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ORIGINAL RESEARCH

# tNGS Reveals Predominant Mixed Viral Infections in a Multicenter Retrospective Study

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**Purpose:** Viral respiratory infections can damage epithelial cells and suppress host immunity, thereby predisposing patients to secondary bacterial or fungal infections. Accurate and timely identification of co-pathogens remains a clinical challenge. We aimed to examine whether tNGS can more comprehensively identify viral and mixed infections in comparison to conventional methods.

**Patients and Methods:** We retrospectively analyzed 834 patients tested using targeted next-generation sequencing (tNGS) and 2263 patients tested using conventional methods across multiple centers. Pathogen profiles—including viral, bacterial, fungal, and atypical microorganisms—were compared between the groups.

**Results:** Compared with conventional diagnostic methods, tNGS detected significantly higher proportions of viral co-infections, including fungal pathogens (eg, *Aspergillus* and *Mucor*), bacterial pathogens, *Mycobacterium* spp., herpesviruses, and multiple viral combinations (such as two distinct respiratory viruses, respiratory viruses with herpesviruses, or two non-herpesviruses). The most frequently identified viruses by tNGS were Epstein–Barr virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), herpes simplex virus type 1, influenza A virus, and rhinovirus. Commonly detected bacterial pathogens included *Klebsiella* spp. *Fusobacterium nucleatum*, and *Streptococcus mitis*, while fungal pathogens frequently identified included *Aspergillus* spp. and *Mucor* spp. In addition, the detection rates of *Mycoplasma* spp., *Mycobacterium tuberculosis*, and nontuberculous mycobacteria were significantly higher with tNGS than with conventional testing (all $P < 0.05$).

**Conclusion:** Targeted next-generation sequencing identified a higher prevalence and broader spectrum of viral co-infections and secondary bacterial/fungal infections compared with conventional methods. These findings highlight the complexity of respiratory infections in the post–COVID-19 era and support the potential of tNGS as a comprehensive diagnostic approach in clinical practice.

**Plain Language Summary:** Respiratory viruses can damage the airways and weaken the immune system, making people more likely to develop bacterial or fungal infections. Identifying these additional "co-infections" is important for treatment but can be difficult with standard lab tests. In this study, researchers compared two testing approaches: targeted next-generation sequencing (tNGS) and conventional diagnostic methods. They reviewed data from 834 patients tested with tNGS and 2263 patients tested with conventional methods across multiple centers. The results showed that tNGS detected a much wider range of infections. It identified not only common respiratory viruses but also multiple simultaneous viral infections, herpesviruses, bacteria, and fungi that were often missed by conventional testing. Frequent viruses found included Epstein–Barr virus, SARS-CoV-2, herpes simplex virus type 1, influenza A, and rhinovirus. Bacteria, such as *Klebsiella, Fusobacterium nucleatum*, and *Streptococcus mitis*, were commonly seen, along with fungal pathogens like *Aspergillus* and *Mucor*. tNGS also had higher detection rates for *Mycoplasma, Mycobacterium tuberculosis*, and nontuberculous mycobacteria. Overall, tNGS provided a more complete picture of mixed respiratory infections than conventional methods, suggesting it may be a valuable tool for improving diagnosis and patient care in the post–COVID-19 era.

**Keywords:** *Aspergillus*, mixed viral infections, *Mucor*, post-COVID-19, tNGS, virus



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# Introduction

Respiratory viral infections, including influenza, respiratory syncytial virus (RSV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are well-established triggers of secondary bacterial and fungal infections, which can significantly worsen clinical outcomes.[1–3] Virus-induced airway epithelial damage, impaired mucociliary clearance, and dysregulation of innate and adaptive immunity increase susceptibility to opportunistic pathogens, such as *Streptococcus pneumoniae, Staphylococcus aureus*, and *Aspergillus* spp.[4–6] For example, influenza-associated pulmonary aspergillosis and coronavirus disease 2019 (COVID-19)–associated pulmonary aspergillosis have emerged as serious complications in critically ill patients, associated with high morbidity and mortality.[7–9]

Despite the clinical significance of these mixed infections, conventional microbiological methods—such as culture, antigen testing, and polymerase chain reaction (PCR)—are often insufficient for early and comprehensive detection because of limited sensitivity, prolonged turnaround times, and the inability to simultaneously identify multiple pathogens.[10–12] Furthermore, these methods are designed to target specific suspected pathogens and may overlook atypical or coexisting organisms, particularly in complex polymicrobial infections.

Targeted next-generation sequencing (tNGS) is a novel high-throughput molecular diagnostic technique capable of simultaneously identifying a broad range of pathogens—including viruses, bacteria, fungi, and atypical organisms—in clinical samples with high sensitivity and specificity.[13,14] By enabling unbiased multiplex detection, tNGS shows strong potential for uncovering the spectrum of mixed infections, particularly in critically ill or immunocompromised patients.[15]

Recent studies published since 2021 have applied next-generation sequencing (NGS) and metagenomic approaches to investigate respiratory infections, highlighting their potential for detecting viral, bacterial, and fungal co-infections in both immunocompetent and immunocompromised patients.[16–18] However, most of these reports have focused on specific patient groups, such as critically ill individuals with COVID-19 or influenza, or have evaluated metagenomic NGS (mNGS) rather than targeted NGS (tNGS). Furthermore, limited data are available on the comprehensive microbiological profiles of suspected pulmonary infections across multiple centers, particularly regarding the spectrum and prevalence of viral and mixed infections in routine clinical practice.

Given the limitations of conventional diagnostics and the growing clinical burden of post-viral secondary infections, a deeper understanding of the microbiological profiles identified by tNGS is urgently needed. In this multicenter retrospective study, we compared pathogen detection using tNGS with conventional microbiological methods in patients with suspected pulmonary infections, with particular focus on the prevalence and patterns of viral and mixed infections in the post–COVID-19 era.

In this work, we aimed to investigate whether tNGS can more comprehensively identify viral and mixed infections than conventional methods. We hypothesized that tNGS would detect a higher prevalence and broader spectrum of viral co-infections, thereby providing additional insights into the complexity of respiratory infections in the post–COVID-19 clinical context.

# Materials and Methods

## Study Design and Patient Involvement

Between March 2023 and March 2025, we retrospectively enrolled 834 patients (>14 years of age) diagnosed with viral pneumonia who underwent tNGS testing at China-Japan Friendship Hospital, Tangshan Workers' Hospital, Weifang Second People's Hospital, and Renqiu Youyi Hospital. In parallel, we included 2263 patients (>14 years of age) with viral pneumonia who underwent conventional pathogen detection between August 8, 2016, and March 31, 2025. For each patient, we collected comprehensive microbiological data, including tNGS results (tNGS group) and conventional diagnostic results, such as viral nucleic acid testing, bacterial and fungal cultures from sputum, galactomannan (GM) testing, *Aspergillus*-specific IgG antibody testing, and mycobacterial culture results.

## Diagnostic Criteria

tNGS was employed for microbial diagnosis. Previous studies have demonstrated the diagnostic efficacy of tNGS in pulmonary infections, showing >88% concordance with reverse transcription (RT)-qPCR results for COVID-19 and influenza virus detection,[19,20] and higher sensitivity than conventional methods for fungal detection.[16,21]

In the conventional diagnostic group, viral pneumonia was diagnosed by viral nucleic acid detection. Fungal diagnoses were based on the 2020 consensus definitions of invasive fungal diseases by the European Organization for Research and Treatment of Cancer–Mycoses Study Group Education and Research Consortium, which address proven and probable invasive mold disease and were modified to include patients at risk of mucormycosis.[22]

## Data Collection

Clinical data were extracted from medical records and included laboratory and microbiological results from blood, sputum, and bronchoalveolar lavage fluid (BALF) analyses; bacterial, fungal, and mycobacterial cultures; viral nucleic acid detection; nucleic acid tests for *Mycoplasma, Chlamydia*, and *Legionella*; GM and (1,3)-β-D-glucan assays; and *Aspergillus*-specific IgG antibody testing.

## Pathogen Detection by tNGS

Samples were processed at KingMed Biotechnology Co., Ltd. (Guangzhou, China). Automated nucleic acid extraction was performed using the MagPure Viral DNA/RNA Kit (IVD5412; Magen Biotechnology, Guangzhou, China) on a KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA). A non-template control (nuclease-free water; Invitrogen, Waltham, MA, USA) was included in each run to monitor contamination.

Reverse transcription, multiplex PCR preamplification, and library preparation were performed using the Respiratory Pathogen Microorganism Multiplex Testing Kit (KingCreate, Guangzhou, China). Libraries were quantified using the Equalbit DNA HS Assay Kit (Vazyme Biotech Co., Ltd., Nanjing, China) with the Invitrogen™ Qubit™ 3.0/4.0 fluorometer (Thermo Fisher Scientific) to ensure a library density ≥0.5 ng/μL; otherwise, libraries were reconstructed.

DNA fragment analysis was conducted using the Standard Cartridge Kit (BiOptic Inc., Jiangsu, China) on the Qsep100 Capillary Electrophoresis System. Sequencing was performed on the KM MiniSeq Dx-CN Platform (KingCreate).

Bioinformatics analysis included base calling (bcl2fastq), adaptor trimming and quality filtering (fastp), and mapping to a tNGS-specific, curated database using Bowtie2 in "very sensitive" mode. Reads aligned to target amplicon regions were quantified, normalized to 100,000 reads, and reported as reads per kilobase. Final results were displayed in the KingCreate Report System and interpreted by a clinical microbiology expert.[23]

## Conventional Microbiology

A viral etiology was confirmed if respiratory viruses—including SARS-CoV-2, cytomegalovirus, Epstein–Barr virus (EBV), RSV, influenza A and B, parainfluenza virus (PIV), human rhinovirus (HRV), human metapneumovirus (HMPV), adenovirus (AdV), human coronavirus (HCoV), herpes simplex virus type 1 (HSV-1), *Mycoplasma, Chlamydia*, and *Legionella*—were detected in sputum, endotracheal aspirates, BALF, or nasopharyngeal swabs using RT-PCR (Shanghai Bojie Medical Technology or Zhijiang Biological Technology, Shanghai, China). Bacterial and fungal pathogens were analyzed in sputum and BALF samples. *Aspergillus* GM and IgG antigen testing was performed using the Platelia *Aspergillus* ELISA (Bio-Rad Laboratories, Marnes-la-Coquette, France) and Dana Tianjin Biotechnology (Tianjin, China).

## Statistical Analysis

Patient demographics, clinical characteristics, and pathogen detection results are expressed as means (±standard deviations), medians (interquartile ranges), or frequencies (percentages). Comparisons of continuous variables were performed using parametric or non-parametric tests depending on distribution. Categorical variables were compared using the $\chi^2$-test. Statistical analyses were conducted with SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Two-sided $P$ values <0.05 were considered statistically significant.

# Results

A total of 834 patients were included in the tNGS group and 2263 in the conventional pathogen detection group.

The proportion of viral infections with *Aspergillus or Mucor* was significantly higher in the tNGS group compared with the conventional group (17.6% vs 11.2%). Similarly, the prevalence of viral co-infections with bacteria (56.8% vs

21.8%), *Mycobacterium* spp. (6.2% vs 1.1%), two different viruses (45.9% vs 6.1%), herpesviruses (50.6% vs 24.8%), respiratory viruses combined with herpesviruses (40.4% vs 4.9%), two viruses other than herpesviruses (10.0% vs 1.8%), and *Mycoplasma* or *Chlamydia* (5.4% vs 1.5%) were all significantly higher in the tNGS group (Table 1).

## Viral Detection

In the tNGS group, the most frequently identified viruses were EBV (38.1%), SARS-CoV-2 (23.14%), HSV-1 (19.0%), influenza A virus (FLUA, 18.47%), HRV (14.39%), AdV (8.63%), PIV (7.31%), HHV-7 (7.31%), HCoV (6.71%), RSV (6.47%), HMPV (5.64%), influenza B virus (FLUB, 2.28%), enterovirus (EV, 2.04%), varicella-zoster virus (VZV, 0.60%), HHV-6 (0.48%), and parvovirus B19 (0.48%).

In the conventional pathogen detection group, the most commonly detected viruses were SARS-CoV-2 (47.95%), FLUA (14.14%), RSV (6.98%), EBV (6.14%), FLUB (3.62%), PIV (3.31%), AdV (2.87%), HRV (2.47%), HSV-1 (0.84%), HMPV (0.22%), HCoV (0.09%), and B19 (0.04%). The detection rate of SARS-CoV-2 in the conventional group was not lower than that in the tNGS group, and no significant differences were observed in the detection of RSV or FLUB. For all other viruses, detection rates were significantly lower in the conventional group compared with tNGS ($P$ <0.05) (Table 1 and Figure 1).

## Bacterial Detection

In the tNGS group, the most frequently identified bacteria were *Klebsiella* spp. (19.30%), *Fusobacterium nucleatum* (13.43%), *Streptococcus mitis* (12.59%), *Pseudomonas aeruginosa* (11.15%), *Acinetobacter baumannii* (10.79%), *Haemophilus influenzae* (8.15%), *Streptococcus anginosus* (6.24%), *Micrococcus luteus* (5.40%), *Streptococcus pneumoniae* (5.04%), *Staphylococcus aureus* (4.44%), *Escherichia coli* (4.08%), and *Enterobacter cloacae* (3.96%).

In the conventional group, the most frequently identified bacterium was *A. baumannii* (6.94%), followed by *P. aeruginosa* (3.09%), *Klebsiella* spp. (2.39%), *S. aureus* (1.68%), *Bacteroides fragilis* (1.50%), *Brevundimonas maltophilia* (1.28%), *Enterococcus* spp. (1.02%), *E. coli* (0.84%), *E. cloacae* (0.75%), *H. influenzae* (0.62%), *S. pneumoniae* (0.53%), and *Nocardia* spp. (0.53%). Across all major bacterial taxa, detection rates were significantly lower in the conventional group compared with tNGS ($P$ <0.05) (Table 2 and Figures 2–3).

## Fungal Detection

The tNGS group demonstrated significantly higher detection rates of fungal pathogens compared with the conventional group, including *Aspergillus* spp. (22.30% vs 10.20%), *Candida albicans* (29.02% vs 0.62%), *Candida tropicalis* (7.19% vs 0.40%), *Candida glabrata* (3.48% vs 0.31%), *Candida parapsilosis* (3.12% vs 0.27%), *Rhizopus oryzae* (1.32% vs 0.13%), and *Rhizopus microsporus* (1.32% vs 0). No significant differences were observed between groups for *Pneumocystis jirovecii* or *Cryptococcus* spp. (Table 2 and Figure 4).

**Table 1** The Comparison of Viral Co-Infections Between the tNGS Group and the Conventional Pathogen Detection Group

| Pathogen | tNGS n=834 | Traditional Pathogen Detection n=2263 | P value |
|---|---|---|---|
| Virus-Aspergillus (Rhizopus) co-infection | 147 (17.6) | 253 (11.2) | <0.001 |
| Virus-Bacteria co-infection | 474 (56.8) | 494 (21.8) | <0.001 |
| Virus-Mycobacteria co-infection | 52 (6.2) | 26 (1.1) | <0.001 |
| Two or more viruses | 383 (45.9) | 137 (6.1) | <0.001 |
| Herpesvirus | 422 (50.6) | 561 (24.8) | <0.001 |
| Co-infection of respiratory viruses and herpesviruses | 337 (40.4) | 112 (4.9) | <0.001 |
| Co-infection with two respiratory viruses | 83 (10.0) | 41 (1.8) | <0.001 |
| Co-infection of virus and Mycoplasma/Chlamydia | 45 (5.4) | 35 (1.5) | <0.001 |

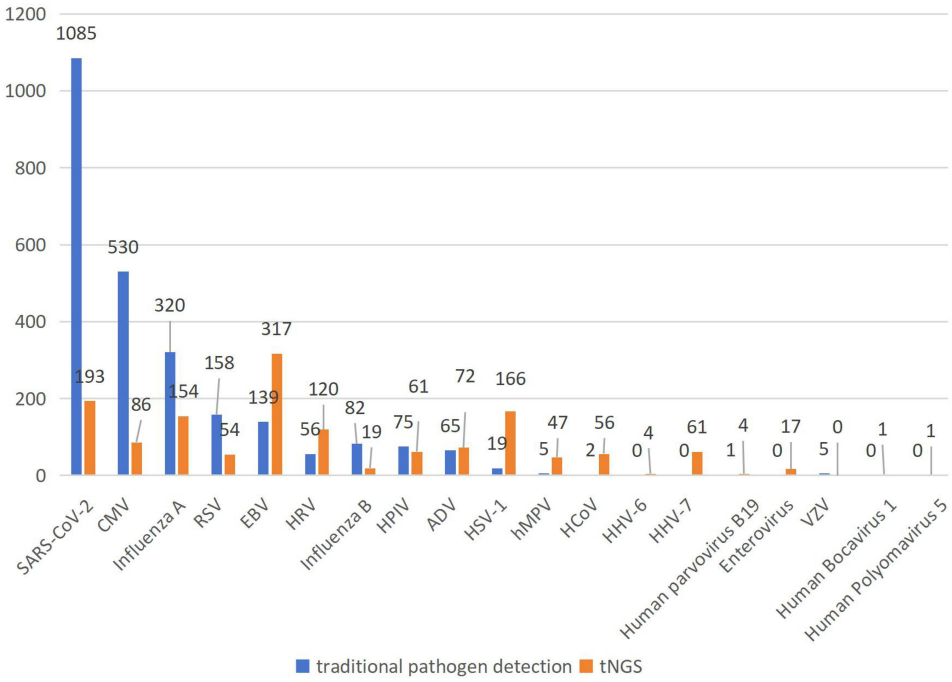


**Figure 1** Viral detection in the tNGS group and the conventional pathogen detection group.

## Atypical and Mycobacterial Pathogens

tNGS also detected higher rates of atypical bacterial infections, including *Mycoplasma* spp. (4.80% vs 1.77%), *Chlamydia pneumoniae* (0.24% vs 0), *Chlamydia psittaci* (0.72% vs 0.04%), and *Legionella* spp. (1.08% vs 0.22%). Detection rates of *Mycobacterium tuberculosis* (4.91% vs 0.75%) and nontuberculous mycobacteria (1.56% vs 0.40%) were also significantly higher in the tNGS group compared with the conventional group (Table 2 and Figure 4).

**Table 2** Comparison of Pathogen Detection Between the tNGS Group and the Conventional Pathogen Detection Group

| Pathogen | tNGS n=834 | Traditional Pathogen Detection n=2263 | P value |
|---|---|---|---|
| Virus | | | |
| SARS-CoV-2 | 193 (23.14) | 1085 (47.95) | <0.001 |
| Influenza A | 154 (18.47) | 320 (14.14) | 0.003 |
| Respiratory Syncytial Virus | 54 (6.47) | 158 (6.98) | 0.620 |
| Epstein-Barr virus | 317 (38.01) | 139 (6.14) | <0.001 |
| Human Rhinovirus | 120 (14.39) | 56 (2.47) | <0.001 |
| Influenza B | 19 (2.28) | 82 (3.62) | 0.062 |
| Human Parainfluenza Virus | 61 (7.31) | 75 (3.31) | <0.001 |
| Adenovirus | 72 (8.63) | 65 (2.87) | <0.001 |
| Herpes Simplex Virus Type 1 | 166 (19.0) | 19 (0.84) | <0.001 |
| Human Metapneumovirus | 47 (5.64) | 5 (0.22) | <0.001 |
| Human Coronavirus | 56 (6.71) | 2 (0.09) | <0.001 |
| Human Herpesvirus 6 | 4 (0.48) | 0 (0) | 0.001 |
| Human Herpesvirus 7 | 61 (7.31) | 0 (0) | <0.001 |
| Human parvovirus B19 | 4 (0.48) | 0 (0) | 0.001 |
| Enterovirus | 17 (2.04) | 0 (0) | <0.001 |
| Varicella-Zoster Virus | 5(0.60) | 0 (0) | <0.001 |

(*Continued*)


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**Table 2** (Continued).

| Pathogen | tNGS n=834 | Traditional Pathogen Detection n=2263 | P value |
|---|---|---|---|
| Bacteria | | | |
| Klebsiella spp | 161 (19.30) | 54 (2.39) | <0.001 |
| Fusobacterium nucleatum | 112 (13.43) | 0 (0) | <0.001 |
| Streptococcus mitis | 105 (12.59) | 0 (0) | <0.001 |
| Pseudomonas aeruginosa | 93 (11.15) | 70 (3.09) | <0.001 |
| Acinetobacter baumannii | 90 (10.79) | 157 (6.94) | <0.001 |
| Haemophilus influenzae | 68 (8.15) | 14 (0.62) | <0.001 |
| Streptococcus anginosus | 52 (6.24) | 0 (0) | <0.001 |
| Micrococcus luteus | 45 (5.40) | 0 (0) | <0.001 |
| Streptococcus pneumoniae | 42 (5.04) | 12 (0.53) | <0.001 |
| Staphylococcus aureus | 37 (4.44) | 38 (1.68) | <0.001 |
| Escherichia coli | 34 (4.08) | 19 (0.84) | <0.001 |
| Enterobacter cloacae | 33 (3.96) | 17 (0.75) | <0.001 |
| Brevundimonas maltophilia | 31 (3.72) | 29 (1.28) | <0.001 |
| Bacteroides fragilis | 26 (3.12) | 34 (1.50) | 0.004 |
| Enterococcus | 23 (2.76) | 23 (1.02) | <0.001 |
| Nocardia | 16 (1.92) | 12 (0.53) | <0.001 |
| Moraxella catarrhalis | 15 (1.80) | 1 (0.04) | <0.001 |
| Tropheryma whipplei | 11 (1.32) | 0 (0) | <0.001 |
| Streptococcus constellatus | 10 (1.20) | 1 (0.04) | <0.001 |
| Serratia marcescens | 8 (0.96) | 6 (0.27) | 0.011 |
| Elizabethkingia miricola | 8 (0.96) | 0 (0) | <0.001 |
| Bordetella pertussis | 7 (0.84) | 0 (0) | <0.001 |
| Proteus mirabilis | 6 (0.72) | 1 (0.04) | <0.001 |
| Streptococcus nonlactis | 6 (0.72) | 0 (0) | <0.001 |
| Streptococcus intermedius | 5 (0.60) | 1 (0.04) | 0.002 |
| Streptococcus pyogenes | 3 (0.36) | 0 (0) | 0.004 |
| Burkholderia cepacia | 3 (0.36) | 0 (0) | 0.004 |
| Burkholderia pseudomallei | 2 (0.24) | 0 (0) | 0.020 |
| Bacteroides vulgatus | 2 (0.24) | 0 (0) | 0.020 |
| Xanthomonas oxytoca | 2 (0.24) | 0 (0) | 0.020 |
| Morganella morganii | 1 (0.12) | 0 (0) | 0.099 |
| Enterobacter hormaechei | 1 (0.12) | 0 (0) | 0.099 |
| Ralstonia mannitolilytica | 1 (0.12) | 4 (0.18) | 0.727 |
| Fungus | | | |
| Aspergillus | 186 (22.30) | 232 (10.25) | <0.001 |
| Pneumocystis jirovecii | 63 (7.55) | 155 (6.85) | 0.496 |
| Candida albicans | 242 (29.02) | 14 (0.62) | <0.001 |
| Candida tropicalis | 60 (7.19) | 9 (0.40) | <0.001 |
| Candida albicans | 29 (3.48) | 7 (0.31) | <0.001 |
| Candida parapsilosis | 26 (3.12) | 6 (0.27) | <0.001 |
| Rhizopus oryzae | 11 (1.32) | 3 (0.13) | <0.001 |
| Rhizopus microsporus | 11 (1.32) | 0 (0) | <0.001 |
| Cryptococcus | 4 (0.48) | 3 (0.13) | 0.071 |
| Trichosporon asahii | 3 (0.36) | 0 (0) | 0.004 |
| Lichtheimia ramosa | 2 (0.24) | 0 (0) | 0.020 |
| Marinifilum fragile | 1 (0.12) | 0 (0) | 0.099 |
| Scedosporium apiospermum | 1(0.12) | 0 (0) | 0.099 |

(*Continued*)

**Table 2** (Continued).

| Pathogen | tNGS n=834 | Traditional Pathogen Detection n=2263 | P value |
|---|---|---|---|
| Atypical pathogens | | | |
| Mycoplasma | 40 (4.80) | 40 (1.77) | <0.001 |
| Chlamydia pneumoniae | 2 (0.24) | 0 (0) | 0.020 |
| Chlamydia psittaci | 6 (0.72) | 1 (0.04) | <0.001 |
| Legionella pneumophila | 9 (1.08) | 5 (0.22) | 0.002 |
| Mycobacterium tuberculosis | 41 (4.91) | 17 (0.75) | <0.001 |
| Nontuberculous Mycobacteria | 13 (1.56) | 9 (0.40) | 0.001 |

## Discussion

Our study demonstrated that tNGS significantly improved the detection of respiratory pathogens, particularly co-infections, compared with conventional diagnostic methods. Patients in the tNGS group exhibited markedly higher detection rates for viral, bacterial, and fungal pathogens, underscoring the superior sensitivity and broad-spectrum coverage of this technique.

Recent studies have emphasized the clinical burden of viral co-infections with fungal or bacterial pathogens, particularly in critically ill or immunocompromised patients. Salazar et al reported invasive pulmonary aspergillosis rates of 19%–35% in intensive care unit (ICU) patients with severe influenza and COVID-19.[24] Oliva and Terrier observed bacterial co-infection rates of 11%–35% in influenza and up to 30% in COVID-19 ICU cases, both associated with worse outcomes.[25] In our study, viral–*Aspergillus* or *Mucor* co-infections were significantly more frequent in the tNGS group compared with the conventional group (17.6% vs 11.2%), and viral–bacterial co-infections were higher (56.8% vs 21.8%). Although these rates are slightly lower than those reported in ICU cohorts, our broader study population suggests that tNGS offers superior sensitivity in detecting early or subclinical fungal co-infections and complex mixed infections often missed by conventional diagnostics.

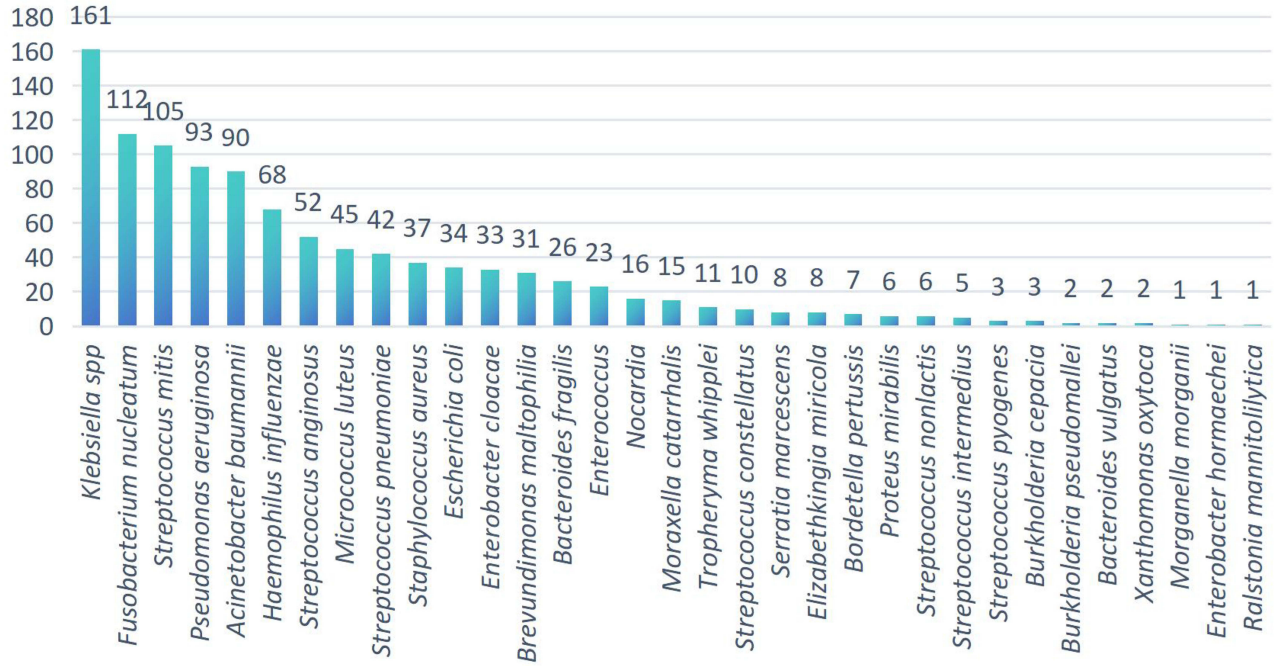


**Figure 2** Detection of viral and bacterial co-infections in the tNGS group.


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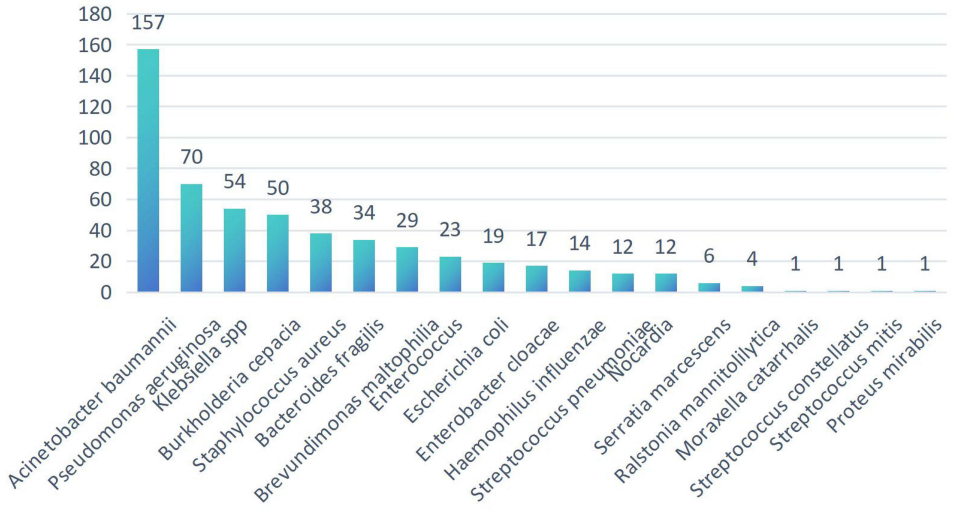


**Figure 3** Detection of viral and bacterial co-infections in the conventional pathogen detection group.

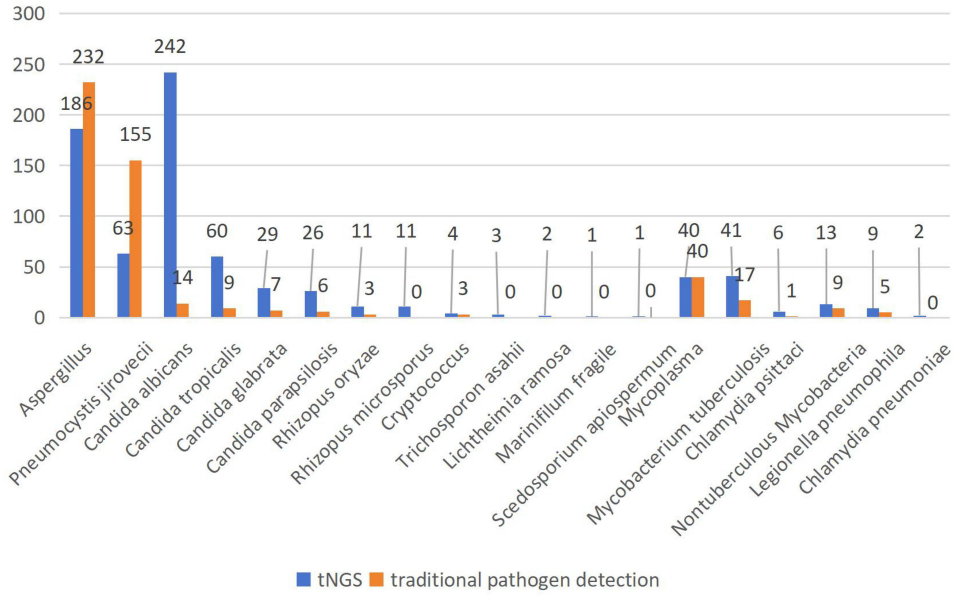


**Figure 4** Detection of fungi, tuberculosis, and atypical pathogens in the tNGS group and the conventional pathogen detection group.

Herpesviruses, particularly EBV and HSV-1, were among the most frequently detected viruses in the tNGS group. Since the onset of COVID-19, herpesvirus reactivation has become increasingly recognized, likely driven by SARS-CoV-2–induced immune dysregulation, including lymphopenia, T-cell exhaustion, and cytokine imbalance, which may reactivate latent viruses in immune-privileged sites.[26–29] In our data, herpesvirus detection was more than twice as frequent as in the conventional group, supporting this association. Other respiratory viruses, such as influenza, parainfluenza, and RSV, may also trigger herpesvirus reactivation by transiently suppressing antiviral immunity, particularly in hospitalized or immunocompromised patients.[30,31] Clinically, herpesvirus reactivation not only reflects immune suppression but may also promote secondary bacterial or fungal infections, aggravate tissue injury, and prolong hospitalization. EBV, for instance, has been linked to enhanced pulmonary inflammation and predisposition to invasive pulmonary aspergillosis.[32,33]

Respiratory viral co-infections are also common, especially in children, immunocompromised patients, and during epidemics, with reported prevalence ranging from 5% to >15% depending on population and diagnostic method.[34,35]

COVID-19 meta-analyses report co-infection rates around 5%,[34] whereas pediatric rates may exceed 20%.[36] In our study, tNGS detected co-infections with two or more non-herpes respiratory viruses in 10.0% of cases, compared with only 1.8% detected by conventional methods, highlighting the superior sensitivity and broader detection capacity of tNGS. Viral co-infections may act synergistically, compromising host defenses, exacerbating inflammation, and predisposing to secondary bacterial or fungal infections. Advanced tools, such as tNGS, provide valuable insights into viral interactions and may inform more effective infection control and treatment strategies.

tNGS yielded substantially higher detection rates of bacterial pathogens compared with conventional methods, including *Klebsiella* spp., *Fusobacterium nucleatum*, and *Streptococcus mitis*. *F. nucleatum*, a strict anaerobe rarely identified in routine cultures, was detected in 13.43% of tNGS cases, suggesting a potential role in polymicrobial lower respiratory tract infections.[37] Although *S. mitis* is not typically classified as a primary respiratory pathogen, its frequent and high-abundance detection in BALF samples suggests a role in polymicrobial infections or dysbiosis-associated lung inflammation. Studies have shown that viridans streptococci, including *S. mitis*, can exacerbate lung injury through biofilm formation and immune modulation, particularly in critically ill patients.[38,39]

In the tNGS group, 1.32% of patients exhibited viral co-infections with *Rhizomucor* or *Rhizopus microsporus*, whereas no such cases were identified by conventional methods. This underscores the diagnostic advantage of tNGS for invasive mucormycosis and other occult infections. The rise in mucormycosis following SARS-CoV-2 infection has been associated with immune dysregulation, mucosal damage, and corticosteroid exposure.[40–42] Traditional fungal cultures have low sensitivity for Mucorales due to sampling and growth limitations, whereas tNGS detects pathogen DNA directly, enabling earlier diagnosis. Thus, clinicians should consider mucormycosis in high-risk viral infection cases and utilize tNGS for prompt detection, particularly during epidemics.

The enhanced detection of mixed infections by tNGS highlights the need to re-evaluate current diagnostic algorithms. Conventional methods often fail to identify multiple or unexpected pathogens, leading to missed opportunities for targeted antimicrobial therapy. Incorporating tNGS into clinical workflows may enable clinicians to tailor treatment more effectively, reduce unnecessary broad-spectrum antibiotic use, and improve surveillance for emerging pathogens.[43,44] This is especially important for immunocompromised patients and those with underlying pulmonary disease, who are highly vulnerable to viral and fungal co-infections. In the post–COVID-19 era, the epidemiology of respiratory infections continues to evolve, underscoring the importance of multiplex and metagenomic diagnostics.

This study has several limitations. First, due to its retrospective design, complete clinical data were not available for all patients, particularly regarding comorbidities, which may have influenced the analysis of mixed infections. Second, some patients in the conventional diagnostic group did not undergo every routine test, although overall microbiological data were relatively comprehensive and systematic, providing robust support for analysis. Finally, the clinical significance of low-abundance or commensal organisms detected by tNGS remains uncertain. Future prospective studies should integrate detailed comorbidity data with standardized microbiological testing to better define the diagnostic and clinical management value of NGS in mixed infections.

## Conclusion

Consistent with our research question and hypothesis, tNGS identified a higher prevalence and broader spectrum of viral co-infections as well as secondary bacterial and fungal infections compared with conventional methods. These findings provide important insights into the complexity of respiratory infections in the post–COVID-19 era and underscore the potential of tNGS as a comprehensive diagnostic tool for the detection of mixed infections in clinical practice.

## Abbreviations

ADV, Adenovirus; BALF, Bronchoalveolar Lavage Fluid; COVID-19, Coronavirus Disease 2019; DTI, Diffusion Tensor Imaging; EBV, Epstein–Barr Virus; EV, Enterovirus; FLUA, Influenza A Virus; FLUB, Influenza B Virus; GM, Galactomannan; HCOV/HCoV, Human Coronavirus; HMPV, Human Metapneumovirus; HRV, Human Rhinovirus; HSV-1, Herpes Simplex Virus Type 1; ICU, Intensive Care Unit; IVD, In Vitro Diagnostic; NGS, Next-Generation Sequencing; PCR, Polymerase Chain Reaction; PIV, Parainfluenza Virus; RSV, Respiratory Syncytial Virus; RT-PCR,

Reverse Transcription Polymerase Chain Reaction; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; tNGS, Targeted Next-Generation Sequencing; VZV, Varicella-Zoster Virus.

## Data Sharing Statement

The datasets used and/or analysed in the current study are available from the corresponding author upon reasonable request.

## Ethics Approval and Informed Consent

This study was conducted in accordance with the principles of the Declaration of Helsinki. The study was approved by the Ethics Committee of the China-Japan Friendship Hospital (Approval No. 2024-KY-409-1). Given the retrospective design of the study, the requirement for informed consent was waived by the Ethics Committee.

## Author Contributions

Study design: LL. Data Collection: RT, XZ, and XL. Statistical analysis: LL. Writing: RT and LL. All the au-thors take full responsibility for the study design, data analysis, interpretation, and manuscript preparation. The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and each author believes that the manuscript represents honest work.

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## Disclosure

The authors report no conflicts of interest in this work.

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