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ORIGINAL RESEARCH

Targeted Next-Generation Sequencing in Pneumonia: Applications in the Detection of Responsible Pathogens, Antimicrobial Resistance, and Virulence

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Background: Targeted next-generation sequencing (tNGS) is a high-throughput and cost-effective diagnostic alternative for pneumonia, with the ability to simultaneously detect pathogens, antimicrobial resistance genes, and virulence genes. We aimed to explore the applicability of tNGS in the co-detection of the responsible pathogens, antimicrobial resistance (AMR) genes, and virulence genes in patients with pneumonia.

Methods: A prospective study was conducted among patients with suspected pneumonia at Ruijin Hospital from March 1 to May 31, 2023. Bronchoalveolar lavage fluid (BALF) or sputum samples were collected and sent for tNGS, metagenomic next-generation sequencing (mNGS), and conventional microbiological tests (CMTs).

Results: In total, 67 BALF and 11 sputum samples from 78 patients were included in the analyses. According to the composite reference standards, the accuracy of tNGS in the detection of responsible pathogens was 0.852 (95% confidence interval 0.786–0.918), which resembled that of mNGS and remarkably exceeded that of CMTs. In addition, 81 AMR genes associated with responsible pathogens were reported, and 75.8% (25/33) priority drug-resistant pathogens could be directly identified. A total of 144 virulence genes were detected for four common pathogens. And patients with virulence genes detected were of higher proportions of severe pneumonia (95.0% vs 42.9%, P = 0.009), acute respiratory distress syndrome (55.0% vs 0%, P = 0.022), and neutrophils (82.3% vs 62.2%, P = 0.026) than those not.

Conclusion: In patients with pneumonia, tNGS could detect the responsible pathogens, AMR genes, and virulence genes simultaneously, serving as an efficient and cost-effective tool for the diagnosis, treatment, and severity indication of pneumonia.

Keywords: targeted next-generation sequencing, pneumonia, pathogen, antimicrobial resistance, virulence

Introduction

Pneumonia has been a leading cause of death in the world for years.[1] A wide variety of microorganisms are able to cause pneumonia, and the presentations can be atypical in many cases.[2,3] Failures in the identification of responsible pathogens

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could result in unnecessary antibiotic usage, development of drug resistance, additional medical costs, and deteriorating prognoses.[3,4] It is imperative to accurately identify the responsible pathogens for pneumonia and promptly initiate appropriate antibiotic treatment.[4] However, conventional microbiological tests (CMTs) are limited by their low sensitivity, narrow detection range, and vulnerability to antibiotic exposure.[5]

Metagenomic next-generation sequencing (mNGS) has been increasingly used in the pathogenic characterization of infectious diseases owing to its high sensitivity, reduced susceptibility to antimicrobial treatment, and short turnaround time.[6] In mNGS, all nucleic acids extracted from samples are sequenced without selection, and the pathogen detection rate of pneumonia has been reported to be markedly improved.[7] However, the processing of sample and sequencing data in mNGS is relatively complicated as a result of the dominant proportion of human genes.[6,7] In addition, the cost of mNGS is high, and a more simple and cost-effective sequencing method with enough throughput is needed.

Targeted next-generation sequencing (tNGS) emerges as the times require. In tNGS, multiple target genes are first amplified using one or more specific primers, and high-throughput next-generation sequencing (NGS) is subsequently performed to sequence the amplified products.[8] The targeted preamplification prior to targeted sequencing can enable sequencing at lower depths, thus simplifying procedures and reducing cost. To date, the costs of tNGS-based tests are about one-fourth of the costs of mNGS-based tests. Besides, the detection range of tNGS is highly adaptable, as various primers can be combined and introduced into the amplification pool prior to the NGS procedure. Currently, tNGS with various detection ranges has been reported to assist in the pathogen detection of pneumonia in several previous studies.[9–13] However, data concerning the detection performance of tNGS for antimicrobial resistance (AMR) genes and virulence genes in patients with pneumonia are still limited. A previous study conducted on bronchoalveolar lavage fluid (BALF) attempted to explore the utilization of tNGS in the detection of AMR and made a preliminary genotype-phenotypic association analysis.[14] As AMR and virulence are two crucial traits of bacteria closely related to clinical treatment and severity of infection, it would be beneficial if tNGS could provide clues to AMR and virulence characteristics at the time of reporting pathogens.[15]

In this study, we attempted to explore the applications of tNGS in pneumonia for the co-detection of responsible pathogens, AMR genes, and virulence genes.

Materials and Methods

Study Population and Sample Collection

A prospective study was conducted among adult inpatients with suspected pneumonia in Ruijin Hospital from March 1 to May 31, 2023. For these patients, CMTs or in-hospital mNGS was ordered by attending physicians according to medical needs. For patients whose culture and mNGS were both conducted on the same samples, residual samples were stored at −80°C for further tNGS. Only one sample from each patient was saved for further tNGS to avoid duplication. Demographic and medical information of patients was collected from medical records. The diagnoses of pneumonia and severe pneumonia were made according to the Infectious Diseases Society of America/American Thoracic Society Criteria.[16,17] To specify, pneumonia was defined as the presence of new lung infiltrate plus clinical evidence that the infiltrate was of an infectious origin, which included the new onset of fever, purulent sputum, leukocytosis, and decline in oxygenation.[16] And severe pneumonia was classified when one major criterion or no less than three minor criteria were meet: 1) minor criteria included respiratory rate $\geq$ 30 breaths/min, PaO_2/FiO_2 ratio $\leq$ 250, multilobar infiltrates, confusion/disorientation, uremia (blood urea nitrogen level $\geq$ 20 mg/dL), leukopenia due to infection alone (white blood cell count $<$ 4000 cells/μL), thrombocytopenia (platelet count $<$ 100000/μL), hypothermia (core temperature $<$ 36°C), and hypotension requiring aggressive fluid resuscitation; 2) major criteria contained septic shock with need for vasopressors and respiratory failure requiring mechanical ventilation.[17] The study was conducted in compliance with the Declaration of Helsinki and approved by the Ethics Committee of Ruijin Hospital (No. 2022–320). Written informed consents were obtained from all patients enrolled accordingly.

CMTs and Antimicrobial Susceptibility Test (AST)

CMTs performed in this study included culture, polymerase chain reaction (PCR)/real-time quantitative PCR (RT-qPCR) based nucleic acid testing, and serological testing. All BALF and sputum samples were cultured for both bacteria and fungi. When mycobacterial infection was suspected, mycobacterial culture was also conducted. Cultured isolates were identified by the VITEK MS system (bioMérieux, Marcy l'Etoile, France). ASTs were performed with the disk diffusion method or the VITEK 2 system (bioMérieux, Marcy l'Etoile, France) according to the recommendations of the Clinical and Laboratory Standards Institute in 2023.[18] Additionally, (1,3)-β-D-glucan tests and galactomannan tests, T-cell spot tests, and Xpert MTB/RIF were made to assist the diagnosis of fungal and mycobacterial infections. Serological antibody detections were performed for atypical pathogens (*Chlamydia pneumoniae, Mycoplasma pneumoniae, Coxiella burneti*, and *Legionella pneumophila*). For viruses, multiplex PCR for 15 viruses (namely influenza A, influenza B, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, parainfluenza virus 4, coronavirus OC43, coronavirus 229E/NL63, respiratory syncytial virus A, respiratory syncytial virus B, adenovirus, human metapneumovirus, rhinovirus, enterovirus, and human bocavirus), and RT-qPCR for Severe acute respiratory syndrome coronavirus 2 were conducted on nasopharyngeal or oropharyngeal swabs. And PCR for Epstein–Barr virus and cytomegalo virus were conducted using blood samples.

mNGS and Bioinformatic Analysis

mNGS was conducted in the clinical laboratory for both DNA and RNA pathogens. The sequencing procedures assembled those described in our previous study.[19] An average of 20 million reads were generated for each sample in the mNGS workflow. For the bioinformatic analysis, adapter contaminants and low-quality reads were filtered by fastp (version 0.19.5). And reads belonging to humans were discarded with reference to the human genome assembly GRCh38 by Bowtie2 (version 2.3.4.3). The remaining reads were aligned to the microorganism database with kraken2 (version 2.1.2).[19] The relative abundances of certain microorganisms were calculated for each sample.

tNGS and Bioinformatic Analysis

Multiplex PCR-based tNGS was conducted to identify 420 microbial species, 93 AMR genes, and 190 virulence genes (Supplementary Table 1). DNA extraction was performed using the TIANamp Micro DNA Kit (Tiangen, Beijing, China), and RNA extraction was performed utilizing the QIAamp Viral RNA Mini Kit (Qiagen, Dusseldorf, Germany). After the DNA and RNA were ready, the sequencing library was constructed with the Pathogeno One 400 Plus Kit (Pathogeno, Gungdong, China) following the manufacturer's instructions. For library quality control, the sizes and concentrations of the amplified DNA fragments were checked by agarose gel electrophoresis and a Qubit 4.0 fluorometer (Invitrogen, California, USA), respectively. The qualified library fragment should be around 350 bp in size and no less than 1 ng/μL in concentration. Sequencing was performed on the Illumina MiSeq platform (Illumina, CA, USA) with 50-bp single-end sequencing. The average number of reads per library was approximately 0.03 million to 0.05 million.

In the bioinformatics analysis, low-quality reads and adapters were first removed via fastp (version 0.19.5). The data were subsequently aligned to the microorganism database by kraken2 (version 2.1.2), the Comprehensive Antibiotic Research Database (CARD, https://card.mcmaster.ca) by RGI (version v5.2.0), and the Virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/) by BLASTn (version 2.9.0+).[19,20] Since one or more pairs of primers were used to amplify the targeted genes, the number of reads was normalized based on the number of primer pairs. The concentrations of targeted pathogens, AMR genes, and virulence genes were calculated according to the normalized reads, amplification efficiency, and reference genes in the bioinformatic pipeline.

Interpretation of Pathogens, AMR Genes, and Virulence Genes

Microorganisms identified in tNGS and mNGS procedures were first interpreted with reference to the Johns Hopkins ABX Guide and the reporting criteria in a previous study to identify potential pathogens.[14] All *torque teno viruses* were taken as no-pathogenic and not included in further analysis. Then, potential pathogens were interpreted as definite, probable, possible, or unlikely pathogens according to the composite reference standards by three experienced physicians.[13,21,22] The composite reference standards consisted of clinical adjudication (including clinical presentations, images, and treatment responses) and all microbiological tests (including CMTs, mNGS, and tNGS results).[13,21,22] The

classification criteria were as follows: 1) Definite: microbes detected by CMTs, or by both tNGS and mNGS; 2) Probable: microbes detected by either tNGS or mNGS, consistent with clinical feedback, and considered as the responsible pathogens in pneumonia; (3) Possible: microbes detected by either tNGS or mNGS, maybe pathogenic, but not responsible for the main clinical characteristics of pneumonia; (4) Unlikely: microbes detected by either tNGS or mNGS in patients without infection or inconsistent with the clinical manifestations of pneumonia. Next, pathogens of definite were further categorized as responsible and non-responsible pathogens with reference to clinical data.

To evaluate the performance of tNGS in the detection of responsible pathogens, pathogens of true positive (TP), false positive (FP), true negative (TN), and false negative (FN) were determined (Supplementary Table 2). According to composite reference standards, if a detection method reported a pathogen responsible for pneumonia, it was taken as TP. When a pathogen of clinical importance was reported by a certain detection method but not interpreted as a responsible pathogen according to the composite reference standards, it was considered as FP. Similarly, TN indicated that no clinically important pathogens were reported in patients without pneumonia, and FN indicated that a responsible pathogen was under-reported.

For AMR genes, the AMR genotypes they confer were interpreted according to the CARD. Associations of AMR genes with corresponding bacteria were made with reference to the CARD and previous literature. Likewise, both the VFDB and published literature were utilized in the explanation of the virulence genes detected.

Statistical Analysis

Continuous variables were presented as median (interquartile range, IQR) and categorical variables as counts (percentages) or ratios (95% confidence intervals, 95% CIs). Data between groups were compared by chi-square test or nonparametric test. A two-sided significance level of 0.05 was selected for all analyses. SPSS 26.0 (IBM, New York, USA) was used for data analyses. GraphPad Prism 8.0.1 (GraphPad, Santiago, USA), R 4.4.0 (R Foundation for Statistical Computing), and Adobe Illustrator 27.3.1 (Adobe, California, USA) were utilized for data visualization.

Results

Baseline Characteristics of the Study Population

From March 1 to May 31, 2023, 102 BALF or sputum samples of inpatients with suspected pneumonia were collected. After the exclusion of 16 duplicate samples from the same patients, 5 samples without adequate volume for testing, and 3 samples of poor sequencing quality, 67 BALF and 11 sputum samples from 78 patients were ultimately included in the study (Table 1).

Detection of Pathogens

Totally, 49 kinds of microorganisms were reported as potential pathogens by tNGS, mNGS, and CMTs (Figure 1A and Supplementary Table 2). And multiple pathogens were detected in 61 (78.2%) patients. More kinds (38 vs 17, $P < 0.001$) and more numbers (189 vs 55, $P < 0.001$) of pathogens were reported by tNGS than CMTs, and no significant difference was found in those between tNGS and mNGS (38 vs 40 in terms of type, 189 vs 195 in terms of number). According to composite reference standards, responsible pathogens were selected from among the potential pathogens (Supplementary Table 2).

The accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of tNGS were 0.852 (95% CI 0.786–0.918), 0.845 (95% CI 0.772–0.919), 0.889 (95% CI 0.728–1.050), 0.976 (95% CI 0.943–1.009), and 0.516 (95% CI 0.330–0.702), respectively (Figure 1B and C). Compared with tNGS, the sensitivity of mNGS [0.887 (95% CI 0.822–0.951), $P = 0.399$] was similar, whereas the sensitivity of CMTs was lower [0.505 (95% CI 0.404–0.606), $P < 0.001$]. And the accuracy of tNGS was equal to that of mNGS [0.835 (95% CI 0.766–0.904), $P = 0.717$], and higher than that of CMTs [0.583 (95% CI 0.491–0.674), $P < 0.001$].

Detection of AMR Genes

With regard to AMR genes, 125 AMR genes were reported in 40 of the 78 samples by tNGS (Figure 2 and Supplementary Table 3). Among them, 64.8% (81/125) of AMR genes detected could be associated with responsible

Table 1 Demographic and Clinical Characteristics of the Study Population

Baseline Characteristic	Patients (n=78)
Age, years, median (IQR)	67.5 (59.5–75.0)
Gender, n (%)	
Male	55 (70.5)
Female	23 (29.5)
Body mass index, kg/m^2, median (IQR)	22.2 (19.6–24.4)
Comorbidities, n (%)	
Chronic lung diseases	27 (34.6)
Cardiovascular disease	16 (20.5)
Diabetes mellitus	19 (24.4)
Stroke	12 (15.4)
Malignancy	19 (24.4)
Immunocompromised patients, n (%)	23 (29.5)
Use of antimicrobials before sample collection, n (%)	56 (71.8)
Anti-bacteria	55 (70.5)
Anti-fungi	10 (12.8)
Anti-virus	2 (2.6)
Length of hospital stay	10 (6–23)
Pneumonia, n (%)	
Severe pneumonia	38 (48.7)
Non-severe pneumonia	26 (33.3)
Non pneumonia	14 (17.9)
Department, n (%)	
ICU	38 (48.7)
Non-ICU	40 (51.3)
PaO_2/FiO_2 at sample collection, mmHg, n (%)	
< 200	21 (26.9)
≥ 200	57 (73.1)
Sample type, n (%)	
BALF	67 (85.9)
Sputum	11 (14.1)

Note: *Immunocompromised patients included those with organ transplantation, stem cell transplantation, connective tissue disorder, receipt of chemotherapy or radiation therapy, and chronic steroid use.

Abbreviations: BALF, bronchoalveolar lavage fluid; FiO_2, fraction of inspired oxygen; PaO_2, partial pressure of oxygen; ICU, intensive care unit; IQR, interquartile range.

pathogens, and the rest with resident microbiota (Figure 2). These genes could confer resistance to β-lactams, aminoglycosides, macrolides, fluoroquinolones, phenicols, tetracyclines, and multi-antibiotics. Among all AMR genes associated with responsible pathogens, those conferring resistance to β-lactam antibiotics were the most frequently detected (47/81, 58.0%). Among the background AMR genes, tetracycline resistance genes accounted for the largest proportion (30/44, 68.2%).

Recently, the World Health Organization released the list of drug-resistant bacteria most threatening to human health, named the Bacterial Priority Pathogens List (BPPL).[23] Therefore, with the AMR genes detected, we further explored the applicability of tNGS in the identification of BPPL pathogens (Table 2). There turned out to be 7 kinds of BPPL bacteria in all the samples according to conventional culture and AST results, which were carbapenem-resistant *A. baumannii* (CRAB) (n = 9), carbapenem-resistant *K. pneumoniae* (CRKP) (n = 7), third-generation cephalosporin-resistant *K. pneumoniae* (3GCephRKP) (n = 7), third-generation cephalosporin-resistant *E. coli* (3GCephREC) (n = 2), carbapenem-resistant *P. aeruginosa* (CRPA) (n = 5), methicillin-resistant *S. aureus* (MRSA) (n = 2), and macrolide-resistant

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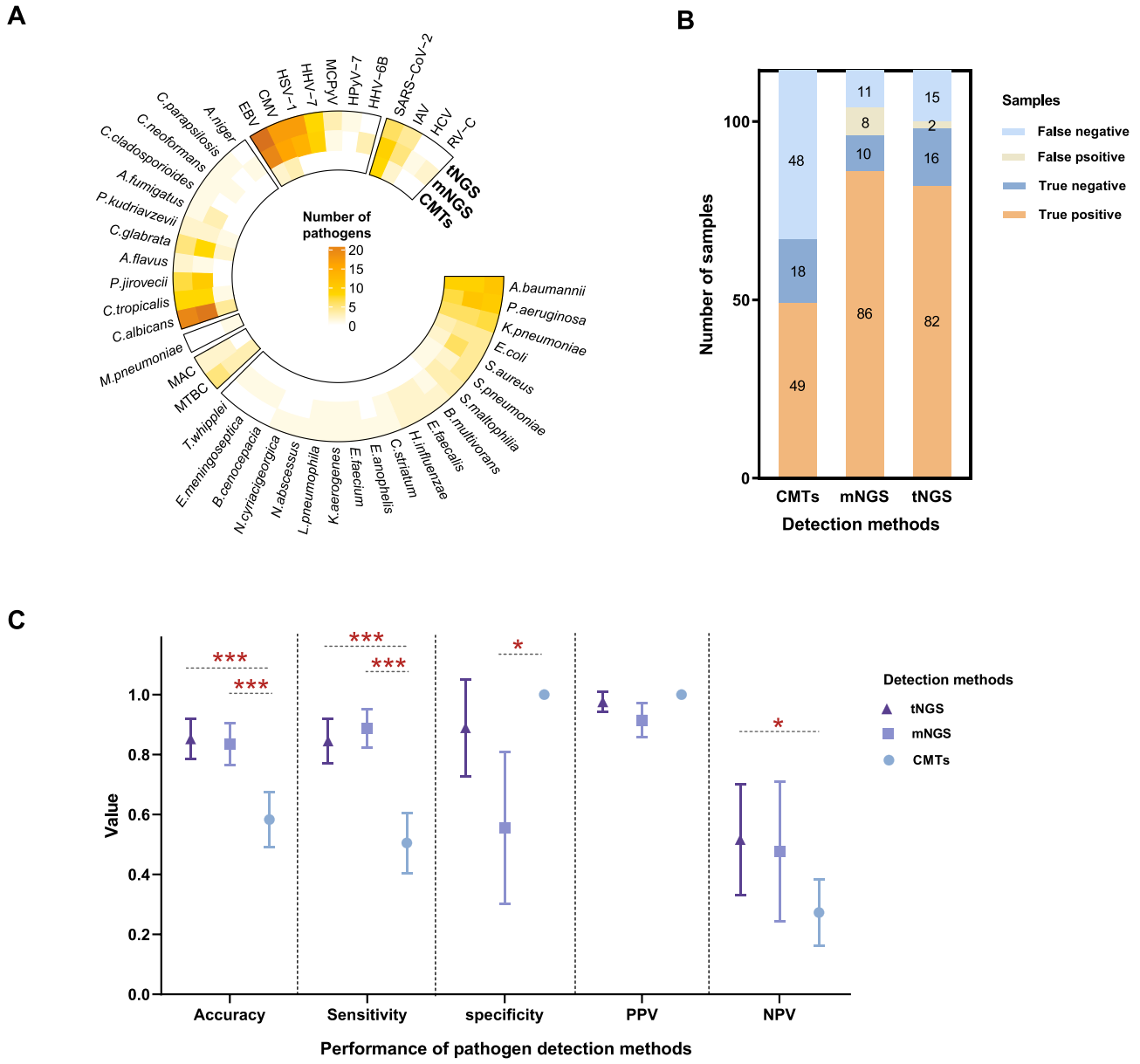

Figure 1 Detection of pathogens by tNGS, mNGS, and CMTs in respiratory samples. (**A**) Potential pathogens reported by tNGS, mNGS, and CMTs. (**B**) Number of samples with true positive, true negative, false positive, and false negative detection for responsible pathogens by tNGS, mNGS, and CMTs. (**C**) Accuracy, sensitivity, specificity, PPV, and NPV for the detection of responsible pathogens of tNGS, mNGS, and CMTs. Circles, squares, and triangles represented the values; Bars represented the 95% CI; ***represented P<0.001; *represented P<0.05.

Abbreviations: CMV, human herpesvirus 5; EBV, Epstein–Barr virus; HCV, hepatitis C virus; HHV-6B, human herpesvirus 6B; HHV-7, human herpesvirus 7; HPV-147, human papillomavirus 147; HPV-49, human papillomavirus 49; HPV-6, human papillomavirus 6; HPyV-7, human polyomavirus 7; HSV-1, human herpesvirus 1; IAV, influenza A virus; MAC, mycobacterium avium complex; MCPyV, Merkel cell polyomavirus; MTBC, mycobacterium tuberculosis complex; RV-C, rhinovirus C; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; PPV, positive predictive value; NPV, negative predictive value.

S. pneumoniae (MRSP) (n = 2). With AMR genes detected by tNGS, 75.8% (25/33) of priority drug-resistant pathogens were directly identified (Table 2 and Supplementary Table 3).

Detection of Virulence Genes

Resistance and virulence are the two major pathogenic characteristics of bacteria. In this study, we also analyzed the probability of tNGS in virulence identification while reporting pathogens and their resistance. In the tNGS panel, primers were developed for virulence factors (VFs) in four pathogens of heavy AMR and mortality burden, which were

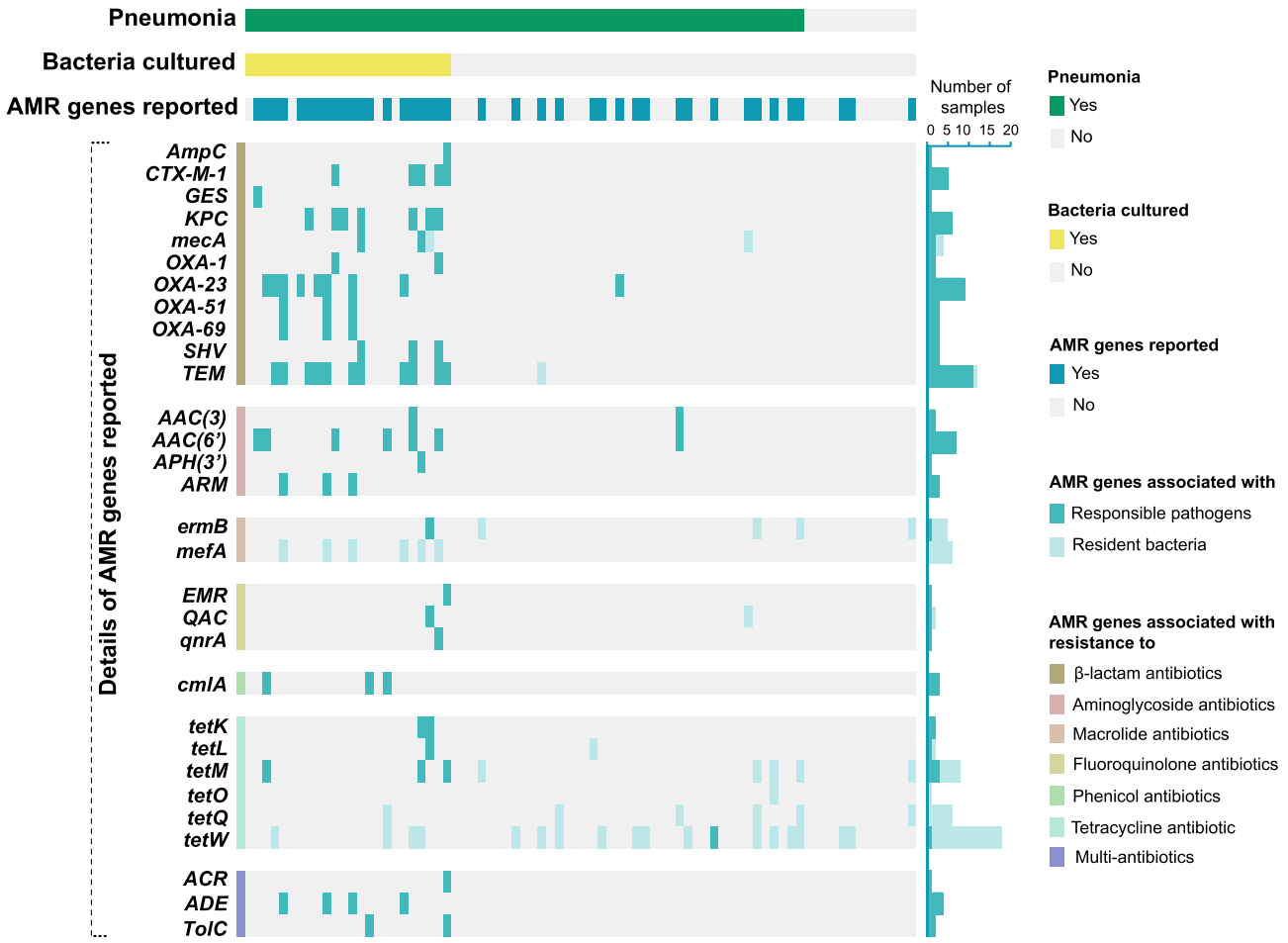

Figure 2 AMR genes detected by tNGS.
Abbreviation: AMR, antimicrobial resistance.

A. baumannii, P. aeruginosa, K. pneumoniae, and *E. coli*.[3,23,24] After sequencing, 125 VF genes of 31 kinds were detected for the four pathogens, with a median normalized copy number of 1297 per millilitre (IQR 374–9272) (Figure 3A and Supplementary Table 4). In the correlation analysis between VF genes detected and disease severity

Table 2 Priority Drug-Resistant Pathogens Detected by tNGS

Patients' Identity	Priority Pathogens	Priority Categories	Responsible AMR Genes Detected
1	CRPA	High priority	–
2	CRPA	High priority	*GES*
3	CRPA	High priority	–
3	CRAB	Critical priority	*OXA-23*
7	CRAB	Critical priority	*OXA-23*
8	CRAB	Critical priority	*OXA-23, OXA-51, OXA-69*
9	MRSP	Medium priority	–
17	CRAB	Critical priority	*OXA-23*
20	CRKP	Critical priority	*KPC*
20	3GCephRKP	Critical priority	*KPC, TEM*
30	CRAB	Critical priority	*OXA-23*
47	CRAB	Critical priority	*OXA-23, OXA-51, OXA-69*

(*Continued*)

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Table 2 (Continued).

Patients' Identity	Priority Pathogens	Priority Categories	Responsible AMR Genes Detected
48	CRKP	Critical priority	*KPC*
48	3GCephRKP	Critical priority	*KPC, OXA-1, CTX-M-1*
51	CRPA	High priority	*KPC*
69	CRAB	Critical priority	*OXA-23, OXA-51, OXA-69*
70	CRKP	Critical priority	*KPC, SHV*
70	3GCephRKP	Critical priority	*KPC, SHV, TEM*
70	MRSA	High priority	*mecA*
81	3GCephREC	Critical priority	–
92	CRAB	Critical priority	–
92	CRKP	Critical priority	–
92	3GCephRKP	Critical priority	–
93	CRAB	Critical priority	*OXA-23*
95	CRKP	Critical priority	*KPC, SHV*
95	3GCephRKP	Critical priority	*KPC, CTX-M-1, SHV, TEM*
97	CRPA	High priority	–
97	MRSA	High priority	*mecA*
99	CRKP	Critical priority	*KPC*
99	3GCephRKP	Critical priority	*KPC*
100	CRKP	Critical priority	*KPC, SHV*
100	3GCephRKP	Critical priority	*KPC, SHV, CTX-M-1, OXA-1, TEM*
102	3GCephREC	Critical priority	*CTX-M-1, TEM, AmpC*

Notes: AMR genes encoding multidrug efflux pumps were not considered responsible for the explanation of AMR phenotypes here, for only the overexpression of these pumps could confer resistance to multi-drugs in the current opinion.
Abbreviations: CRAB, carbapenem-resistant *A. baumannii*; CRKP, carbapenem-resistant *K. pneumoniae*; CRPA, carbapenem-resistant *P. aeruginosa*; MRSA, methicillin-resistant *S. aureus*; MRSP, macrolide-resistant *S. pneumoniae*; 3GCephRKP, third-generation cephalosporin-resistant *K. pneumoniae*; 3GCephREC, third-generation cephalosporin-resistant *E. coli*.

among patients with pneumonia owing to these four pathogens, patients with VF genes detected presented a greater proportion of severe pneumonia (95.0% vs 42.9%, P = 0.009) and acute respiratory distress syndrome (55.0% vs 0%, P = 0.022) (Figure 3B). With respect to inflammation indicators, neutrophil percentages in those with VF genes detected were higher than those without VF genes detected (82.3% vs 62.2%, P = 0.026) (Figure 3C). No significant differences were found in the levels of C-reactive protein, erythrocyte sedimentation rate, or serum amyloid A between the two groups.

Discussion

With a flexible detection range, high-throughput, and relatively low cost, tNGS could be quite helpful in the diagnosis and characterization of infectious diseases. In this study, we demonstrated the benefits of tNGS in the detection of pathogens, AMR, and virulence genes in patients with suspected pneumonia. Briefly, we determined the following findings: 1) The performance of tNGS in the detection of responsible pathogens was superior to that of CMTs and comparable with that of mNGS; 2) 75.8% of BPPL pathogens could be directly identified with AMR genes detected by tNGS; 3) The virulence genes reported by tNGS could be well correlated with the severity of pneumonia and the inflammation level.

tNGS is quite sensitive in the detection of microorganisms, greatly improving the identification of the etiology of pneumonia. In this study, the diagnostic rate of pathogens responsible for pneumonia was greatly improved from 50.5% by CMTs to around 84.5% by tNGS (P < 0.001), in line with previous studies conducted on pneumonia or respiratory samples.[9–12] tNGS also remarkably outperformed CMTs in the diagnosis of other infectious diseases or the pathogen identification with other sample types in most previous studies.[25,26] However, a study on periprosthetic joint infection with 31 samples included reported a non-significantly increased sensitivity of tNGS when compared with culture, which might be due to the relatively small sample size.[27]

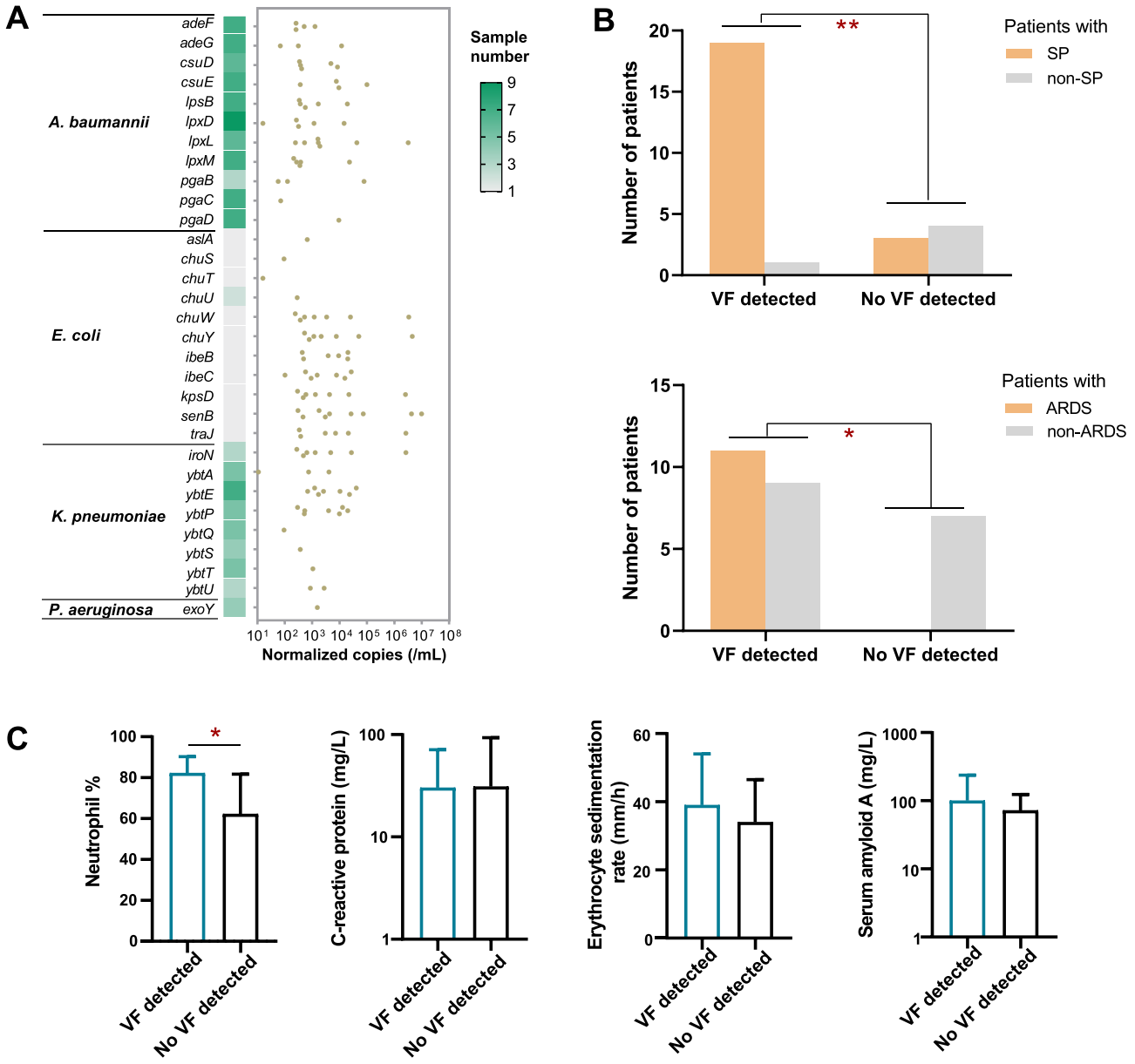

Figure 3 Virulence genes detected by tNGS and their correlations with clinical characteristics. (**A**) Virulence genes detected by tNGS for *A. baumannii, P. aeruginosa, K. pneumoniae*, and *E. coli*, and their normalized copies. (**B**) Virulence genes detected by tNGS in patients with different disease severities. (**C**) Inflammation indicators in patients with virulence genes detected or not. **represented P<0.01, *represented P<0.05.

Abbreviations: ARDS, acute respiratory distress syndrome; SP, severe pneumonia; VF, virulence factor.

tNGS and mNGS are both high-throughput NGS methods that appear successively. In the performance comparison between tNGS and mNGS, the accuracy, sensitivity, specificity, PPV, and NPV of tNGS were on a par with those of mNGS according to our data. Recent studies also presented matched diagnosis or pathogen-detection rates of pneumonia and bloodstream infection.[10,11,25,26] A superior performance of tNGS over mNGS was demonstrated in the diagnostic efficiency of meningitis and encephalitis.[28] To summarize, tNGS is not inferior to mNGS in the identification of pathogens responsible for infection.

Theoretically, mNGS has an unparalleled advantage in pathogen detection, especially for uncommon or newly emerging pathogens, owing to its unlimited detection range over tNGS. While the spectrum of pathogens causing certain kinds of infection is relatively fixed in most clinical practices. With an appropriate panel set, tNGS could perform similarly to mNGS in pathogen detection and infection diagnosis.[10,11,25,26] Besides, as the tNGS workflow involves an

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additional gene amplification process compared with mNGS, the proportion of sequencing reads belonging to pathogens in tNGS could be greatly improved. It was reported that fewer than 10% of the raw mNGS reads were derived from microbial genes,[7] and the median proportion of raw reads that could be attributed to targeted microorganisms was 79.60% (IQR 45.39%–92.9%) in our study. Though the ratio of microorganism reads was improved, the reported limit-of -detection ranges between tNGS and mNGS remained unchanged in BALF.[14] This might be explained by the fact that any nucleotide sequence on a certain gene could be detected and aligned in mNGS workflows, but only primer-containing nucleotide sequences could be amplified and sequenced in tNGS workflows. Besides, tNGS workflows enable the simultaneous detection of DNA and RNA nucleic acids without price doubled, facilitating its clinical implementation with a reduced economic burden.

tNGS was shown to effectively detect AMR genes from clinical samples in this study. A variety of AMR genes were reported, and with genotype–phenotype associations according to the CARD and former reports, 75.8% of BPPL pathogens could be correctly reported directly from clinical samples. A former study explored the possibility of tNGS in AMR detection from BALF samples.[14] AMR genes detected by the Respiratory Pathogen ID/AMR (RPIP) tNGS workflow were demonstrated to be consistent with AST results in 53.8% of the samples.[14] In a study conducted with blood samples, 80.6% carbapenem-resistant or extended-spectrum β-lactamase (ESBL) producing bacteria were successfully identified by a targeted nanopore sequencing method.[26] Since nanopore sequencing and NGS perform similarly in figuring out the existence of AMR genes,[19] targeted nanopore sequencing and tNGS might perform equally as well. It should also be noted that current targeted sequencing workflows, both in this study and in previous ones, could report only the existence of AMR genes but not the gene variations associated with AMR phenotype.[14,26] By redesigning the primers to amplify the full-length of AMR genes or gene parts containing the mutation sites, AMR-associated gene variations might also be detected as described in a previous study.[29]

The applicability of tNGS in the prediction of pathogen virulence was explored in *A. baumannii, P. aeruginosa, K. pneumoniae*, and *E. coli*, which are the most critical gram-negative bacteria notorious for their heavy burdens of prevalence, AMR, and mortality.[3,23,24] *A. baumannii* and *K. pneumoniae* were the main pathogens detected in this study. Among the 11 samples with *A. baumannii* reported in our study, VF genes were co-detected in 7 samples. VF genes regarding biofilm formation (*adeF, adeG, csuD, csuE, pgaB, pgaC*, and *pgaD*) favor the adherence to host cells and growth on abiotic surfaces, thus leading to the persistence of *A. baumannii* in the host and the hospital environment.[30,31] And lipopolysaccharide (LPS) synthesis-related genes (*lpsB, lpxD, lpxL*, and *lpxM*) were involved in the evasion of the host immunity and inflammatory signaling.[30] Besides, three hypervirulent *K. pneumoniae* were identified by tNGS in three samples.[32] Yersiniabactin (encoded by *ybt*) and salmochelin (encoded by *iro*) are two common siderophore systems in *K. pneumoniae* associated with invasive infections.[33–35] The *ybt* and *iro* loci are located within mobile genetic elements and could be transferred among different *K. pneumoniae* clones.[33,34] As to *P. aeruginosa* and *E. coli*, VFs associated with type III secretion system (*exoY*), invasion (*aslA, ibeB, ibeC, kpsD*, and *traJ*), iron uptake (*chuS, chuT, chuU, chuW*, and *chuY*), and exotoxin (*senB*) were detected in this study.[36] After association analysis between VF genes detected and clinical characteristics, it turned out that VF genes reported by tNGS could be well correlated with the severity of pneumonia and the inflammation level.

The targeted preamplification and the subsequently high-throughput sequencing have made tNGS an accurate, broad-spectrum, and cost-effective tool in the etiological characterization of pneumonia and other infectious diseases. However, there remain some challenges in the application of tNGS in clinical practice.[13] tNGS cannot detect genes out of its previously set spectrum, making it unavailable in case of novel or rare pathogens. And the detection range of a certain tNGS panel need periodic update with reference to online databases and published literature. Also, the preamplification process makes it more prone to contamination from adjacent specimens or laboratory environment.

There are some limitations in this study. First, the sample size was relatively small. The results presented in this study could be further validated with a larger sample size. Second, as the pathogenicity of a certain pathogen is strongly influenced by host immunity and other coexistent microorganisms, there might be some misidentification of responsible pathogens from colonized ones. Third, AMR-associated gene variations could not be detected by the tNGS workflow utilized in this study, which could be settled by designing primers to amplify the potential mutation sites.

Conclusion

In summary, we explored the clinical value of tNGS in the diagnosis and characterization of pneumonia. The tNGS workflows utilized in this study could detect the responsible pathogens, AMR genes, and virulence genes simultaneously, rendering it an efficient and cost-effective tool for the diagnosis, treatment, and severity indication of pneumonia.

Ethics Approval and Informed Consent

The study received approval from the Ethics Committee of Ruijin Hospital (No. 2022-320) and was conducted in line with the Declaration of Helsinki. Written informed consents were obtained from all patients involved according to the requirements of the Ethics Committee of Ruijin Hospital.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declared no competing interest.

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