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ORIGINAL RESEARCH

Distribution Patterns of Pathogens Causing Lower Respiratory Tract Infection Based on Metagenomic Next-Generation Sequencing

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Purpose: Lower Respiratory Tract Infection (LRTI) is a leading cause of morbidity and mortality worldwide. In this study, the distribution patterns of causative pathogens in LRTI were evaluated within a city-level hospital by combining conventional microbiological tests (CMT) with metagenomic next-generation sequencing (mNGS).

Patients and Methods: This retrospective cohort study involved 160 patients suspected of having LRTI in a single center. Specimens, including bronchoalveolar lavage fluid (BALF), blood, tissue, sputum, and pus were utilized to identify pathogens. The seasonal prevalence of pathogens and co-pathogens involved in multiple infections was analyzed.

Results: A total of 137 patients with 156 samples were included in this study. *Pseudomonas aeruginosa, Corynebacterium striatum*, *Klebsiella pneumoniae, Candida*, and *human herpesvirus* were the top prevalent pathogens. We observed seasonal dynamic variation in the top prevalent bacteria (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and *herpesvirus* (Epstein-Barr virus and Human herpesvirus-7). The majority of patients had single bacterial infections, followed by instances of bacterial-viral co-infections, as well as mixed infections involving bacteria, fungi, and viruses. Notably, the spectrum of co-infecting pathogens was broader among the elderly population, and positive Spearman correlations were observed among these co-infecting pathogens.

Conclusion: Co-infections were prevalent among patients with LRTI, and the pathogens displayed distinct seasonal distribution patterns. The findings underscored the significance of comprehending pathogen distribution and epidemic patterns, which can serve as a basis for early etiological identification.

Keywords: metagenomic next-generation sequencing, low respiratory tract infection, co-pathogens, seasonal prevalence

Introduction

Lower respiratory tract infection (LRTI) remains one of the leading causes of death worldwide.[1] Several well-known pathogens, including *Streptococcus pneumoniae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida, Herpesvirus*, and others, have been identified as significant causes of infection.[2] Nonetheless, nearly half of the cases still have an undetermined etiology,[3,4] despite the extensive application of clinical microbiology tests. In certain scenarios, LRTI can progress to severe pneumonia, multiple organ dysfunction syndrome, and septic shock.[5]

Rapid and precise microbiological diagnoses are essential for the appropriate use of antibiotics and reduce LRTI mortality.[6] However, current clinical diagnosis methods face various challenges, including prolonged turnaround time and low sensitivity of cultures,[6,7] as well as narrow spectrum of smear microscopy, serology tests, PCR tests and multiplex PCR.[4] Recently, metagenomic next-generation sequencing (mNGS) is widely developed and applied as an effective pathogen detection technology due to its capacity to overcome complications from antibiotic exposure[8] and to detect unexpected pathogens.[9] The mNGS approach becomes a valuable supplement to conventional microbiological test (CMT).

mNGS allows for the comprehensive analysis of pathogen profile. Previous studies have demonstrated that the age of the patients as well as regional/seasonal features might affect the distribution of pathogens.[10,11] For example, seasonal

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variation is often observed in human bacterial and viral infections.[11–13] *Pseudomonas aeruginosa, Enterobacter cloacae, Acinetobacter baumannii* and *Klebsiella pneumoniae* were more prevalent during the warmer months of the year.[13–15] Thus, a comprehensive analysis of the pathogens among LRTI patients at a city-level hospital can enhance our comprehension of these infections.

Therefore, we conducted a retrospective study at the Shijiazhuang People's Hospital of Hebei Province between April 7, 2021, and November 13, 2022, which enrolled 160 hospitalized patients with suspected LRTI. The lower respiratory tract sample or blood was collected and tested by both mNGS and CMT methods. Pathogens identified by mNGS and CMT methods were compared, and their seasonal prevalence was further analyzed.

Materials and Methods

Ethics Statement

This study was carried out in accord with adherence to the principles of the Declaration of Helsinki and approved by the Ethics Review Committee of Shijiazhuang People's Hospital of Hebei Province. Patients' approval and informed consent were waived because of the retrospective design. Patient anonymity was preserved.

Patient Population and Study Design

This retrospective observational study enrolled patients with suspected LRTI who were hospitalized at the Shijiazhuang People's Hospital of Hebei Province between 7 April 2021 and 13 November 2022. The enrollment criteria were (1) new-onset shadows on chest X-ray or computed tomography (CT) and (2) presence of at least one of the following typical symptoms: a) cough, sputum production, dyspnea, chest pain, or exacerbation of existing respiratory symptoms; b) fever; c) clinical signs of lung consolidation or moist rales; d) peripheral leukocytosis ($> 10\times10^9$ /L) or leucopenia ($< 4\times10^9$ /L). The exclusion criteria were (1) patients with incomplete clinical data and (2) patients with no confirmed clinical diagnosis results.

Bronchoalveolar lavage fluid (BALF), blood, tissue, sputum, and pus samples of patients were collected to identify pathogens by conventional microbiological tests (CMT) and mNGS simultaneously. The CMT methods included bacteria and fungi culturing, PCR of eight respiratory tract pathogens (including *Mycoplasma pneumoniae, Chlamydia pneumoniae,* Respiratory syncytial virus, Human adenovirus, Coxsackievirus B, Influenza A/B virus, and Parainfluenza virus) were performed using virus nucleic acid detection Kit (PCR-Fluorescence Probing) (HXD-01, Coyote, Beijing, China) and *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* nucleic acid combined detection kit (Fluorescence PCR) (Liferiver, Shanghai, China). Serum 1, 3-beta-D-glucan level (G test) for fungi, as well as T-spot and GeneXpert MTB/RIF assay for *Mycobacterium tuberculosis* (TB) identification were also performed. A proportion of 97.4% (152/156) cases had performed culture to detect bacteria and fungi and patients with CMT test were detailed in Supplementary Table 1.

mNGS assays were performed within 24 h after sample collection, including body fluids (2–3 mL), the lung biopsy tissues ($\geq 3 \times 3 \times 3$ mm) and whole blood (≥ 4 mL) samples in EDTA tubes.

Sample Preparation and mNGS Sequencing

Blood samples were centrifuged at 1900 × g and 4°C for 10 min to get plasma for subsequent processing. Sputum samples underwent a liquefaction treatment, while tissues were initially fragmented into small pieces and subsequently homogenized. Plasma cell-free DNA (cfDNA) was extracted using PathoXtract® cell-free Nucleic Acid Kit (WYXM03010S, WillingMed Corp, Beijing, China). DNA from BALF, sputum, tissue, and pus were extracted using PathoXtract® Basic Pathogen Nucleic Acid Kit (WYXM03211S, WillingMed Corp, Beijing, China) according to the manufacturer's protocol. RNA was extracted using PathoXtract® Virus DNA/RNA Isolation Kit (WYXM03009S, WillingMed Corp, Beijing, China). Extracted RNA was reverse transcribed to get the first strand cDNA using SuperScript® Double-Stranded cDNA Synthesis Kit (11917020, Invitrogen, United States).

cfDNA libraries were prepared using KAPA DNA HyperPrep Kit (KK8504, KAPA, Kapa Biosystems, Wilmington, MA, United States), and DNA libraries were constructed using the Illumina® DNA Prep, (M) Tagmentation (20018705, Illumina, San Diego, USA). The quality of the libraries was evaluated on an Agilent 2100 Bioanalyzer (Agilent

Technologies), and qualified libraries were sequenced on the NextSeq™ 550Dx sequencer (Illumina, San Diego, USA) using a 75-bp single-end method. Nuclease free water was used as negative control (NTC) and was set for each sequencing run to control contaminating DNA.

Bioinformatic Analysis

The Trimmomatic v0.40[16] was used to filter out low-quality sequences, contaminated adapters, duplicated reads and reads shorter than 36 bp. Then, the sequences were compared with the human reference genome GRCh37 (hg19) using Bowtie2 to remove human sequences.[17] For taxonomic classification and identification of microbial reads, we utilized Kraken2 with non-redundant nucleotide sequence database of National Center for Biotechnology Information (NCBI).[18]

The following criteria were applied to report the positive pathogens. Reads per ten million (RPTM) was used to quantify pathogen abundance. Bacteria and fungi with RPTM ≥ 20, viruses with RPTM ≥ 3, and special pathogens (including *Cryptococcus, Mycobacterium, Mycoplasma, Chlamydia*, and parasites) with RPTM ≥ 1 were identified as positive.[19,20]

Statistical Analysis

The Wilcoxon-Mann-Whitney test was used for comparison between groups, and Fisher's exact test was used for categorical variables. Statistical analyses were performed using Prism 9 (GraphPad, La Jolla, CA). *P*-values below 0.05 were considered statistical significance. The correlation between patient characteristics associated with the presence of multiple pathogen types and the representative microorganism was assessed using Spearman's rank correlation coefficient.

Results

General Characteristics of the Patients

A total of 160 suspected LRTI patients were enrolled in this study. After excluding 21 patients who did not undergo CMT testing and 2 patients with undiagnosed disease, 156 samples from 137 patients were used for subsequent analysis (Figure 1). Seventeen patients provided ≥ 2 samples for mNGS testing, which from different locations and/or at different time. Among them, 15 patients provided 2 samples, 2 patients provided 3 samples (Supplementary Table 1). The samples analyzed in the study included 129 BALF, 10 blood, 9 sputum, 7 tissue, and 1 pus. The age of the patients ranged from 14 to 87 years, with a median of 63 years (60.1 ± 15.1). Males made up 62.8% of the patients (Table 1). The most common diagnoses were pneumonia (54.7%) and severe pneumonia (25.5%).

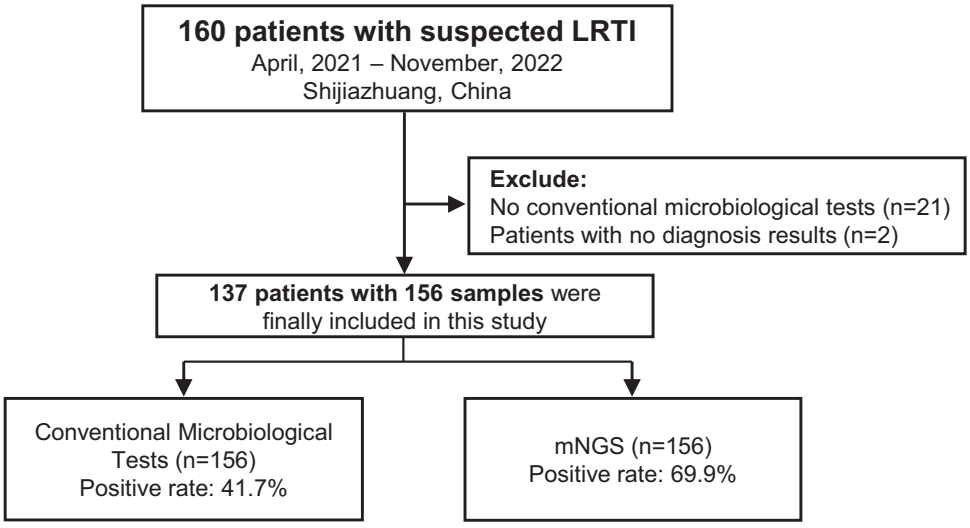

Figure 1 Flow diagram of patient's inclusion in this study.

Table 1 Characteristics of the Patients

Characteristics	Patients[#], n (%)
Age, years, mean ± standard deviation	60.1 ± 15.0
Gender	
Male (%)	86 (62.8%)
Female (%)	51 (37.2%)
Infection types (n=137)	
Pneumonia	75 (54.7%)
Severe pneumonia	35 (25.5%)
Tuberculosis	9 (6.6%)
Lung abscess	7 (5.1%)
AECOPD	5 (3.6%)
Bronchitis	1 (0.7%)
Other pulmonary infection	13 (9.5%)
Non-infection	3 (2.2%)
Any comorbidity, n (%)	
Bronchiectasis	4 (2.9%)
COPD	1 (0.7%)

Notes: [#]Individual patients may have multiple diagnosis. Other pulmonary infection including interstitial lung disease, pulmonary shadow patients with pathogens detected, and finally diagnosed with pulmonary infection patients. Non-infection represents patients had aberrant lung imaging, negative pathogen findings from mNGS and CMT testing, and a diagnose of unclear infection type.
Abbreviations: AECOPD, acute exacerbation chronic obstructive pulmonary disease; COPD, chronic obstructive pulmonary disease.

Pathogens Detected by CMT and mNGS

An overall positive rate of 69.9% (109/156) was detected by mNGS. Among all sample types, the positive rate of sputum was 88.9% (8/9), BALF was 74.4% (96/129), tissue was 28.6% (2/7) and blood was 20% (2/10). The only one pus samples also showed positive result (Figure 2A). For the CMT results, 41.7% (65/156) samples showed positive results (Figure 2B). Further analysis showed that mNGS and CMT were both negative in 24% cases and both positive in 36% samples. Among the double-positive cases, 12.5% samples showed complete match pathogens, 53.6% showed partial match and the rest 33.9% demonstrated mismatch (Figure 2C).

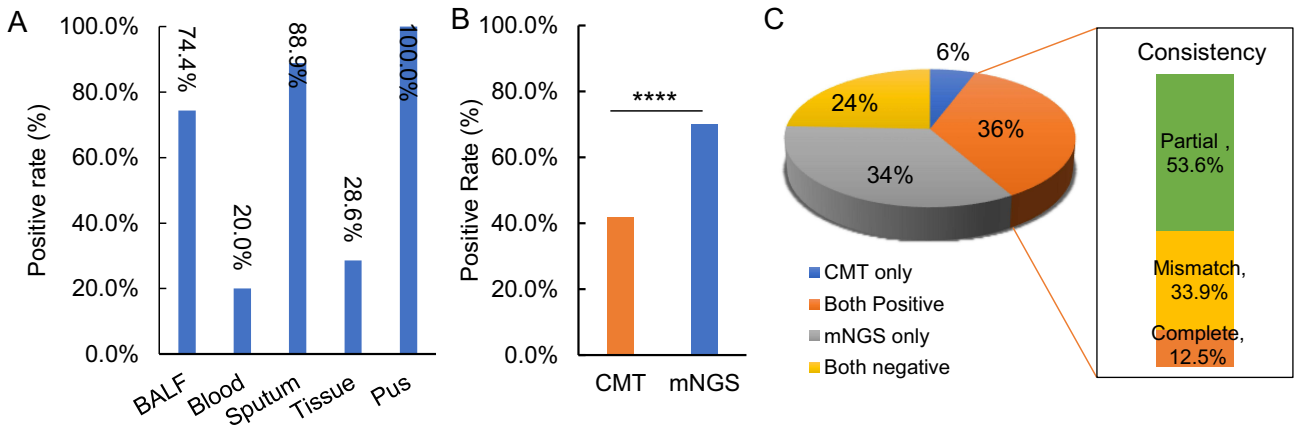

Figure 2 Pathogen detection rate and concordance between CMT and mNGS. (**A**) The positive rate of mNGS for different kinds of specimen. (**B**) The positive rate of CMT and mNGS results. *P* value present with ****Indicating less than 0.0001. (**C**) Concordance of detected pathogens between mNGS and CMT.

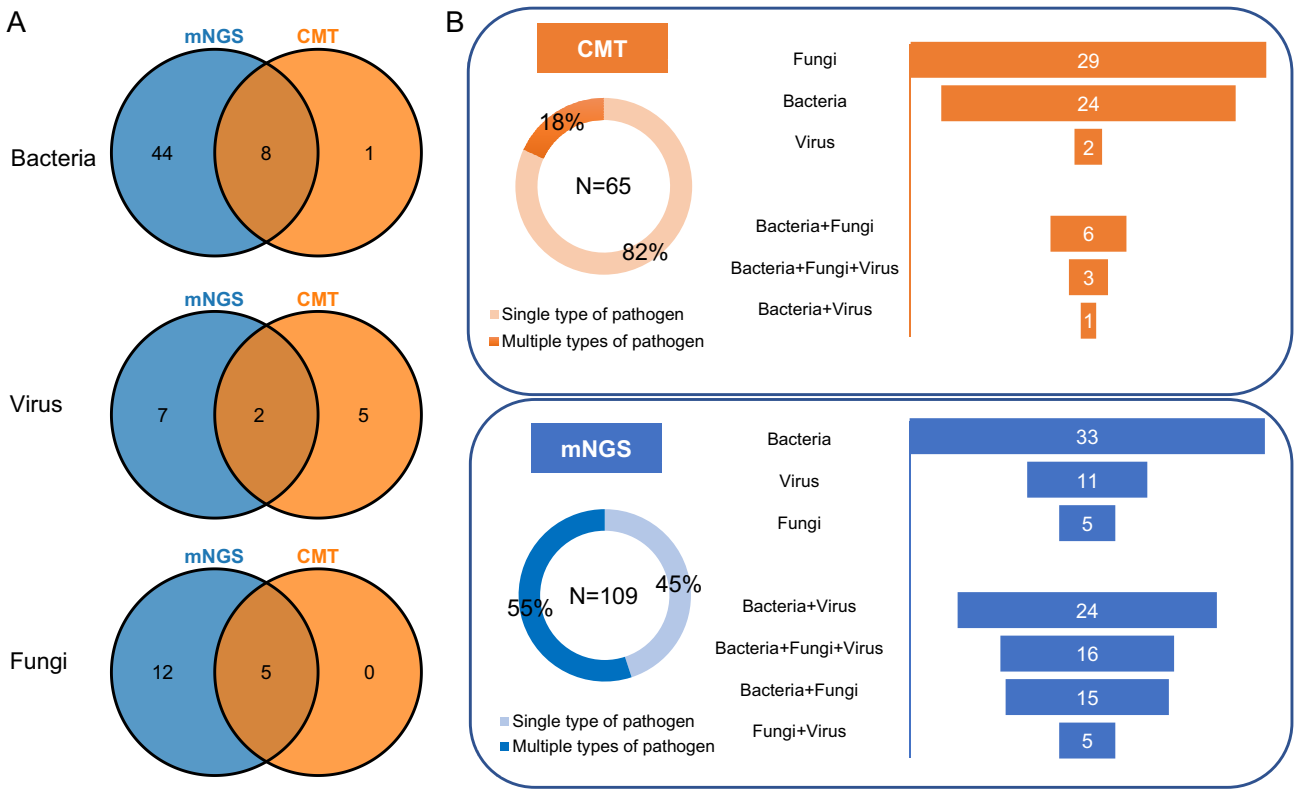

Figure 3 Pathogens identified by CMT and mNGS. (**A**) Veen diagram showed the overlapped numbers of pathogen species detected by mNGS and CMT. (**B**) The type of infected pathogens for the patients based on the CMT and mNGS results.

Among the detected pathogens, there were 8 species of bacteria, 2 viruses and 5 fungi were identified by both mNGS and CMT methods. Additionally, 44 species of bacteria, 7 viruses and 12 fungi were only detected by mNGS, while another 1 species of bacteria, and 5 viruses were solely detected by CMT. Almost all of the bacteria and fungi detected by CMT were also detected by the mNGS technique (Figure 3A). Furthermore, 9 samples had positive results by CMT but negative by mNGS, including *Candida albicans* (n = 5), fungi (n = 2), Epstein-Barr virus (EBV, n = 1), and *Pseudomonas aeruginosa* (n = 1) in these samples (Table 2).

Table 2 The Samples with Only Positive Pathogen Results by CMT

Sample ID	Diagnosis	mNGS Specimen	CMT Specimen	CMT Results[#]
10	Pulmonary infection	Blood	BALF	EBV
12	Pneumonia	BALF	Sputum	*Candida albicans*
17	Severe pneumonia	Blood	Sputum	*Candida albicans*
20	Pneumonia	BALF	BALF	*Pseudomonas aeruginosa*
23	Pneumonia	Tissue	Sputum	*Candida albicans*
37	Severe pneumonia	BALF	Sputum	*Candida albicans*
56	Severe pneumonia	Blood	Sputum	*Candida albicans*
75	Severe pneumonia	Blood	Serum	Fungi
103	Pneumonia	BALF	Serum	Fungi

Note: Fungi means the pathogen was detected by G test.
Abbreviation: [#]EBV, Epstein-Barr virus.

We classified the samples into two categories based on the types of pathogens identified: single type of pathogen infection and multiple types of pathogen infection. Among the mNGS findings, the proportion of the two categories were close (45% vs 55%, $P > 0.05$). Bacterial infection, bacteria and virus co-infections, and mixed bacteria-fungi-virus infections were the most common kinds of infection (Figure 3B). While in CMT results, only 18% of cases identified multiple types of pathogens. The top three prevalent infection types were single fungi infection, bacterial infection, and bacteria and fungi co-infections (Figure 3B).

Detailed pathogen profiles were shown in Figure 4. In both mNGS and CMT results, *Pseudomonas aeruginosa* (n = 24), *Corynebacterium striatum* (n = 16) and *Klebsiella pneumoniae* (n = 14) were the leading bacterial pathogens. *Candida* was the most frequently detected fungi, primarily *Candida albicans* (n = 31), *Candida tropicalis* (n = 10) and *Candida glabrata* (n = 7). *Human herpesvirus* was the predominantly virus detected, including EBV, *Human herpesvirus-7* (HHV-7) and Cytomegalovirus (CMV). Additionally, the mNGS approach identified some special bacterial pathogens, including *Mycoplasma pneumoniae* (n = 6) and *Chlamydia psittaci* (n = 2). Among the mNGS results, only 9.6% (5/52) bacteria species were not detected from BALF samples, including *Chroococcidiopsis thermalis* (detected from tissue), *Fusobacterium nucleatum* (tissue), *Filifactor alocis* (Pus), *Bilophila wadsworthia* (sputum) and *Mycolicibacterium smegmatis* (sputum). Furthermore, *herpesvirus* has been found in both blood and sputum samples, and one instance of *Candida tropicalis* was identified in sputum samples.

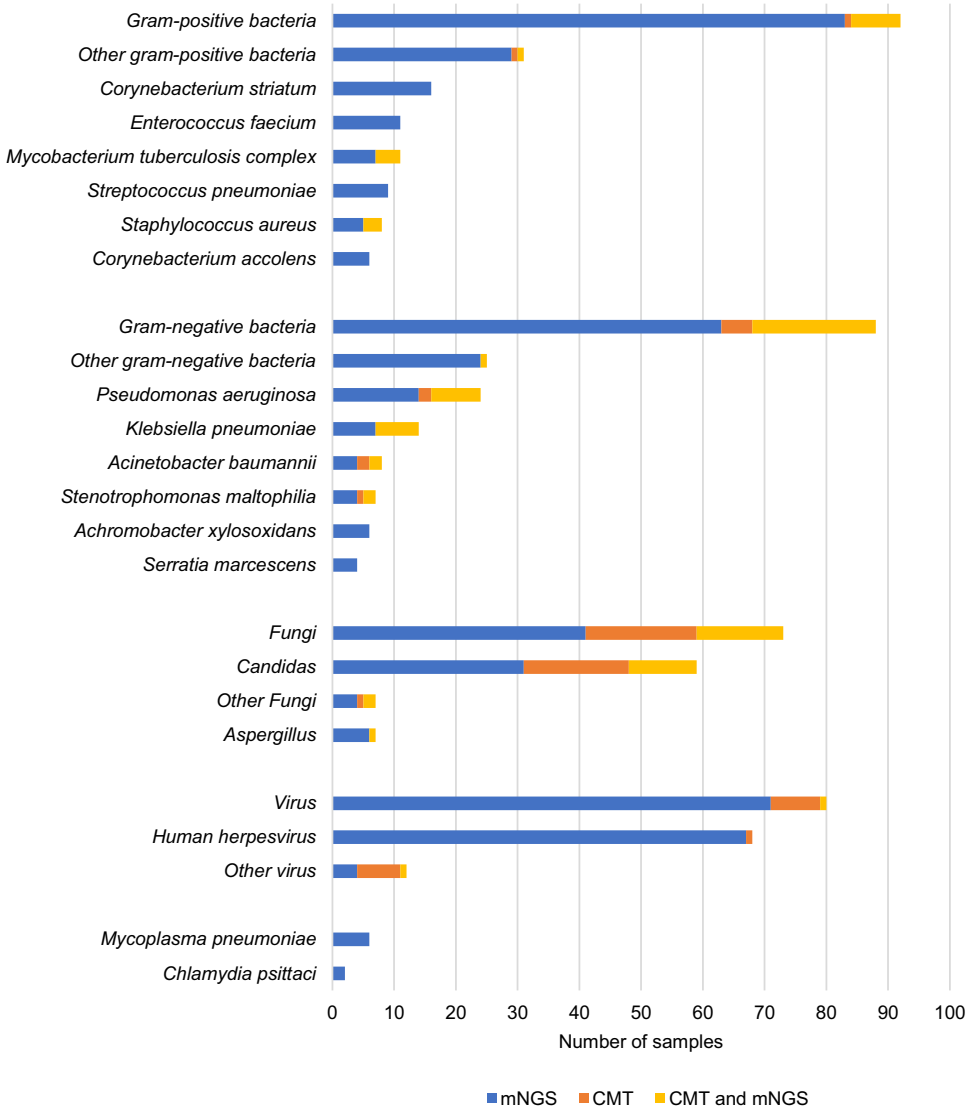

Figure 4 Pathogen profiles detected by mNGS and CMT.

Seasonal Prevalence of the Pathogens

Pathogens found in both mNGS and CMT tests were used to investigate seasonal prevalence of infections. Of the 37 samples with consistent positive results (Figure 2C), 7, 14, 9 and 7 samples were obtained in spring, summer, autumn, and winter, respectively. TB was the most common pathogen in spring, while *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* exhibited higher prevalence in the summer and autumn months. Moreover, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were more likely to be identified in the winter. *Candida albicans* was consistently the most prevalent fungus across all seasons. Virus (Influenza B virus) was detected only in the winter season (Figure 5).

Furthermore, we analyzed the seasonal distribution of all the microorganisms identified by mNGS. There were 33, 50, 37 and 36 samples were collected in spring, summer, autumn, and winter, which achieved 63.6%, 70%, 75.7% and 69.4% positive rate, respectively. Bacteria that have been detected more than once are shown in Figure 6A and B. Gram-negative bacteria like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, as well as gram-positive bacteria including *Corynebacterium striatum* and *Enterococcus faecium*, were detected more frequently during the spring seasons. The types of fungi detected in summer and winter were more diverse (Figure 6C). Additionally, the prevalence of EBV was highest during the spring and summer, while a higher detection of HHV-7 was observed in autumn and winter (Figure 6D).

Characteristics of Co-Infections with Multiple Types of Microorganisms

More than half of the samples had co-infection with multiple types of pathogens (Figure 3B). Only pathogens which have consistent results in both mNGS and CMT were applied to co-infection analysis. In total, 29 samples from 28 patients,

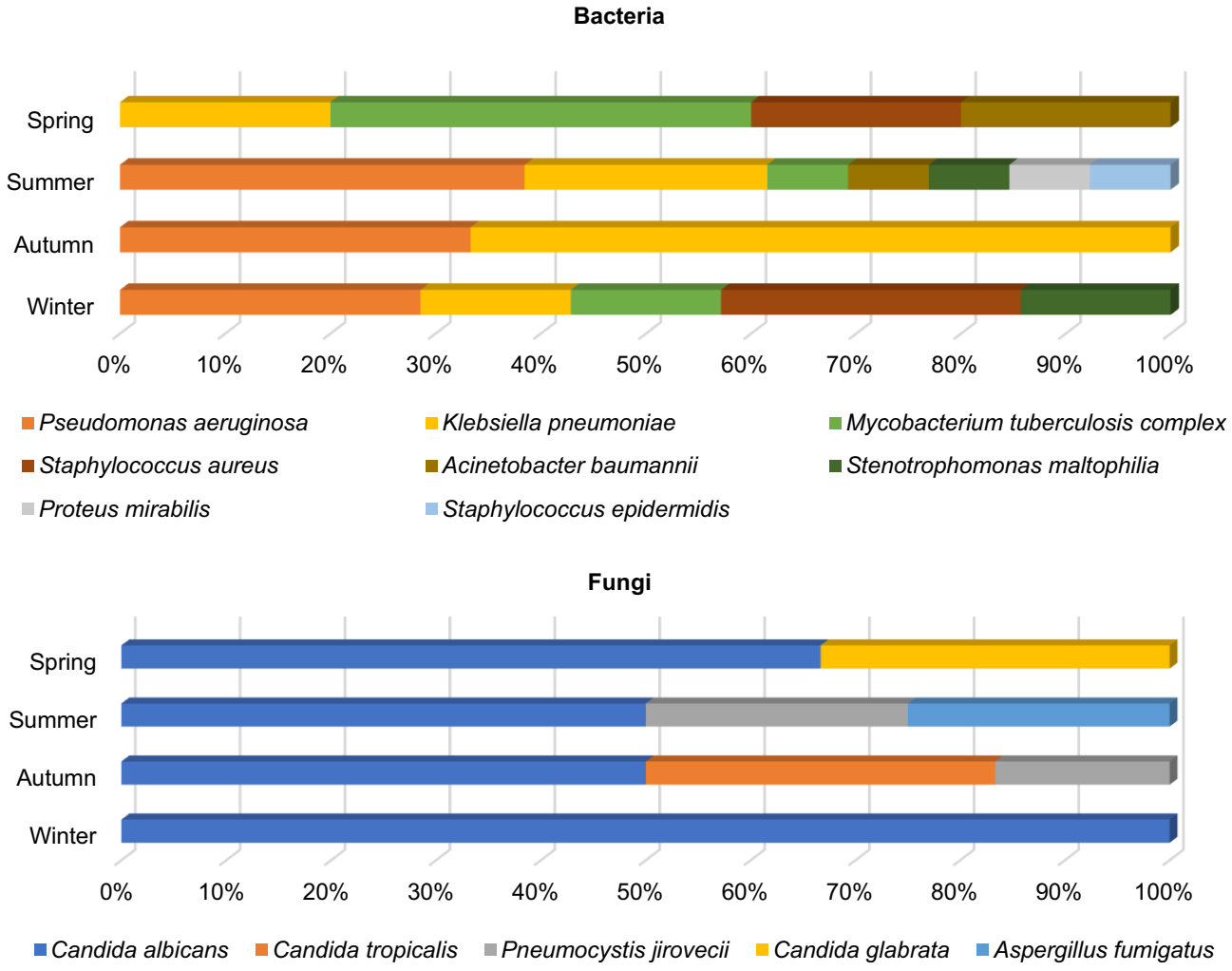

Figure 5 Seasonal prevalence of the pathogens detected by both mNGS and CMT.

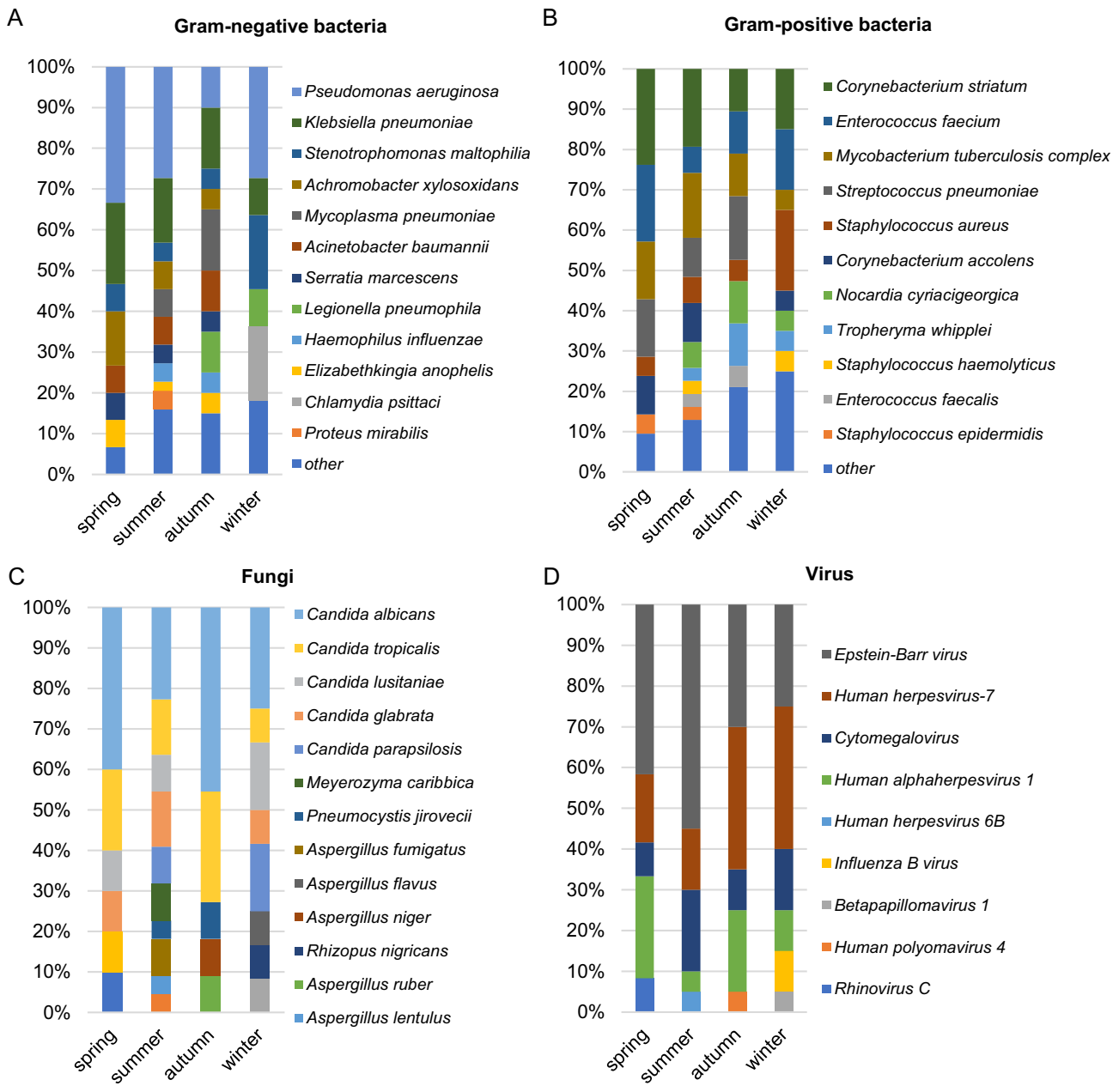

Figure 6 Seasonal prevalence of the pathogens detected by mNGS. (**A**) The distribution of gram-negative bacteria. (**B**) The distribution of gram-positive bacteria. (**C**) The seasonal prevalence of fungi. (**D**) Virus distribution based on seasons. "Other" represents the set of bacteria present in only one sample.

comprising 27 species of bacteria, 12 species of fungi, and 6 viruses were used for the analysis. Male patients accounted for 64.3% of the total cohort, with a median age of 70 (range 37-87) years.

The findings revealed a broader range of co-infecting pathogens in elderly individuals, particularly involving gram-positive bacteria (Figure 7). Moreover, the prevalent forms of infection were attributed to a co-infection of bacteria, fungi, and viruses. No significant correlation was observed between the distribution of co-pathogens and the infection seasons of patients ($P > 0.05$, Supplementary Figure 1). Age exhibited a positive correlation only with *Corynebacterium striatum, Stenotrophomonas maltophilia*, and *Candida lusitaniae* ($P < 0.05$). Notably, all the correlations between the pathogens were positive. CMV and EBV were correlated with *Aspergillus fumigatus* ($P < 0.05$), and *Aspergillus fumigatus* also correlated with *Pneumocystis jirovecii* ($P < 0.001$). HHV-7 was correlated with *Mycoplasma pneumoniae* ($P < 0.001$). *Human alphaherpesvirus* 1 (HSV-1) was significantly correlated with two gram-negative bacteria (*Achromobacter xylosoxidans*

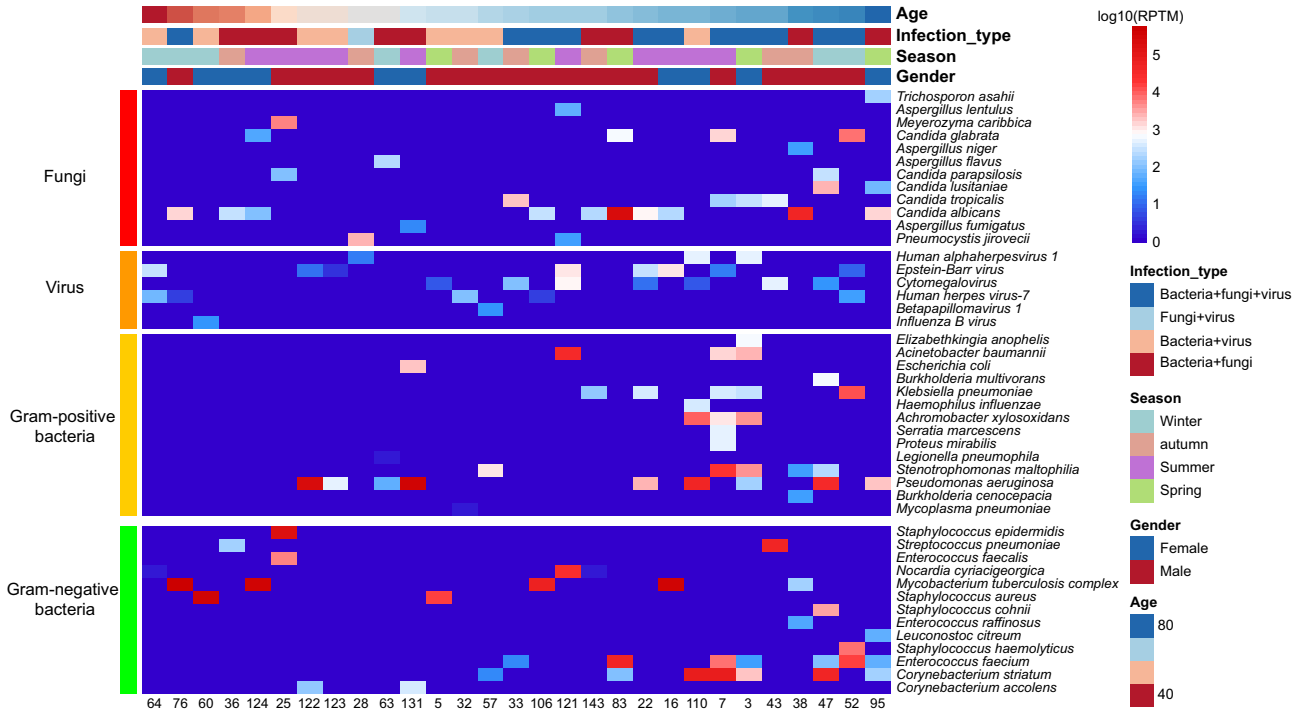

Figure 7 Heatmap of pathogens in samples with at least one consistent pathogen between mNGS and CMT.

and *Elizabethkingia anopheles,* $P < 0.001$). *Candida glabrata* and *Candida tropicalis* were more frequently co-infected with gram-negative bacteria, while other *Candida* species were more likely to coexist with gram-positive bacteria. *Aspergillus* and gram-negative bacteria were more typical co-pathogens (Supplementary Figure 1).

Discussion

Timely and accurately identification of pathogens is critical for treating LRTIs, particularly for critically ill patients or those infected with atypical pathogens. In this study, the seasonal prevalence of pathogens and potential co-pathogens in multiple infection events were analyzed.

Effective control and prevention of infectious diseases rely on a full understanding of their prevalence pattern and the factors that influence transmission.[21] Bacterial infections showed seasonal fluctuation.[11–13] The most prevalent bacterial pathogens discovered in this study were *Pseudomonas aeruginosa, Corynebacterium striatum*, and *Klebsiella pneumoniae* (Figure 4), which was consistent with previous studies.[2,22] Additionally, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were more prevalent during the warmer months of the year.[13–15] We found that *Pseudomonas aeruginosa* are most common in spring and summer, and *Klebsiella pneumoniae* was more prevalent in autumn (Figures 5 and 6). Tuberculosis is one of the most important infectious diseases in humans, and it is difficult to diagnose based on clinical signs and symptoms alone. Among the 9 TB patients diagnosed in this study, 4 cases were identified by both CMT and mNGS, and the remaining 5 were detected by mNGS only (Figure 4). TB was the most common pathogen in spring (Figure 5), which is consistent with the previous studies.[23]

Viral infections also demonstrated seasonal prevalence pattern.[11–13] *Human herpesviruses* were the most frequently detected viruses, including EBV, HHV-7 and CMV (Figure 4). EBV was most frequently detected in spring and summer, while HHV-7 was more frequently detected in autumn and winter (Figure 6D). *Herpesviruses* are generally considered as noncausative pathogens. *Herpesviruses* are known to persist in the host in a latent state following infection, and the mechanisms governing their establishment, maintenance, and reactivation can vary among different subfamilies.[24] Previous studies found that CMV, EBV and HSV-1 were the most commonly reactivated viruses, and their reactivation leading to an 2.052-fold increase in mortality.[25] In addition, reactivation of CMV and EBV is also frequently seen in

severe COVID-19 patients.[26] Twenty samples from 18 patients underwent DNA and RNA mNGS for pathogen detection, and 2 of the cases identified Influenza B virus, 1 of which was further confirmed by PCR; another 2 cases tested by PCR have got negative results. Of the 136 remaining samples, only 4 RNA viruses were detected by PCR (Figure 3A). However, these samples were not performing RNA-sequencing by mNGS. Physicians did not perform RNA sequencing on all the samples using mNGS due to the low detection rate of RNA viruses in mNGS.[27]

Mixed infections were frequently observed among patients with LRTI (Figure 3), and there existed a noteworthy correlation between the pathogens in these instances (Supplementary Figure 1). The presence of polymicrobial pulmonary infection adds to the complexity of antibiotic spectrums and clinical manifestation. Conventional broad-spectrum antibiotics using may yield side effects before identifying the causative pathogens.[28] However, research on mixed infections or co-pathogens is limited. The minimum inhibitory concentration test revealed that *Acinetobacter baumannii* cross-protects *Klebsiella pneumoniae* against cefotaxime, which helps elucidate the basis for their co-existence in polymicrobial infections and provides guidance for clinical medication.[29] Competitive and cooperative interactions between *Staphylococcus aureus* and *Pseudomonas aeruginosa* influence the survival, antibiotic susceptibility, and persistence, consequently the disease progression of cystic fibrosis.[30] The co-existing of *Citrobacter freundii* exacerbated the pathogenicity of *Pseudomonas aeruginosa*.[31] Therefore, improving the understanding of the relationship between co-infectious pathogens and comparing the therapeutic effects of various antibiotics may provide a basis for reducing the use of antibiotics and promoting precision treatment.

This research has some limitations that should be acknowledged, such as the lack of in-depth analysis of the patients' characteristics and clinical symptoms. Additionally, the data was obtained solely from a single hospital, which may have resulted in inherent bias.

Conclusion

In conclusion, this study indicates that mNGS holds great promise for detecting potential pathogens in the clinical samples of LRTI patients. Furthermore, the seasonal prevalence and co-pathogen patterns of the identified pathogens could facilitate the early and precise guidance of treatment procedures.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest in this work.

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