

# Application of mNGS in the Etiological Analysis of Lower Respiratory Tract Infections and the Prediction of Drug Resistance

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ABSTRACT Lower respiratory tract infections (LRTIs) have high morbidity and mortality rates. However, traditional etiological detection methods have not been able to meet the needs for the clinical diagnosis and prognosis of LRTIs. The rapid development of metagenomic next-generation sequencing (mNGS) provides new insights for the diagnosis and treatment of LRTIs; however, little is known about how to interpret the application of mNGS results in LRTIs. In this study, lower respiratory tract specimens from 46 patients with suspected LRTIs were tested simultaneously using conventional microbiological detection methods and mNGS. Receiver operating characteristic (ROC) curves were used to evaluate the performance of the logarithm of reads per kilobase per million mapped reads [lg(RPKM)], genomic coverage, and relative abundance of the organism in predicting the true-positive pathogenic bacteria. True-positive viruses were identified according to the lg(RPKM) threshold of bacteria. We also evaluated the ability to predict drug resistance genes using mNGS. Compared to that using conventional detection methods, the false-positive detection rate of pathogenic bacteria was significantly higher using mNGS. It was concluded from the ROC curves that the lg(RPKM) and genomic coverage contributed to the identification of pathogenic bacteria, with the performance of lg(RPKM) being the best (area under the curve [AUC] = 0.99). The corresponding lg(RPKM) threshold for identifying the pathogenic bacteria was -1.35. Thirty-five strains of true-positive virus were identified based on the lg(RPKM) threshold of bacteria, with the detection of human gammaherpesvirus 4 being the highest and prone to coinfection with Pseudomonas aeruginosa, Acinetobacter baumannii, and Stenotrophomonas maltophilia. Antimicrobial susceptibility tests (AST) revealed the resistance of bacteria containing drug resistance genes (detected by mNGS). However, the drug resistance genes of some multidrug-resistant bacteria were not detected. As an emerging technology, mNGS has shown many advantages for the unbiased etiological detection and the prediction of antibiotic resistance. However, a correct understanding of mNGS results is a prerequisite for its clinical application, especially for LRTIs.

**IMPORTANCE** LRTIs are caused by hundreds of pathogens, and they have become a great threat to human health due to the limitations of traditional etiological detection methods. As an unbiased approach to detect pathogens, mNGS overcomes such etiological diagnostic challenges. However, there is no unified standard on how to use mNGS indicators (the sequencing reads, genomic coverage, and relative abundance of each organism) to distinguish between pathogens and colonizing microorganisms or contaminant microorganisms. Here, we selected the mNGS indicator with the best identification performance and established a cutoff value for the identification of pathogens in LRTIs using ROC curves. In addition, we also evaluated the accuracy of antibiotic resistance prediction using mNGS.

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ower respiratory tract infections (LRTIs) are prevalent worldwide, with high morbidity and mortality, especially in children, elderly, and immunocompromised populations (1, 2). The timely and accurate determination of infectious pathogens in LRTIs is difficult, as LRTIs are caused by hundreds of pathogens, including bacteria, virus, and fungi. In immunocompromised patients, almost all bacteria or fungi can be considered potential pathogens in pulmonary infections (3).

At present, conventional pathogen testing includes microbial cultures, microscopic smears, histopathology, polymerase chain reactions (PCR), nucleic acid hybridization, and serological antibody testing. However, because of the limitations of microbial cultures and microscopic smears in terms of their detection rates, speeds, and available assay targets, it is difficult to satisfy the needs of clinicians (4).

Histopathological analysis is the diagnostic gold standard for invasive fungal infections, but it is time-consuming and lacks pathogen specificity (5). PCR assays require the design of specific primers or probes for microbial pathogens, and thus, the ability to detect pathogens is limited (6). Additionally, it is difficult to accurately predict the window period for serum antibody detection (7).

Due to the lack of microbiological diagnostic methods, clinicians often prescribe antibiotics empirically to patients with negative etiological test results, which can exacerbate a reinfection and drive the emergence of antibiotic resistance and multidrug-resistant pathogens (8). Due to the above reasons, it is necessary to identify a fast and accurate method to detect multiple pathogenic microorganisms from LRTIs.

Advancements in genome sequencing technologies and bioinformatics approaches, such as metagenomic next-generation sequencing (mNGS) (9), provide powerful options for overcoming such clinical diagnostic challenges. The mNGS (also termed high-throughput sequencing technology) has high efficiency and short turnaround time and is an unbiased approach to detect all pathogens in a clinical sample. In addition to the above advantages, mNGS can provide genetic information for evolutionary tracing, the prediction of antibiotic resistance, and the presence of virulence factors and can be used to infer the concentration of pathogens based on the number of reads (10). There have been several reports on the use of mNGS for the detection of pathogenic microorganisms in infections of the central nervous system, blood, respiratory tract, and focal tissue (11-13). However, there are still many challenges preventing its use in clinical applications. One of the primary challenges is that there is no unified standard for interpreting mNGS results. There are three main indicators in the mNGS report, including the sequencing reads, the genomic coverage, and the relative abundance of each organism. It has not yet been reported which indicator is the best for distinguishing between pathogens and colonizing microorganisms or contaminant microorganisms that are present in the sample, reagents, or laboratory environment. This is particularly true for infections of the respiratory tract, which is not a sterile environment (14).

In this study, we first evaluated the performance of the above three indicators to identify pathogenic bacteria in LRTIs using receiver operating characteristic (ROC) curves and established a cutoff value for the identification of pathogens. Using the cutoff value, we analyzed the true-positive viruses in LRTIs and identified which viruses were prone to coinfection with bacteria. In addition, we also analyzed the accuracy of antibiotic resistance prediction using mNGS.

#### RESULTS

**Patient demographics and detection results of different methods.** The 46 patients enrolled in our study were mainly from the respiratory department, geriatrics department, and intensive care unit (ICU), and the average age was 70.67  $\pm$  13.33 years old. Most of the patients were male (*n* = 33, 72%); female patients accounted for 28% (*n* = 13). Among

Patient IDSexAge (yr)Underlying disease(s)Conventional detectionmNGS testP1Male56HypertensionPcePce/HV-7P2Male56HypertensionPac/Ab/PmaPac/Ab/PmaP3Male81AnemiaPac/Ab/PmaPac/Ab/PmaP3Male80COPDPaePae/PmaPae/Ba/KV-AP5Male70HypertensionPae/PmaPae/Pma/Kpn/HU-/FW/CMV/HCov-NL63P6Male62Hypertension, type 2 diabetesPae/PmaPae/Pst/Bca/Eco/EBWP7Male60CorDpol InfarctionPaePae/Su/Ka/RAba/Pma/Kpn/HEWP10Male62COPD/hypertension, type 2 diabetesPae/Pst/Bca/Eco/KDWPae/Pst/Bca/Eco/KBWP11Male90Hypertension, type 2 diabetesPae/PmaAba/Pma/Kpn/EBWP11Male90HypertensionAba/Pma/Kpn/EBWAba/Pma/Kpn/EBWP13Female95Coronary heart diseaseKpnAba/Pma/Kpn/EBWP14Female95Coronary heart diseaseAbaAba/Pma/Kpn/EBWP15Female95Coronary heart diseaseAbaAba/Pma/Kpn/EBWP14Female94COPDAba/Pma/Pae/Eco/Kpn/Sma/EBWP15Female95NoneSau/FmaSau/PmaP16Male94COPDAba/Pma/Pae/Eco/Kpn/Sma/EBWP17Male94COPDAba/Pma/Pae/Eco/Kpn/Sma/EBWP18Male				Detection results for:				
P1MaleMateCecherbain/infarction, hypertensionPcePce/Mba/KpnP2MaleS6HypertensionPac Aba/PmaPac/Aba/PmaP3Male73Senile dementiaPac/PmaPac/Aba/PmaP5Male73Senile dementiaPac/PmaPac/Rbi/AP5Male73Senile dementiaPac/PraPac/Pit/BcaPac/Pit/BcaP6Male62Hypertension, type 2 diabetesPac/Pat/BcaPac/Pit/BcaP6Male94CO/D/hypertension, type 2 diabetesPac/Pat/BcaPac/Pat/BcaP10Male94Hypertension, type 2 diabetesAba/Pma/KpnAba/Pma/KpnP11Male94Hypertension, type 2 diabetesAba/Pma/KpnAba/Pma/KMP12Female80Hypertension, type 2 diabetesAba/Pma/KpnAba/Pma/KMP13Female80HypertensionAba/Pma/KpnAba/Pma/KpnP14Female81Type 2 terspiratory failureSau/Sma/Pae/Sau/Fma/Tae/Ea/Mpi/Sma/EBVP15Female82Live cirthosisSau/Sma/PaeAba/Pma/Pae/SauP16Male65NoneSau/Pma/PaeAba/Pma/RaeP17Male64NopeSau/Pma/Pae/Sau/Sph/MPVP18Male74HypertensionSau/Pma/Pae/Sau/Sph/MpVP19Male74HypertensionSau/Pma/Pae/Sau/Sph/MpVP17Male65NoneSau/Sma/Pae/Sau/Sph/MpVP18Male74 </th <th>Patient ID</th> <th>Sex</th> <th>Age (yr)</th> <th>Underlying disease(s)</th> <th>Conventional detection</th> <th>mNGS test</th>	Patient ID	Sex	Age (yr)	Underlying disease(s)	Conventional detection	mNGS test		
P2MaleMateMateMappendicationPace/Map/MappendicationP3Male81AnemiaPac/Map/MappendicationPac/Map/MappendicationP4Male73Senile dementiaPac/ParaPac/Map/MappendicationP5Male70Hypertension, type 2 diabetesPac/ParaPac/Para/Kap/HHV-1/EBV/CMV/HCov-NL63P7Male60Cerebrai InfactionPacPac/Para/Kap/MappendicationPacP3Male60Cerebrai InfactionPacPac/StaCaCoEVP4Male70Hypertension, type 2 diabetesEcoEco/KapP10Male78Hypertension, type 2 diabetesAba/PmarA(Apn/EBVP11Male78Hypertension, type 2 diabetesAba/Para/KapP12Female80Putomary heart diseaseKpnAba/Para/SapP13Female80Putomary heart diseaseKpnAba/Pac/SauP14Female82Live cirrhosisSau/ParaSau/Sam/Apac/SauP15Female85Coronary heart diseaseSau/ParaSau/Sap/MaVP14Male85Live cirrhosisSau/ParaSau/Sam/MavpP17Male85Ive cirrhosisSau/ParaSau/Sau/MavP18Male94COPDAba/Para/SauSau/Sau/Para/Sau/Eon/Kap/EdV/MappedicationP17Male85Ive cirrhosisSau/ParaSau/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/Sau/Para/S	P1	Male	78	Cerebral infarction, hypertension	Pce	Pce/HHV-7		
P3MaleMaleAnemiaPae/Aba/PmaPae/Aba/PmaPae/Aba/PmaP4Male80COPDPaePae/Pma/Kpu/Hu/Hsu/Hsu/Hsu/Hsu/Hsu/Hsu/Hsu/Hsu/Hsu	P2	Male	56	Hypertension	Pce	Pce/Aba/Kpn		
P4MaleMaleCOPDPaePae/Pna/Kpn/HHV-A55Male73Senile dementiaPae/PnaPae/Pna/Kpn/HHV-1/EBV/CMV/HCov-NL63P6Male62Hypertension, type 2 diabetesPae/Pst/Bca/Eco/EBVP7Male60Cerebrai InfractionPaePae/Sau/Kpn/EcoP9Male73Hypertension, type 2 diabetesEcoEco/KpnP10Male74Hypertension, type 2 diabetesAba/Pma/Kpn/EWP11Male79Hypertension, type 2 diabetesAba/Pma/Kpn/EWP11Male76Hypertension, type 2 diabetesKpn/Sgr/HW-1/HCV-HKU1/EW/HHV-7/RhV-AP13Female80Hypertension, type 2 diabetesAba/Pma/Kpn/EWP14Female80Hypertension, type 2 diabetesAbaAba/Pma/Kpn/EWP15Female80Liver cirrhosisSau/Sma/PaeAba/Paa/Fae/Cov/Kpn/EWP16Male85Liver cirrhosisSau/PmaSau/Sma/Pae/SauP17Male65NoneSau/Pma/PaeSau/Pma/Pae/Eo/Kpn/Sma/EBVP18Male94COPDAba/Pma/PaeSau/Pma/Pae/Eo/Kpn/Sma/EBVP19Male53NoneHUT-JiEW/RhV-2/RhV-3/RhV-	P3	Male	81	Anemia	Pae/Aba/Pma	Pae/Aba/Pma		
P5Male73Senile dementiaPac/PmaPac/Pma/Kyn/HW-1/EBV/CMV/HCov-NLG3P6Male70Hypertension, type 2 diabetesPac/Pst/BcaPac/Ssu/Kpn/ECoP7Male92CorD/hypertension, type 2 diabetesEcoPac/Ssu/Kpn/ECoP10Male92CorD/hypertension, type 2 diabetesEcoEco/KpnP11Male93Hypertension, type 2 diabetesKpnAba/Pma/KpnP10Male94Hypertension, type 2 diabetesKpnAba/Pma/CMVP11Male95Hypertension, type 2 diabetesKpnAba/Pma/CMVP12Female86HypertensionAba/Pma/KpnAba/Pma/CMVP14Female96Vumonary heart diseaseKpnKpn/Sac/Kpn/EBVP15Female92Type 2 respiratory failureSau/SmaSau/PmaP16Male65NoneSau/SmaSau/Spn/MeVP18Male64COPDAba/Pma/PacSau/Spn/MeVP19Male47HypertensionSpn/Eco/KPNSP19Male43NoneAba/Pma/PacP20Male53NoneSpn/Eco/KPNSP21Female63NoneSpn/Eco/KPNSP22Male54NoneSpn/Eco/KPNSP23Male73NopediditisAsperdillis fumigatusP24SauSau/Hero/TMBV/HCov-NLG3/Asperdillus fumigatusSau/Hero/TMBV/HCov-NLG3/Asperdillus fumigatusP23Male	P4	Male	80	COPD	Pae	Pae/RhV-A		
P6Male70Hypertension, type 2 diabetesPae, Pae, Pae, Pae, Pae, Pae, Pae, Pae,	P5	Male	73	Senile dementia	Pae/Pma	Pae/Pma/Kpn/HHV-1/EBV/CMV/HCov-NL63		
P7Male62Hypertension, type 2 diabetesPae/Pst/BcaPae/Pst/Bca/Eco/EBVP8Male60Cerebral informationPaePae/Sau/Kgn/Eco/EBVP9Male92COPD/hypertension, type 2 diabetesEcoEco/KpnP10Male59Hypertension, type 2 diabetesAba/Pma/KpnAba/Pma/Kpn/EBVP11Male59Hypertension, type 2 diabetesAba/PmaAba/Pma/Kpn/EBVP12Female80Pulmoary heart diseaseKpnAba/Hu-/1EBV/RhV-AP14Female82Type 2 respiratory failureSau/Sma/PaeSau/PmaP15Female52Coronary heart diseaseAbaAba/Pma/Pae/Eco/Kpn/EBVP16Male85Liver cirrhosisSau/PmaSau/PmaP17Male65NoneSau/PmaSau/Pma/PaeP18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP18Male74HypertensionSpr/Eco/KN/MARSV-B/N-M/ARV3P20Male53NoneAcid-fast bacillusMtu/Sau/HiV-7P21Female63Type 2 diabetesNoneEBVP22Male55Pleural effusionNoneSau/HuV-1/EBV/HiV-7/EptP23Male64HypertensionNoneSau/HuV-1/EBV/HiV-7/EptP24Male65HypertensionNoneSau/HuV-1/EBV/HiV-7/EptP23Male62NoneKpn/EinSau/HuV-1/EBV/HiV-7/EptP24	P6	Male	70	Hypertension	Spn	Spn/RSV-B		
P8Male60Cerebral infarctionPaePae's MachinePae's Machine<	P7	Male	62	Hypertension, type 2 diabetes	Pae/Pst/Bca	Pae/Pst/Bca/Eco/EBV		
P9Male92COPD/hypertension, type 2 diabetesEcoEco/(pnP11Male78Hypertension, type 2 diabetesAba/Pma/KpnAba/Pma/CMVP11Male59HypertensionAba/PmaAba/Pma/CMVP12Female80Pulmonary heart diseaseKpnKpn/SU/HW-1/HKV-7/RHV-AP14Female80Type 2 respiratory failureSau/Sma/PaeSau/Sma/Pae/Eco/Kpn/EBVP14Female52Type 2 respiratory failureSau/Sma/PaeAba/Pae/SauP15Female52Coronary heart diseaseAbaAba/Pae/SauP16Male53Liver cirrhosisSau/PmaSau/PmaP17Male65NoneSau/Pma/Pae/Eco/Kpn/EBVP18Male54COPDAba/Pma/Pae/Eco/Kpn/SMa/EBVP19Male53NoneAcid fast bacillusMtu/Sau/HHV-7P19Male53NoneAcid fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneEWP23Male57Type 2 diabetes, hypertensionNoneEWP24Male53HypertensionNoneCptP25Female63HypertensionNoneCptP26Female70Type 2 diabetesBrewundimonas diminutaNoneP24Male53HypertensionNoneCptP25Female70Type 2 diabetesNoneCMVP26Fe	P8	Male	60	Cerebral infarction	Pae	Pae/Sau/Kpn/Eco		
P10Male78Hypertension, type 2 diabetesAba/Pma/kpnAba/Pma/kpn/EBVP11Male59HypertensionAba/Pma/kpnAba/Pma/CWVP12Female86HypertensionAbaAba/Pma/CWP13Female86HypertensionAbaAba/Pma/CWP14Female53Coronary heart diseaseAbaAba/Pma/Sec/IHV-1/HCV/HKU1/EBV/HHV-7/RhV-AP15Female55Coronary heart diseaseAbaSau/Sma/PaeP16Male85Liver cirrhosisSau/PmaSau/Pma/Pae/Eou/Kpn/Sma/EBVP17Male65NoneSau/Pma/PaeAba/Pma/Pae/Eou/Kpn/Sma/EBVP18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eou/Kpn/Sma/EBVP19Male47HypertensionSpn/Eco/RSVSpr/Eco/RSVP20Male53NoneRSVRSV-LSV-M/HN-7/CptP21Female63Type 2 diabetesNoneEBVP22Male70Type 2 diabetes, hypertensionNoneCptP24Male70Type 2 diabetesBrevundimonas diminutaNoneP25Female73HypertensionNoneCptP26Female74HypertensionNoneMulti-Xi-MPV/HCov-NL63/Aspergillus fumigatusP27Male62NoneKpnSou/HHV-7/EBVP36Female73HypertensionNoneSou/HHV-7/EBVP37Male62ODPDNone <td>P9</td> <td>Male</td> <td>92</td> <td>COPD/hypertension, type 2 diabetes</td> <td>Eco</td> <td>Eco/Kpn</td>	P9	Male	92	COPD/hypertension, type 2 diabetes	Eco	Eco/Kpn		
P11Male99HypertensionAba/PmaAba/Pma/CMVP12Female90Pulmonary heart diseaseKpnKpn/Sg//HIV-1/HCV/HKU/-HKU//EW/HKU-7/RhV-AP14Female92Type 2 respiratory failureSau/Sma/PaeSau/Sma/Pae/Eor/Kpn/EBVP14Female92Type 2 respiratory failureSau/Sma/PaeSau/Sma/Pae/Eor/Kpn/EBVP15Female92Coronary heart diseaseAbaAba/Pae/SouP16Male83Liver cirrhosisSau/PmaSau/PmaP17Male65NoneSau/PmaSau/Pma/Pae/Eor/Kpn/Sma/EBVP18Male94COPDAba/Pae/Feor/Kpn/Sma/EBVP19Male74HypertensionSpr/Eor/SVSpn/Eor/CMV/Nab/RSV-B/RhV-B/HKV3P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneEbVP23Male77Type 2 diabetes, hypertensionNoneEbVP24Male76HypertensionNoneCptP25Female73HypertensionNoneCptP26Female73HypertensionNoneEbVP27Male74Type 2 diabetesMoneSou/HHV-7/hMPV/HCv-NL63/Aspergillus fumigatusP26Female73HypertensionNoneEbV/CMVP37Male74HypertensionNoneEbV/CMVP38Female75SLENoneS	P10	Male	78	Hypertension, type 2 diabetes	Aba/Pma/Kpn	Aba/Pma/Kpn/EBV		
P12Female90Pulmonary heart diseaseKpnKpn/Sg/rHHV-1/HV-3/PK-V-AP13Female90HypertensionAbaAbaAba/HHV-1/EV/RhV-AP15Female95Coroany heart diseaseAbaSau/Sma/PaeSau/Sma/Pae/Eco/Kpn/EBVP16Male85Liver cirrhosisSau/PmaSau/PmaSau/Sma/Pae/Eco/Kpn/Sma/EBVP17Male65NoneSau/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP19Male53NoneSpn/Eco/RSVSpr/Eco/KNNASV-B/RhV-B/HRV3P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7/CptP21Female63Type 2 diabetsNoneHHV-1/EU/HHV-7/CptP22Male55Pleural effusionRSVSpar/End/KNVP23Male56HypertensionNoneEBVP24Male56HypertensionNoneCMVP25Female63NoneNoneCMVP24Male57PleutensionNoneEBVP24Male50HypertensionNoneCMVP25Female63HypertensionNoneCMVP26Female63HypertensionNoneEBV/CMVP36Female63HypertensionNoneEM/EW/HHV-7P37Male64Aoneinon, COPDNoneEM/EW/HHV-7P3	P11	Male	59	Hypertension	Aba/Pma	Aba/Pma/CMV		
P13Female86HypertensionAbaAba/HH-V-1/EBV/RhV-AP14Female52Type 2 respiratory failureSau/Sma/PaeSau/Sma/Pae/SauP15Female52Coronary heart diseaseAbaAba/Pae/SauP16Male85Liver cirrhosisSau/PmaSau/PmaP17Male65NoneSau/PmaSau/Pma/Pae/Cs/Mp/Sma/EBVP18Male53NoneAba/Pma/PaeAba/Pma/Pae/Cs/Mp/Sma/EBVP19Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P20Male53Pleural effusionRSVRSV-B/MMPVP22Male55Pleural effusionNoneHHV-1/EBV/HHV-7/CptP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male56HypertensionNoneCptP25Female65HypertensionNoneCptP26Female73HypertensionNoneCptP27Male62NoneNoneMoneP28Female73HypertensionNoneEBV/CMVP31Male83Hypertension, COPDNoneSau/HHV-7/HW-7/EBVP33Female70NoneNoneSau/HHV-7P34Male75Stel AnemiaNoneSau/HHV-7P34Male75NpertensionNoneEBV/CMVP35Female70NoneSau/HHV-7P36	P12	Female	90	Pulmonary heart disease	Kpn	Kpn/Sgc/HHV-1/HCoV-HKU1/EBV/HHV-7/RhV-A		
P14     Female     92     Type 2 respiratory failure     Sau/Sma/Pae     Sau/Sma/Pae/Eco/Kpn/EBV       P15     Female     55     Coronary heart disease     Aba     Aba/Pae/Sau       P16     Male     85     Liver cirrhosis     Sau/Pma     Sau/Sma/Pae/Eco/Kpn/Sma/EBV       P16     Male     85     None     Sau/Pma     Sau/Spn/MPV       P18     Male     94     COPD     Aba/Pma/Pae     Aba/Pma/Pae/Eco/Kpn/Sma/EBV       P19     Male     47     Hypertension     Spn/Eco/RSV     Spn/Eco/CMV/Nab/RSV-B/RhV-B/HRV3       P20     Male     53     None     Mtt/Sau/HH-/7     Mtt/Sau/HH-/7       P21     Female     63     Type 2 diabetes, Nypertension     None     BSV       P22     Male     55     Pipeural effusion     None     Sau/HH-/T/MBV/HCov-NL63/Aspergillus fumigatus       P23     Male     63     Ankylosing spondylitis     Aspergillus fumigatus     Sau/HH-/T/MBV/HCov-NL63/Aspergillus fumigatus       P24     Male     64     Ankylosing spondylitis     Aspergillus fumigatus     Sau/HH-/T/MBV/HCov-NL63/Aspergillus fumigatus       P25     Female     70     None     Kpn     CMV       P24     Male     62     None     None     CMV       P27     Male <td>P13</td> <td>Female</td> <td>86</td> <td>Hypertension</td> <td>Aba</td> <td>Aba/HHV-1/EBV/RhV-A</td>	P13	Female	86	Hypertension	Aba	Aba/HHV-1/EBV/RhV-A		
P15Female55Coronary heart diseaseAbaAba/Pae/Sau EBVP16Male85Liver cirrhosisSau/PmaSau/PmaP17Male65NoneSauSau/Syp/hMPVP18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP19Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP19Male53NoneAcid-fast bacillusMtu/Sau/HIV-7P20Male53NoneAcid-fast bacillusMtu/Sau/HIV-7/CptP21Female63Type 2 diabetesNoneEBVP22Male56HypertensionRSVSau/HIV-7/hMPV/HCov-NL63/Aspergillus fumigatusP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitisAspergillus fumigatusSau/HIV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female70Type 2 diabetesBrewindimonas diminutaNoneCPLP26Female73HypertensionNoneEBV/CMVP31Male62COPDNoneEDV/CMVP33Female75SLENoneSau/HIV-7/EBVP34Male75Type 2 diabetesNoneHIV-7/EBVP33Female76AnemiaNoneEcoHPV-819P34Male75Type 2 diabetesNoneBal/CMV/HIV-7P35Male76AnemiaNone	P14	Female	92	Type 2 respiratory failure	Sau/Sma/Pae	Sau/Sma/Pae/Eco/Kpn/EBV		
P16Male85Liver cirrhosisSau/PmaSau/PmaP17Male85NoneSauSau/Spy/hMPVP18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP19Male47HypertensionSpn/Eco/RSVSpr/Eco/KNN/Nab/RSV-B/NN-B/HRV3P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneHHV-1/EBV/HHV-7/CptP22Male55Pleural effusionRSVRSV-B/NMPVP23Male76Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female65HypertensionNoneCptP26Female73HypertensionNoneEN/CMVP30Male62COPDNoneSu/HHV-7/EBVP31Male83Hypertension, COPDNoneSu/CMVP33Female75Type 2 diabetesNoneHPV-1/EBVP34Male75Type 2 diabetesNoneBN/CMVP35Male73Hypertension, COPDNoneEBV/CMV/HIV-7P34Male75Type 2 diabetesNoneHPV-1/EBVP33Female73HypertensionNoneEBV/CMV/HIV-7P34Male73HypertensionNoneEBV/CMV/HIV-7P35Male </td <td>P15</td> <td>Female</td> <td>55</td> <td>Coronary heart disease</td> <td>Aba</td> <td>Aba/Pae/Sau</td>	P15	Female	55	Coronary heart disease	Aba	Aba/Pae/Sau		
P16Male85Liver cirrhosisSau/PmaSau/PmaSau/Spy/MPVP17Male65NoneSauSau/Spy/MPVP18Male94COPDAba/Pma/PaeAb/Pma/Pae/Eco/Kpn/Sma/EBVP19Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneHVI-1/EBV/HHV-1/CPUP22Male55Pleural effusionRSVRSV-B/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male64Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female70Type 2 diabetesBrewundimonas diminutaNoneCptP26Female70Type 2 diabetesBrewundimonas diminutaNoneCMVP27Male62OOPDNoneEBV/CMVSau/HHV-7/hBV/HCov-NL63/Aspergillus fumigatusP28Female73HypertensionNoneEBV/CMVP31Male62OOPDNoneCMVP33Female75SLENoneSau/HHV-7/LEBVP34Male75Type 2 respiratory failureNoneHHV-1/EBV/HHV-7P34Male73HypertensionNoneEBV/CMV/HHV-7P35Male71Type 2 respiratory failureNoneEBV/CMV/HHV-7P34 <td></td> <td></td> <td></td> <td>,</td> <td></td> <td>EBV</td>				,		EBV		
P17Male65NoneSauSau/Spy/hMPVP18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP19Male73HypertensionSpn/Eco/CW/Nab/SV-B/HRV3P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneHHV-1/EBV/HHV-7/CptP22Male57Pleural effusionRSVRSV-B/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitis <i>Aspergillus fumigatus</i> Sau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female63HypertensionNoneCptP26Female70Type 2 diabetes <i>Breundimonas diminuta</i> NoneP27Male62NoneNoneCptP28Female73HypertensionNoneNoneP27Male62OOPDNoneBBV/CMVP38Male62Hypertension, COPDNoneBBV/CMVP31Male83Hypertension, COPDNoneSau/HHV-7/EBVP33Female73Lype 2 diabetesNoneNoneP34Male75SLENoneNoneNoneP34Male73HypertensionNoneEBV/CMV/HV-7P34Male73HypertensionNoneEBV/HW-7P35Male73HypertensionNone <td>P16</td> <td>Male</td> <td>85</td> <td>Liver cirrhosis</td> <td>Sau/Pma</td> <td>Sau/Pma</td>	P16	Male	85	Liver cirrhosis	Sau/Pma	Sau/Pma		
P18Male94COPDAba/Pma/PaeAba/Pma/Pae/Eco/Kpn/Sma/EBVP19Male47HypertensionSpn/Eco/RSVSpn/Eco/RSVP20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneHHV-1/EBV/HHV-7/CptP22Male55Pleural effusionRSVRSV-B/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male56HypertensionNoneCptP25Female65HypertensionNoneCptP26Female70Type 2 diabetesBrevundimonas diminutNoneP27Male62NoneKpnCMVP28Female73HypertensionNoneEBV/CMVP29Male62COPDNoneEBV/CMVP30Male62Hypertension, COPDNoneEBV/CMVP31Male83Hypertension, COPDNoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHIV-1/EBVP36Male75Type 2 respiratory failureNoneHIV-1/EBVP36Male75Type 2 diabetesNoneHIV-1/EBVP37Male73HypertensionNoneEBV/CMV/HIV-7P38Male <td< td=""><td>P17</td><td>Male</td><td>65</td><td>None</td><td>Sau</td><td>Sau/Spv/hMPV</td></td<>	P17	Male	65	None	Sau	Sau/Spv/hMPV		
P19Male47HypertensionSpn/Eco/RSVSpn/Eco/CM//Nab/RSV-B/RhV-B/HRV3P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneHHV-1/EBV/HHV-7/CptP22Male55Pleural effusionRSVRSV-B/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female70Type 2 diabetesBrewundimonas diminutaNoneP26Female73HypertensionNoneCptP27Male62NoneKpnCMVP28Female73HypertensionNoneBBV/CMVP29Male62COPDNoneEBV/CMVP30Male62Hypertension, COPDNoneSau/HHV-1/EBVP31Male75Type 2 diabetesNoneSau/HHV-1/EBVP33Female73Hypertension, COPDNoneSau/HHV-1/EBVP34Male75Type 2 diabetesNoneHIn/HHV-7P34Male75Type 2 diabetesNoneHIn/HV-7P34Male75Type 2 diabetesNoneHIn/HV-7P34Male73HypertensionNoneBV/CMV/HIN-7P35Male71Type 2 diabetesNoneBV/CMV/HIN-7P36Male <td>P18</td> <td>Male</td> <td>94</td> <td>COPD</td> <td>Aba/Pma/Pae</td> <td>Aba/Pma/Pae/Eco/Kpn/Sma/EBV</td>	P18	Male	94	COPD	Aba/Pma/Pae	Aba/Pma/Pae/Eco/Kpn/Sma/EBV		
P20Male53NoneAcid-fast bacillusMtu/Sau/HHV-7P21Female63Type 2 diabetesNoneHHV-1/EBV/HHV-7/CptP22Male55Pleural effusionRSVRSV-8/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female65HypertensionNoneCptP26Female70Type 2 diabetesBrevundimonas dimuntaNoneP27Male62NoneNoneCMVP28Female73HypertensionNoneNoneP29Male62COPDNoneEBV/CMVP31Male83Hypertension, COPDNoneEBV/CMVP31Male83Hypertension, COPDNoneSau/HHV-1/EBVP33Female70NoneEcoHPV-819P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHIn/HU-7/EBVP36Male85Type 2 diabetesNoneHIV-1/EBVP37Male75Type 2 diabetesNoneHU-1/EBVP38Male75Type 2 diabetesNoneEBV/CMV/HIV-7P38Male51Type 2 diabetesNoneEBV/LWV/HIV-7P38Male51Type 2 diabetesNoneEBV/L	P19	Male	47	Hypertension	Spn/Eco/RSV	Spn/Eco/CMV/Nab/RSV-B/RhV-B/HRV3		
P21Female63Type 2 diabetesNoneHHV-1/EBV/HHV-7/CptP22Male55Pleural effusionRSVRSV-B/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male6Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female65HypertensionNoneCptP26Female70Type 2 diabetesBrevundimonas diminutaNoneP27Male62NoneKpnCMVP28Female73HypertensionNoneNoneP29Male62COPDNoneEBV/CMVP30Male62Hypertension, COPDNoneKpn/EBV/HHV-7P31Male83Hypertension, COPDNoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male73HypertensionNoneHIV-1/EBVP36Male51Type 2 respiratory failureNoneHIV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Female71Type 2 diabetesNoneEBV/HIV-7P39Female84COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HIV-7P42Male60Pulmonary heart diseaseNone <t< td=""><td>P20</td><td>Male</td><td>53</td><td>None</td><td>Acid-fast bacillus</td><td>Mtu/Sau/HHV-7</td></t<>	P20	Male	53	None	Acid-fast bacillus	Mtu/Sau/HHV-7		
P22Male55Pleural effusionRSVRSV-B/hMPVP23Male77Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female60HypertensionNoneCptP26Female70Type 2 diabetesBrevundimonas diminutaNoneP27Male62NoneKpnCMWP28Female73HypertensionNoneBEV/CMVP29Male62OPDNoneEBV/CMVP30Male62Hypertension, COPDNoneKpn/EBV/HIV-7P31Male83Hypertension, COPDNoneKpn/EBV/HIV-7P32Female70NoneEcoHV>-1/EBVP33Female70NoneKoneNoneP34Male75Type 2 diabetesNoneNoneP35Male73HypertensionNoneHIV-1/EBVP36Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneEBV/CMV/HHV-7P41Male90NoneNoneEBV/HIV-7 </td <td>P21</td> <td>Female</td> <td>63</td> <td>Type 2 diabetes</td> <td>None</td> <td>HHV-1/FBV/HHV-7/Cpt</td>	P21	Female	63	Type 2 diabetes	None	HHV-1/FBV/HHV-7/Cpt		
P23Male77Type 2 diabetes, hypertensionNoneEBVP24Male36Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female65HypertensionNoneCptP26Female70Type 2 diabetesBrevundimonas diminutaNoneP27Male62NoneKpnCMVP28Female73HypertensionNoneEBV/CMVP29Male62COPDNoneEBV/CMVP30Male62Hypertension, COPDNoneKpn/EBV/HHV-7P31Male83Hypertension, COPDNoneKpn/EBV/HHV-7P32Female70NoneEcoHPV-B19P34Male75SLENoneSau/HHV-1/EBVP35Male76AnemiaNoneHin/HV-7/EBVP36Male85Type 2 diabetesNoneHin/BcaP37Male73HypertensionNoneEBV/CMV/HV-7P38Male71Type 2 diabetesNoneSau/HHV-7P39Female84COPD, hypertensionNoneSBV/EN/HIV-7P43Male71Type 2 diabetesNoneHW-7P44Female67COPD/HypertensionNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7 <t< td=""><td>P22</td><td>Male</td><td>55</td><td>Pleural effusion</td><td>RSV</td><td>RSV-B/hMPV</td></t<>	P22	Male	55	Pleural effusion	RSV	RSV-B/hMPV		
P24Male36Ankylosing spondylitisAspergillus fumigatusSau/HHV-7/hMPV/HCov-NL63/Aspergillus fumigatusP25Female65HypertensionNoneCptP26Female70Type 2 diabetesBrewindimonas diminutaNoneP27Male62NoneKpnCMVP28Female73HypertensionNoneNoneP29Male62COPDNoneEBV/CMVP30Male62HypertensionNoneEBV/CMVP31Male83Hypertension, COPDNoneKpn/EBV/HV-7P32Female70NoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male73HypertensionNoneHIN/HEVP36Male85Type 2 respiratory failureNoneHin/BcaP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female68<	P23	Male	77	Type 2 diabetes hypertension	None	FRV		
P25Female65HypertensionNoneCptP26Female70Type 2 diabetesBrevundimonas diminutaNoneP27Male62NoneKpnCMVP28Female73HypertensionNoneNoneP29Male62COPDNoneEBV/CMVP30Male62Hypertension, COPDNoneKpn/EBV/HHV-7P31Male83Hypertension, COPDNoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHHV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneEBV/CMV/HHV-7P39Female86COPDRhizobium radiobacterEBVP41Male90NoneNoneHIV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female67COPD/HypertensionNoneNoneP45Female67COPD/Hypertension	P24	Male	36	Ankylosing spondylitis	Asperaillus fumiaatus	Sau/HHV-7/hMPV/HCov-NI 63/Asperaillus fumigatus		
P26Female70Type 2 diabetesBrewndimonas diminutaNoneP27Male62NoneKpnCMVP28Female73HypertensionNoneNoneP29Male62COPDNoneEBV/CMVP30Male62HypertensionNoneKin/HV-7/EBVP31Male83Hypertension, COPDNoneKin/HV-1/EBVP32Female57SLENoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneHHV-1/EBVP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneBEV/CMV/HHV-7P38Male71Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneEBV/HHV-7P40Male86COPDRhizobium radiobacterEBVP41Male70NoneNoneHU-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female67COPD/HypertensionNoneNoneP45Male74Coronary heart diseaseNoneNoneP46Male74Coronary heart diseaseNoneNoneP46Male74Coronary heart diseaseNone	P25	Female	65	Hypertension	None	Cnt		
P27Male62NoneKpnCMVP28Female73HypertensionNoneEBV/CMVP29Male62COPDNoneEBV/CMVP30Male62HypertensionNoneHin/HHV-7/EBVP31Male83Hypertension, COPDNoneKpn/EBV/HHV-7P32Female57SLENoneSau/HHV-1/EBVP33Female70NoneKpn/EBV/HHV-7P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHin/BcaP36Male85Type 2 respiratory failureNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRh/-AP45Female68NoneNoneNoneP46Male74Coropary heart diseaseNoneNoneP45Female68NoneNoneNoneP46Male74Coropary heart diseaseNoneNone<	P26	Female	70	Type 2 diabetes	Rrevundimonas diminuta	None		
P28Female73HypertensionNoneNoneP29Male62COPDNoneEBV/CMVP30Male62HypertensionNoneHin/HHV-7/EBVP31Male83Hypertension, COPDNoneKpn/EBV/HHV-7P32Female57SLENoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHHV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male70NoneNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female68NoneNoneNoneP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNoneP46Male74Coronary heart diseaseNoneNone	P27	Male	62	None	Knn	CMV		
P29Male62COPDNoneBBV/CMVP30Male62HypertensionNoneHin/HHV-7/EBVP31Male83Hypertension, COPDNoneKpn/EBV/HHV-7P32Female57SLENoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHHV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P43Male71Type 2 diabetesNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P45Female68NoneNoneNoneP46Male74COPD/HypertensionNoneNoneP46Male74Coropary heart diseaseNoneNone	P28	Female	73	Hypertension	None	None		
P30Male62Cor DNoneHin/HHV-7/EBVP31Male83Hypertension, COPDNoneKpn/EBV/HHV-7P32Female57SLENoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHHV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNoneP46Male74Coronary heart diseaseNoneNone	P20	Malo	62	COPD	None	FRV/CMV		
NoteNoteNoneNoneP31Male83Hypertension, COPDNoneKpn/EBV/HHV-7P32Female57SLENoneSau/HHV-1/EBVP33Female70NoneEcoHPV-819P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHHV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P43Male71Type 2 diabetesNoneHHV-1/HHV-7P44Female67COPD/HypertensionNoneHHV-1/HHV-7P44Female68NoneNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNoneNoneNoneNoneNoneNone	P30	Male	62	Hypertension	None	Hin/HHV-7/FBV		
P31Male83Hypertension, Cor DNoneNoneNoneP32Female57SLENoneSau/HHV-1/EBVP33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHin/BcaP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneHHV-7P44Female68NoneNoneRhizobium radiobacterHHV-7P44Female68NoneNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNoneP46Male74Coronary heart diseaseNoneNone	D31	Malo	02 93	Hypertension COPD	None	Kpp/EBV/HHV_7		
P33Female70NoneEcoHPV-B19P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHIN/BcaP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/CMV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P42Male60Pulmonary heart diseaseNoneHHV-1/HHV-7P44Female67COPD/HypertensionNoneRhiV-AP45Female68NoneNoneNoneRhV-AP46Male74Coronary heart diseaseNoneNoneNone	121	Fomalo	57	SI F	None	Sau/HHV/_1/EBV		
P34Male75Type 2 diabetesNoneNoneP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneHHV-1/EBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneHHV-7P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	D33	Fomalo	70	None	Fco			
P34Male73Type 2 diabetesNoneHHV-1/EBVP35Male76AnemiaNoneHHV-1/EBVP36Male85Type 2 respiratory failureNoneEBV/CMV/HHV-7P37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneRhizobium radiobacterEBV/HHV-7P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	D34	Malo	75	Type 2 diabetes	None	Nope		
P35Male70AnennaNoneHHV-1/LBVP36Male85Type 2 respiratory failureNoneHin/V-1/LBVP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneNoneEBV/HHV-7P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	D25	Male	75	Anomia	None			
P30MaleS3Type 2 respiratory railineNoneFM//BCAP37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneRhizobium radiobacterEBV/HHV-7P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	F 3 3 D 2 6	Male	70 05	Type 2 respiratory failure	None			
P37Male73HypertensionNoneEBV/CMV/HHV-7P38Male51Type 2 diabetesNoneSpn/HinP39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneEBV/HHV-7P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	r 30 D 27	Male	0J 72		None			
P38MaleS1Type 2 diabetesNoneEBV/HHV-7P39Female84COPD, hypertensionNoneSpn/HinP40Male86COPDRhizobium radiobacterEBVP41Male90NoneEBV/HHV-7P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	P3/	Male	75	Typertension	None			
P39     Permare     64     COPD, hypertension     None     Spin/Init       P40     Male     86     COPD     Rhizobium radiobacter     EBV       P41     Male     90     None     EBV/HIV-7       P42     Male     60     Pulmonary heart disease     None     HHV-7       P43     Male     71     Type 2 diabetes     None     HHV-7       P44     Female     67     COPD/Hypertension     None     None       P45     Female     68     None     None     None       P46     Male     74     Coronary heart disease     None     None	P 20	Fomalo		COPD hypertension	None	EDV/HHV-/		
P40     Male     86     COPD     Rhizbolum radiobacter     EBV       P41     Male     90     None     EBV/HHV-7       HCov-NL63       P42     Male     60     Pulmonary heart disease     None     HHV-7       P43     Male     71     Type 2 diabetes     None     HHV-1/HHV-7       P44     Female     67     COPD/Hypertension     None     RhV-A       P45     Female     68     None     None     None       P46     Male     74     Coronary heart disease     None     None	P 39	remaie	04 06	COPD, hypertension	None Rhizahiwa zadiahaataz			
P41     Male     90     None     None     EBV/HHV-/ HCov-NL63       P42     Male     60     Pulmonary heart disease     None     HHV-7       P43     Male     71     Type 2 diabetes     None     HHV-1/HHV-7       P44     Female     67     COPD/Hypertension     None     RhV-A       P45     Female     68     None     None     None       P46     Male     74     Coronary heart disease     None     None	P40	Male	80	COPD	Rhizobium radiobacter			
P42Male60Pulmonary heart diseaseNoneHHV-7P43Male71Type 2 diabetesNoneHHV-1/HHV-7P44Female67COPD/HypertensionNoneRhV-AP45Female68NoneNoneNoneP46Male74Coronary heart diseaseNoneNone	P41	male	90	None	None	EBV/HHV-/		
P43     Male     71     Type 2 diabetes     None     HHV-1/HHV-7       P44     Female     67     COPD/Hypertension     None     RhV-A       P45     Female     68     None     None     None       P46     Male     74     Coronary heart disease     None     None	P42	Male	60	Pulmonary heart disease	None	HCOV-NL63 HHV-7		
P44     Female     67     COPD/Hypertension     None     RhV-A       P45     Female     68     None     None     None       P46     Male     74     Coronary heart disease     None     None	P43	Male	71	Type 2 diabetes	None	HHV-1/HHV-7		
P45 Female 68 None None None P46 Male 74 Coronary heart disease None None	P44	Female	67	COPD/Hypertension	None	RhV-A		
P46 Male 74 Coronary heart disease None None	P45	Female	68	None	None	None		
	P46	Male	74	Coronary heart disease	None	None		

<sup>a</sup>COPD, chronic obstructive pulmonary disease; Aba, Acinetobacter baumannii; Eco, Escherichia coli; Pce, Burkholderia cenocepacia; Kpn, Klebsiella pneumoniae; Pma, Stenotrophomonas maltophilia; Spn, Streptococcus pneumoniae; Pae, Pseudomonas aeruginosa; Pst, Providencia stuartii; Sau, Staphylococcus aureus; Bca, Moraxella catarrhalis; Sma, Serratia marcescens; HHV-7, human betaherpesvirus 7; RhV-A, rhinovirus A; RhV-B, rhinovirus B; HHV-1, human alphaherpesvirus 1; EBV, human gammaherpesvirus 4; CMV, human betaherpesvirus 5; HCov-NL63, human coronavirus NL63; RSV-B, human respiratory syncytial virus B; Sgc, Streptococcus agalactiae; HCoV-HKU1, human coronavirus HKU1; Spy, Streptococcus pyogenes; hMPV, human metapneumovirus; Nab, Nocardia abscessus; Mtu, Mycobacterium tuberculosis; SLE, systemic lupus erythematosus; Cpt, Chlamydia psittaci; Hin, Haemophilus influenzae; HPV-B19, human parvovirus B19.

the underlying diseases, hypertension (n = 17, 37%), type 2 diabetes (n = 9, 20%), and chronic obstructive pulmonary disease (COPD) (n = 8, 17%) were most prevalent. A total of 38 strains of bacteria, 1 strain of *Aspergillus fumigatus*, and 2 strains of human respiratory syncytial virus (RSV) were detected using conventional methods, but 63 strains of bacteria, 1 strain of *Aspergillus fumigatus*, and 61 strains of viruses were identified by mNGS (Table 1).

		Sex		Department			Underlying disease(s) presence	
Group	Age (yr)	Male	Female	GER	RES	ICU	Yes	No
Bacterial infections	72.80 ± 14.82	16 (80%)	4 (20%)	4 (20%)	11 (55%)	5 (25%)	18 (90%)	2 (10%)
Nonbacterial infections	69.04 ± 12.09	17 (65.38%)	9 (34.62%)	4 (15%)	19 (73%)	3 (12%)	22 (85%)	4 (15%)
P value	0.348	0.336		0.391			0.684	

TABLE 2 Demographic characteristics of bacterial and nonbacterial infections of the lower respiratory tract<sup>a</sup>

<sup>a</sup>GER, geriatrics department; RES, respiratory department; ICU, intensive care unit.

Identification of bacteria by conventional microbiological detection methods and mNGS. According to the comprehensive evaluation, 20 patients were diagnosed with bacterial infections of the lower respiratory tract, and others were diagnosed with nonbacterial infections. There was no statistical significance in demographic characteristics between the two groups (Table 2). Of the lower respiratory tract samples from these patients, 37 were sputum samples and the others were bronchoalveolar lavage fluid (BALF). All specimens were detected by conventional methods, and a total of 38 bacteria were identified in 24 samples. A total of 34 strains of bacteria were the truepositive pathogenic bacteria (Fig. 1A), and 4 strains (including 1 strain each of Escherichia coli, Klebsiella pneumoniae, Brevundimonas diminuta, and Rhizobium radiobacter) were considered false-positive pathogens, accounting for 10.5% of the 38 bacteria. The majority of specimens (11 cases) contained a single pathogen, while 4 samples had two pathogens, and the remaining 5 cases had three pathogenic bacteria. An acid-fast bacillus was detected using acid-fast staining. The most frequently detected bacteria were Pseudomonas aeruginosa (seven cases), followed by Acinetobacter baumannii (six cases) and Stenotrophomonas maltophilia (six cases) (Fig. 1B).

The lower respiratory tract specimens of the 46 patients were also sequenced by mNGS. A total of 63 bacteria were identified from 28 specimens, while 29 strains (46%) were false-positive pathogenic bacteria. The most commonly detected bacteria were *K. pneumoniae* (nine cases), followed by *Staphylococcus aureus* (eight cases), *Pseudomonas aeruginosa* (eight cases), and *Acinetobacter baumannii* (seven cases) (Fig. 1C).

Evaluating the performance of the sequencing reads, the genomic coverage, and the relative abundance of each organism for the identification of pathogens. With the final clinical diagnosis as the gold standard, ROC curves were used to evaluate the performance of the sequencing reads, the genomic coverage, and the relative abundance of each organism in predicting true-positive pathogenic bacteria. To rule out the effect of sequencing depth and gene length in different pathogens, the number of sequencing reads was replaced with the logarithm of reads per kilobase per million mapped reads [lg(RPKM)], and the calculation for RPKM was gene reads/[the total mapped reads (millions)  $\times$  gene length (KB)]. As shown in Table 3, the lg(RPKM), genomic coverage, and relative abundance of the true-positive pathogenic bacteria group were significantly higher than those of the false-positive pathogenic bacteria group (P < 0.01). From the ROC curve of the three indicators, we found that the area under the curve (AUC) of the lg (RPKM) was the largest (0.99), followed by that for genomic coverage (0.98) and the relative abundance (0.83). The corresponding cutoff values were -1.35, 12.92, and 0.25, respectively (Fig. 2). The correlation between the three indicators of true-positive pathogenic bacteria was also analyzed using Spearman's method, and a significant positive correlation was observed between the lg(RPKM) and genomic coverage (r = 0.951). There was no significant correlation between relative abundance and the other two indicators (Fig. 3).

**Analysis of the true-positive viruses based on mNGS results.** Because of the best identification performance, the Ig(RPKM) was used to identify true-positive viruses. As shown in the Fig. 4A, no statistically significant difference was observed between the Ig(RPKM) of bacteria and viruses, and thus, we used the bacterial Ig(RPKM) threshold to identify the infectious viruses. A total of 61 viruses were identified by mNGS, 35 of which were considered to be true-positive viruses according to the Ig(RPKM) threshold and the rest of which were considered to be false positives. The most frequent true-



**FIG 1** Identification of pathogenic bacteria by conventional methods and mNGS. (A) The pathogen distribution of 34 true-positive pathogenic bacteria. (B) Histogram of conventional methods to detect pathogenic bacteria. (C) Histogram of mNGS detection for pathogenic bacteria.

positive virus was human gammaherpesvirus 4 (10 cases), followed by human alphaherpesvirus 1 (5 cases). Human gammaherpesvirus 4 and human betaherpesvirus 7 accounted for 33% of the false-positive viruses (Fig. 4B). In addition, we studied which viruses were prone to coinfection with particular bacteria. As shown in Fig. 5, human gammaherpesvirus 4 was most likely to be coinfected with bacteria and 6 of 10 (60%) strains of human gammaherpesvirus 4 were coinfected with 13 strains of bacteria.

**TABLE 3** Comparison of three indicators between true- and false-positive pathogenic bacteria (median [25th percentile, 75th percentile])

Group	lg(RPKM)	Coverage (%)	Relative abundance (%)
True positive	0.35 (-0.63, 1.04)	78.92 (40.02, 85.87)	14.10 (1.85, 43.60)
False positive	-2.15 (-2.55, -1.55)	2.94 (0.60, 7.02)	0.20 (0.10, 2.20)
P value	0.00	0.00	0.00

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**FIG 2** Evaluating the performance of (A) Ig(RPKM), (B) genomic coverage, and (C) relative abundance for distinguishing between the true- and false-positive pathogenic bacteria groups using ROC curves.

**Performance of mNGS in antimicrobial resistance prediction.** The resistance genes of bacteria were predicted based on the mNGS data. We also investigated the consistency between antibiotic resistance genes and the results of antimicrobial susceptibility tests (AST). As shown in Table 4, the drug resistance genes  $bla_{OXA-23}$ ,  $bla_{OXA-51}$ , and  $bla_{TEM}$  were detected in four strains of *A. baumannii*, while only  $bla_{OXA-23}$  and  $bla_{OXA-51}$  were detected in one strain of *A. baumannii*. Enzymes encoded by  $bla_{OXA-23}$  and  $bla_{OXA-51}$  belong to carbapenem-hydrolyzing class D  $\beta$ -lactamases, which can inactivate penicillin, cephalosporin, and carbapenem and cannot typically be inhibited by clavulanic acid, sulbactam, or tazobactam. The results of the drug sensitivity tests showed that all five strains of *A. baumannii* (CAZ), cefepime (FEP), ceftriaxone (CRO), and imipenem (IPM).

The class A  $\beta$ -lactamase resistance genes were detected in four of the six strains of *Enterobacteriaceae*, including  $bla_{CTX-M}$ ,  $bla_{SHV}$ ,  $bla_{TEM}$ , and  $bla_{KPC}$ . The enzymes encoded by  $bla_{CTX-M}$ ,  $bla_{SHV}$ , and  $bla_{TEM}$  are extended-spectrum-lactamases (ESBLs), which can hydrolyze penicillin (ampicillin [AMP]), cephalosporin (cefazolin [CZO], CAZ, CRO), and monobactam (aztreonam [ATM]) and can be inhibited by enzyme inhibitors. KPC carbapenemases encoded by  $bla_{KPC}$  are resistant to penicillin, cephalosporin, monobactam, and carbapenems; however, newer  $\beta$ -lactamase inhibitors can inhibit them, such as avibactam, relebactam, and vaborbactam. As shown in Table 5, the presence of antibiotic resistance genes was consistent with the results of drug sensitivity tests.

The *mecA* gene encodes a low-affinity penicillin-binding protein (PBP2a) that confers resistance to the entire class of  $\beta$ -lactams, except for ceftaroline and ceftobiprole. *mecA* was detected in all three strains of *S. aureus*, and those strains were resistant to penicillin (PEN) and oxacillin (OXA) (Table 6).

# DISCUSSION

The detection of pathogenic bacteria by traditional methods is limited due to the use of broad-spectrum antibiotics, as well as the presence of bacteria that are fastidious or slow growing. By directly sequencing the DNA or RNA from samples, mNGS



**FIG 3** The correlation among the three indicators was analyzed using Spearman's method. (A) A significant positive correlation was observed between lg(RPKM) and genomic coverage. (B) No significant correlation was observed between the relative abundance and lg(RPKM). (C) No significant correlation was observed between the relative abundance and genomic coverage.

effectively overcomes the deficiencies of traditional detection methods. In this study, an acid-fast bacillus was detected using acid-fast staining, but it was unclear if the bacterium was *Mycobacterium tuberculosis* due to its slow growth phenotype. The bacterium was subsequently identified as *Mycobacterium tuberculosis* using mNGS (634 reads). *Mycobacterium tuberculosis* was considered present if at least one read was aligned at either the species or genus level due to difficulties in DNA extraction and the possibility of contamination (15). The detection rate of false-positive pathogenic bacteria by mNGS was significantly higher than that by conventional methods. Thus, it is important to distinguish between true- and false-positive pathogenic bacteria when interpreting mNGS results.

We used Ig(RPKM), genomic coverage, and relative abundance as tools to identify true-positive pathogenic bacteria from mNGS data and evaluated their identification using ROC curves. An identification cutoff was also established. From our analysis, it was concluded that the Ig(RPKM) and genomic coverage could identify true-positive pathogenic bacteria, with the performance of Ig(RPKM) being the best. It was also seen from the correlation analyses that no significant correlation was present between the relative abundance and Ig(RPKM) of pathogenic bacteria, mainly because the relative abundance was influenced by the total number of bacterial genomes in the sample. Therefore, the relative abundance was not ideal for the identification of true-positive and false-positive pathogenic bacteria.

The traditional methods for detecting respiratory viruses are based on virus-infected cell cultures, immunology-related reactions between antigens and antibodies, or PCR analyses (16). Virus isolation and culture is considered the gold standard for the identification of respiratory viruses (17), while its 4-week time requirement is too long to be used in clinical laboratories. The disadvantages of immunology-related reactions can lead to false-negative results during the window period (18). PCR is a targeted detection method but

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**FIG 4** Analysis of the true-positive viruses based on mNGS results. (A) There was no statistically significant difference between the lg(RPKM) of bacteria and viruses. (B) Identification of the true-positive viruses based on the bacterial lg(RPKM) threshold.

cannot be used for the detection of unknown viruses (19). Thus, the application of mNGS overcomes such problems for the identification of respiratory viruses.

Due to the limitations of detection methods in our study (indirect immunofluorescence), most respiratory viruses were not detected by traditional detection methods. However, mNGS greatly improved the detection rate of respiratory virus and effectively made up for the limitations of traditional detection methods for unknown viruses. In our study, herpesvirus was the predominant true-positive virus, accounting for 54.3% of the total true-positive viruses. It has been reported that rhinovirus, influenza A virus, and adenovirus were the main pathogenic viruses of community-acquired pneumonia (20), and we could conclude from our research that herpesvirus was the main virus detected in hospitalized patients. Twenty-five false-positive viruses. False positives were caused by the different genome lengths of various viruses (genome length of herpesviruses > 150 Kb, genome length of nonherpesviruses  $\leq$  30 Kb). The longer the viral genome, the more reads were produced, and the higher the false-positive rate.

According to previous studies, respiratory viruses can influence the pathogens of pneumonia by altering the carrying structure of bacteria in the upper respiratory tract and the colonization of the lower respiratory tract by specific bacteria (2). Thus, we investigated which viruses were prone to coinfection with particular bacteria. Our findings showed that human gammaherpesvirus 4 was prone to coinfection with *P*.



FIG 5 Heat map showing the trends in coinfections between bacteria and viruses.

*aeruginosa, A. baumannii,* and *S. maltophilia.* Another study also reported that human gammaherpesvirus 4 was associated with fever or acute exacerbation of chronic lung disease (21), possibly due to the bacteria with which the patients were coinfected.

The traditional ASTs include disk diffusion and MIC methods, which are too timeconsuming to be used for precision medicine. Predicting resistance genes by mNGS provides us with a new strategy to infer antimicrobial susceptibility. In our study, the presence of drug-resistant genes was consistent with the results of ASTs. Additionally, the drug resistance genes identified by mNGS were associated with bacterial susceptibility to  $\beta$ -lactam antibiotics, while genes that conferred resistance to other classes of antibiotics were not detected. Additionally, five strains of Pseudomonas aeruginosa that were resistant to carbapenem antibiotics did not contain genes associated with drug resistance. That finding may be due to the following reasons: (i) there is not a standard database that contains all validated antimicrobial resistance genes or point mutations in genes that are associated with antimicrobial resistance, (ii) predicting antimicrobial resistance accurately using mNGS is difficult due to the lack of knowledge of drug resistance caused by genetic mutations and the emergence of new resistance mechanisms, and (iii) increased expressions of intrinsic resistant genes (e.g., those encoding efflux pumps, outer membrane proteins, and intrinsic  $\beta$ -lactamase) can also lead to antimicrobial resistance (22), especially in P. aeruginosa and A. baumannii. Thus, many challenges remain in inferring antimicrobial susceptibility using mNGS data.

**TABLE 4** Drug resistance genes of *A. baumannii* predicted by mNGS and the corresponding AST<sup>a</sup>

		Resistance to drug:					
Patient ID	Resistance genes	SAM	TZP	CAZ	CRO	FEP	IPM
P3	bla <sub>0XA-23</sub> , bla <sub>0XA-51</sub> , bla <sub>TEM</sub>	R	R	R	R	R	R
P11	bla <sub>OXA-23</sub> , bla <sub>OXA-51</sub> , bla <sub>TEM</sub>	R	R	R	R	R	R
P13	bla <sub>OXA-23</sub> , bla <sub>OXA-51</sub>	R	R	R	R	R	R
P15	bla <sub>OXA-23</sub> , bla <sub>OXA-51</sub> , bla <sub>TEM</sub>	R	R	R	R	R	R
P18	bla <sub>OXA-23</sub> , bla <sub>OXA-51</sub> , bla <sub>TEM</sub>	R	R	R	R	R	R

<sup>a</sup>R, resistant.

			Resistance to drug:								
Patient ID	Bacteria	Resistance gene(s)	SAM	TZP	CAZ	CRO	FEP	IPM	AMP	czo	ATM
P9	E. coli	bla <sub>ctx-M</sub>	R	S	R	R	1	S	R	R	R
P19	E. coli	bla <sub>ctx-M</sub> , bla <sub>tem</sub>	R	S	R	R	R	S	R	R	R
P10	K. pneumoniae	bla <sub>стх-м</sub> , bla <sub>sнv</sub> , bla <sub>тем</sub> , bla <sub>крс</sub>	R	R	R	R	R	R		R	R
P14	Serratia marcescens	bla <sub>CTX-M</sub> , bla <sub>SHV</sub> , bla <sub>TEM</sub>		R	R	R	R	S			R

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<sup>a</sup>R, resistant; I, intermediate; S, susceptible

This study had limitations. Fungal pathogens were not analyzed due to their small sample size, most of the true-positive viruses identified by the bacterial lg(RPKM) threshold could not be confirmed by other methods, and the clinical significance of the herpesvirus detections remained to be determined. However, despite this, our study still provided a new perspective on the applicability of mNGS in LRTIs. Compared with conventional microbiological detection methods, mNGS has multiple advantages. Although many challenges remain necessary to overcome for its use in clinical applications, mNGS will be a revolutionary technology for clinical microbiological diagnostics.

#### **MATERIALS AND METHODS**

**Ethics statement.** This project was reviewed and approved by the Ethical Review Committee of Zhenjiang First People's Hospital. An ethics review was exempted as the study was retrospective and the patients were anonymized.

**Specimen collection and processing.** We retrospectively analyzed the clinical data of 46 patients in our hospital who were suspected to have LRTIs. The lower respiratory tract specimens, including sputum or bronchoalveolar lavage fluid (BALF), from these patients were tested simultaneously by conventional microbiological detection methods and mNGS. The sputum or BALF of each patient was divided into two portions; one was sent to the clinical microbiology laboratory for conventional microbiological detection, and the other was sent to a commercial laboratory for mNGS testing.

**Conventional microbiological detection.** In the clinical microbiology laboratory, specimens were used for special pathological staining and semiquantitative culture. Gram staining (BASO), acid-fast staining (BJ-ec), and lactic acid phenol cotton blue staining (RICH SCIENCE) were performed to identify bacteria, *Mycobacterium tuberculosis* complex, and fungi, respectively. Bacterial culture was performed by inoculating specimens on blood agar plates (CHROMagar), MacConkey agar (CHROMagar), and chocolate agar (CHROMagar) and incubating at 35°C for 24 to 48 h. If a typical colony of pathogenic bacteria grew, bacterial identification and drug sensitivity tests were carried out using the Vitek 2 compact system (bioMérieux). Sabouraud agar plates (CHROMagar) were used for fungi culture at 28°C for 7 days. Filamentous fungi were identified according to their morphology. Influenza A/B, parainfluenza 1/2/3, human respiratory syncytial virus (hRSV), adenovirus, *Mycoplasma pneumoniae, Chlamydia*, and *Legionella pneumophila* were detected by indirect immunofluorescence (Euroimmun).

The mNGS test. Specimens (3 mL) used for mNGS were collected in sterile containers, which were then sealed and placed in a foam box containing an ice pack. Specimens were then immediately transported to a commercial laboratory (Vision Medicals) for pathogen testing. Once the laboratory received the specimens, sample processing, nucleic acid extraction, DNA library preparation, high-throughput sequencing, bioinformatics analysis, and the interpretation of mNGS data were performed according to the laboratory's standard operating procedures. To detect the pathogens as much as possible, DNA and RNA sequencing were performed simultaneously. The total DNA and RNA from all samples were extracted using a QIAamp UCP pathogen DNA kit (Qiagen) and QIAamp viral RNA kit (Qiagen), respectively. Human DNA was removed using Benzonase (Qiagen) and Tween 20 (Sigma), and rRNA was removed by a Ribo-Zero rRNA removal kit (Illumina). cDNA was synthesized by reverse transcription. Libraries were constructed using a Nextera XT DNA library prep kit (Illumina), and the quality of libraries was assessed by Qubit dsDNA HS assay kit and high sensitivity DNA kit (Agilent) on an Agilent 2100 Bioanalyzer. Sequencing was performed using Illumina Nextseq CN500. Peripheral blood mononuclear cell (PBMC) samples with 10<sup>5</sup> cells/mL from healthy donors were used as a negative control in parallel

<b>TABLE O</b> Drug resistance genes of 5, dureus predicted by minos and the conceptioning rist	<b>TABLE 6</b> Drug resistance genes of	f S. <i>aureus</i> predicted l	by mNGS and the corres	ponding AST <sup>a</sup>
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	Resistance to drug:			
Patient ID	PEN	OXA		
P14	R	R		
P16	R	R		
P17	R	R		

<sup>a</sup>All three resistance genes are mecA. R, resistant.

with each batch, and the same protocol was performed alongside the specimens to serve as nontemplate controls (23). The high-quality data were obtained by removing low-quality reads, adapter contamination, duplicate reads, and reads shorter than 50 bp using Trimmomatic (24). Low-complexity reads were removed by Kcomplexity with default parameters (25). Human sequence data were identified and excluded by mapping to a human reference genome (hg38) using Burrows-Wheeler Aligner software (26). A set of criteria similar to the National Center for Biotechnology Information (NCBI) criteria (https:// www.ncbi.nlm.nih.gov/assembly/help/anomnotrefseq/) were designed for selecting representative assembly for microorganisms (bacteria, viruses, fungi, protozoa, and other multicellular eukaryotic pathogens) from the NCBI nucleotide and genome databases. Pathogen lists were selected according to Johns Hopkins ABX Guide, Manual of Clinical Microbiology, and clinical case reports or research articles published in current peer-reviewed journals. The final database included about 13,000 genomes. Microbial reads were then aligned to database with SNAP v1.0 beta.18. Virus-positive was defined as the coverage of three or more nonoverlapping regions on the genome. Other pathogens were considered to be positive detection if the reads per million (RPM) ratio or RPM-r (the RPM of a given species or genus in the clinical sample divided by the RPM in negative control) was  $\geq$ 5 (23). Sequencing reads, genomic coverage, the relative abundance of each organism, and drug resistance genes were included in the mNGS report.

**Diagnosis of lower respiratory tract infections.** The final clinical diagnoses of the 46 patients were confirmed based on the comprehensive evaluation of clinical symptoms, X-ray evidence, traditional microbiological tests, mNGS, serological examinations [including the fungus (1-3)- $\beta$ -D-glucan test, serum cryptococcal capsular polysaccharide antigen test, and *Mycoplasma pneumoniae* serological antibody detection], and other clinical examinations (the galactomannan antigen detection test). The pathogens detected by traditional tests or mNGS were considered true positives only if they were consistent with the final clinical diagnosis; otherwise, they were considered false positives (colonizing microorganism or contaminant microorganism).

**Statistical analysis.** The data were analyzed using SPSS 25.0 software. The Shapiro-Wilk test was used to determine whether the quantitative data conformed to a normal distribution, and the Student's *t* test and the Wilcoxon rank test were used to compare two groups that were in normal distribution or not in normal distribution, respectively. Pearson chi-squared ( $\chi^2$ ) test or Fisher's exact test was used for the comparison of frequencies of the categorical data. The correlation between two different indicators was analyzed and expressed as Spearman's *r* values. An ROC curve was drawn to choose the best indicator of the true-positive specific pathogen. A *P* value of <0.05 was considered statistically significant.

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