unanalyzed), reliance on symptomatic patients, seasonal sampling (patient samples were collected only between September and December 2023) and the absence of clinical correlates. In addition, there is evidence that suggests that ILI definition is not accurate for RSV diagnosis, with only 11% of sensitivity [33] and could also not be the most accurate one for other viruses. Addressing these gaps through broader geographic sampling,

inclusion of asymptomatic individuals, seasonal comparisons and integration of clinical data will be critical for future work.

5 | Conclusion

Our study underscores the value of mNGS as a powerful diagnostic tool in clinical virology. However, its routine application in resource-limited settings remains constrained by reagent costs, infrastructure needs and limited bioinformatics capacity. To strengthen national respiratory disease surveillance, we recommend the adoption of multiplex respiratory panels, which already cover nearly all of the viruses identified in this study and are commercially available. In addition, longitudinal studies are needed to capture seasonal variations in respiratory pathogen circulation and clinical interpretation of mNGS findings.

Author Contributions

Modiyinji Abdou Fatawou: funding acquisition, supervision, writing – review and editing, conceptualization, methodology, validation, writing – original draft, formal analysis, investigation. **Cappy Pierre:** investigation, data curation, formal analysis, writing – review and editing, visualization, validation, software, methodology. **Ly Arnaud:** investigation, data curation, formal analysis, writing – review and editing, visualization, validation, software, methodology. **Cheval Justine:** investigation, data curation, formal analysis, writing – review and editing, visualization, validation, software, methodology. **Moumbeket-Yifomnjou Moise Henri:** resources, data curation, formal analysis, writing – review and editing, investigation. **Tsafack Desmon Toutou:** resources, data curation, formal analysis, writing – review and editing, investigation. **Touoyem Ibrahim:** resources, data curation, formal analysis, writing – review and editing, investigation. **Njankouo Ripa:** resources, data curation, formal analysis, writing – review and editing, investigation. **Monamele Chavely Gwladys:** resources, data curation, formal analysis, writing – review and editing, investigation. **Njouom Richard:** conceptualization, funding acquisition, writing – review and editing, supervision, validation, project administration, resources.

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Artificial intelligence has not been used to write this manuscript.

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Ethics Statement

The study protocol was reviewed and approved by the Ethical and Institutional Committee of the University of Douala (N° 397ICEI-Udo/07/2023/M) and was conducted in accordance with the Declaration of Helsinki. Sample collection, analysis and reporting of influenza cases by Centre Pasteur du Cameroon is supported and approved by the Ministry of Public Health in Cameroon and the World Health Organization (WHO) as part of influenza surveillance activities in Cameroon. The samples and database used in this study were pseudonymized by influenza surveillance team before use and all precautions

were taken to avoid any risk of patient identification. As this study is a programmatic (secondary) database analysis with no identifiable information, informed consent was exempted by the Ethics Committee.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Sequence read data generated in this study has been deposited in the SRA database under Bioproject PRJNA1355199. Partial or nearly complete genome sequences are deposited in Genbank with accession numbers: PZ233698-PZ233732 (rhinovirus); PZ233733-PZ233743 (enterovirus); PZ241094-PZ241119 (respiratory syncytial virus); PZ244837-PZ244844 (respirovirus); PZ239601-PZ239602 (human metapneumovirus); PZ246329-PZ246330 (polyomavirus); PZ255042-PZ255046 (influenza A virus); PZ255047-PZ255049 (influenza B virus); PZ258920-PZ258925 (influenza C virus); PZ233447 (SARS-CoV-2); and PZ256266-PZ256273 (adenovirus, cytomegalovirus, CoV-HKU1, rotavirus, torque teno virus and human papillomavirus).

Peer Review

For transparency, the peer review documents associated with this article are available at <https://doi.org/10.1111/irv.70336>.

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