Profile of Bacteria with ARGs Among Real-World Samples from ICU Admission Patients with Pulmonary Infection Revealed by Metagenomic NGS

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Background: Treatment of pulmonary infections in the intensive care unit (ICU) represents a great challenge, especially infections caused by antibiotic resistance pathogens. A thorough and up-to-date knowledge of the local spectrum of antibiotic resistant bacteria can improve the antibiotic treatment efficiency. In this study, we aimed to reveal the profile of bacteria with antibiotic resistance genes (ARGs) in real-world samples from ICU admission patients with pulmonary infection in Mainland, China, by metagenomic next-generation sequencing (mNGS).

Methods: A total of 504 different types of clinical samples from 452 ICU admission patients with pulmonary infection were detected by mNGS analysis.

Results: A total of 485 samples from 434 patients got successful mNGS results. Among 434 patients, one or more bacteria with ARGs were detected in 192 patients (44.24%, 192/434), and ≥2 bacteria with ARGs were detected in 85 (19.59%, 85/434) patients. The predominant detected bacteria were Corynebacterium striatum (C. striatum) (11.76%, 51/434), Acinetobacter baumannii (A. baumannii) (11.52%, 50/434) and Enterococcus faecium (E. faecium) (8.99%, 39/434). ermX conferred resistance to MSLB and cmx to phenicol were the only two ARGs detected in C. striatum; in A. baumannii, most of ARGs were resistance-nodulation-division (RND)-type efflux pumps genes, which conferred resistance to multi-drug; ermB conferred resistance to MSLB and efmd to multi-drug were the predominant ARGs in E. faecium. Bacteria with ARGs were detected in 50% (140/280) bronchoalveolar lavage fluid (BALF) and 50.5% (48/95) sputum samples, which were significantly higher than in blood and cerebrospinal fluid (CSF) samples.

Conclusion: High level of bacteria with ARGs was observed in clinical samples, especially BALF and sputum samples from ICU admission patients with pulmonary infection in Mainland, China. And C. striatum resistant to MSLB and/or phenicol, multi-drug resistance A. baumannii and E. faecium were the lead bacteria.

Keywords: intensive care units, pulmonary infection, antibiotic resistant bacteria, antibiotic resistance genes

Introduction

Pulmonary infection is one of the most frequent nosocomial infections in the intensive care unit (ICU) admission patients and is associated with significant morbidity and mortality. Patients developing pneumonia while in the ICU have a high crude mortality rate of approximately 30%. Currently, the empirical choice of broad-spectrum antibiotic therapy is standard-of-care therapy for most patients with infection in the ICU,
especially in critically ill patients. Clinical success rates reported for patients who developed pneumonia in the ICU remain low (often <60%), 2 re Multistate hospital epidemiology survey, and despite the widespread use of broad-spectrum antibiotics. Mortality rate is significantly lower in patients who received appropriate antibiotic therapy at the early stage of nosocomial pneumonia compared with the patients who did not receive or delayed appropriate therapy. 7 An exponential increase of antibiotic drug-resistant microorganisms in ICU, especially the multi-drug resistance (MDR) pathogens, is the biggest challenge that encompass antibiotic therapy. An international multi-center study demonstrated that 19% of patients with ICU stays over 24 hours acquired an infection, ranging from 2.3% to 49.2% depending on the hospital unit. 8 The average for infections by multi-resistant gram-negative bacteria in ICU patients was 54.4 per 1000 patients/day. 9 MDR pathogens, Enterococcus faecium (E. faecium), Staphylococcus aureus (S. aureus), Klebsiella pneumonia (K. pneumonia), Pseudomonas aeruginosa (P. aeruginosa), Acinetobacter baumannii (A. baumannii), Enterobacter species have been classified as ESKAPE 2 and are the most common microbial causes of ICU acquired infection including the pulmonary infection, 4 but causative pathogens and antibiotics resistance patterns are highly endemic. A thorough and up-to-date knowledge of the local microbial cause epidemiology might allow better initial empirical antibiotic choice to susceptible microorganisms in ICU, while avoiding the overuse of broad-spectrum antibiotic and the emergence of new drug-resistant organism. 5, 6 Many genes contributing to antibiotic resistance have been identified, and the high concordance of genotype-phenotype for drug resistance have been reflected along with the broader usage of NGS technologies in evaluating the antimicrobial resistance (AMR) genetic determinants. 5, 16 Metagenomic next-generation sequencing (mNGS) has the capacity to characterize all DNA or RNA present in a sample, enabling analysis of all potential pathogens and characterization of antibiotic resistance directly from clinical samples. To date, several studies have demonstrated the utility of mNGS in providing clinically actionable information to predict cause of infection and evaluate disease risk, and the effectiveness of mNGS in guiding patient management. 53-57 In this study, we detected different types of clinical samples from ICU admission patients with pulmonary infection using a validated mNGS assay, in order to reveal the prevalence and distribution of bacteria with antibiotic resistance genes (ARGs) in samples of real-world ICU admission patients with pulmonary infection in Mainland, China.

**Materials and Methods**

**Study Population and Specimens**

This retrospective study included 452 ICU admission patients with pulmonary infection who underwent clinical metagenomics next-generation sequencing (mNGS) testing in CapitalBio Medlab (Beijing, China) from January 2019 to November 2020. All patients were diagnosed as pulmonary infection judged by the board-certified physician on the basis of clinical manifestations and imaging examination results. 59, 60 mNGS analysis was ordered as clinically indicated by the treating physician of patients, and informed consent was obtained from each included patient. Total 504 clinical specimens from the 452 patients were included, including Bronchoalveolar lavage fluid (BALF, n = 284), sputum (n = 97), peripheral blood (PB, n = 97), cerebrospinal fluid (CSF, n = 14), pus (n = 3), pleural fluid (PF, n = 5) and lung biopsy tissue (n = 4). Two different types of specimen were collected from 52 patients simultaneously.

**Sample Collection and Processing**

All body fluid samples (BALF, sputum, pleural fluid) were collected from each patient according to standard procedures. 63 At least 5mL of BALF sample and 1–3mL of each sample of sputum, CSF, pleural fluid or other sterile liquid were collected in sterile tube and stored at −80°C before DNA extraction. Sputum samples were liquefied by dithiothreitol (DTT) for 30 min at room temperature with shaking, centrifuged and washed by sterile water twice before DNA extraction. Other body fluid samples except blood could go directly to the DNA extraction procedures. 3–5mL peripheral blood was collected to Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulant tube with special deoxyribonucleic acid (DNA) protective agent and stored at room temperature. The blood samples were centrifuged at 1600g for 10min to separate plasma, and the plasma separation must carried out within 48 hours after blood draw. Lung biopsy tissue was minced into small pieces and ground with a tissue homogenate machine before DNA extraction. 0.6 mL sample, 20 μL enzyme, and 1g of 0.5mm Zirconium beads in a new, sterile 1.5mL microcentrifuge tube were shaken vigorously by a vortex mixer and 0.5-mL sample was transferred to a new 1.5mL microcentrifuge tube for DNA extraction. DNA was extracted using the MAPMI sample preparation kit (CapitalBio Corporation, Beijing, China) following the manufacturer’s operational manual.

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Library Preparation and Sequencing
DNA libraries were constructed through enzymatically DNA fragmentation (200–300bp), end repair, adapters adding, and PCR amplification. The quality of the DNA libraries was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California) combined with qPCR based on Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher, USA), the primers based on the sequences of the adapters were used for the qPCR. Libraries were out of the range of 300–400bp, or with a concentration < 50pmol/L, considered to have failed the library preparation step. Constructed libraries with confirmed quality were pooled and sequenced with Ion PI chip on BES4000 (Capitalbio Corporation, Beijing, China) platform. Internal, negative and positive controls were included in each run. Internal parameters are specific molecular tag that is placed in the sample before nucleic acid extraction to track the entire process and to control the quality of DNA. Negative control is sterile water to monitor potential contamination and positive control is a mock community comprising genomic DNA prepared from 8 bacteria/fungi species mixed at varying concentrations.

Bioinformatic Analysis
An automated bioinformatic analysis system (Capitalbio Corporation, Beijing, China) based on Ion torrent™ server combined with BES4000 sequencer was used for data analysis. 1) Quality control: The original sequencing data were analyzed if: (i) Sequencing sequences subtracted of human host sequences was above 70%. (ii) Reads of sequence were longer than 100bp. (iii) Total reads without removing the human genome component were >10 million. 2) Data filtering: in order to obtain high-quality sequence data, the reads with length less than 50bp, low-quality, low complexity were removed. Host subtraction was performed by mapping to human reference genome (GRCh38). 3) sequences alignment: The remaining unmapped reads were aligned directly with large reference databases downloaded from NCBI, PATRIC and Comprehensive Antibiotic Research Database (CARD) databases, which cover bacteria, fungi, viruses, parasite and ARGs. According to the final results of microbiorganisms and ARGs comparison, all parameters of detected microorganisms and ARGs were calculated, including sequence number, relative abundance, genome coverage and depth, etc. 4) Report generation: The report generated by the system included all the microbiorganisms and ARGs in the specific microorganisms detected in the test. They classified by bacteria, viruses, fungi, parasites and ARGs, which were ranked from high to low according to their unique reads, and the relative content of the former was higher. In the report, ARG was shown as gene microorganism, such as adeB_A. baumannii. Only bacteria and ARGs were further analyzed in this study. Unique reads are number of sequences strictly mapped at species level or ARGs. 5) Threshold criteria for bacteria and ARGs as a positive result: (i) For bacteria except mycobacterium tuberculosis: bacteria species whose unique reads were of the first rank with the genus unique reads>50, cover length>3000bp. For mycobacterium tuberculosis: unique reads ≥3. (ii) For ARGs: unique reads>50, cover length >200bp, cover percentage >70%. (iii) bacterium was reported as antibiotic resistance bacterium (ARB) in this study, if bacterium and ARG in this bacterium were positive simultaneously according the above criteria. Classification of ARGs resistance mechanism was carried out according to the CARD database.

Statistical Analysis
Prism (version 8.0, GraphPad Software, Inc, La Jolla, California, USA) and SPSS (version 22.0, IBM Corporation, Armonk, NY, USA) statistical software were used. Categorical variables described as n (%) and Chi-square test or Fisher’s exact test were used for categorical variables, as appropriate. Statistical significance was defined as 5% (P < 0.05).

Results
Patient Characteristics
In this study, a total of 504 clinical specimens from 452 patients were collected, 19 specimens (3.77%, 19/504) of 18 patients were failed to get final results for variable reasons: small library fragments (often less than 100bp, considered as adapter dimer), lower library concentration/total reads, poor sequencing quality. The 19 failed specimens included 9 blood (9.27%, 9/97), 6 BALF (2.11%, 6/284), 2 sputum (2.06%, 2/97), 2 CSF (14.28%, 2/14). 434 patients with successful mNGS results, included 312 males and 122 females. Mean age of all patients was 61.03 (±15.18) years, ranging from 0 to 96. Among them, 262 patients (60.37%) had underlying different comorbidities, including cardiovascular disease (64, 14.71%), malignancies (50, 11.49%), respiratory disorders (31, 7.13%), renal/liver disease (23, 5.29%), etc.; 377 patients (86.67%) were treated with one or different combinations of antibiotic prior to sample collection (Table 1).
A total of 329 bacteria with ARGs of 30 species were identified in 485 clinical samples with mNGS results from 434 patients, one or more bacteria with ARGs were detected in samples from 192 patients (44.24%, 192/434). Among those, poly bacteria with ARGs were detected in samples from 85 (19.59%, 85/434) patients. The bacteria with ARGs positive result was associated with the ICU department ($P < 0.014$) but gender, age, antibiotic history and comorbidities (Table 1).

The predominant detected bacteria with ARGs were *Corynebacterium striatum* (*C. striatum*) (11.76%, 51/434), *A. baumannii* (11.52%, 50/434) and *E. faecium* (8.99%, 39/434) (Figure 2). Two *Mycobacterium tuberculosis* (*M. tuberculosis*), one with *efpA* and *mtrA*, another with *efpA* alone, and one *Legionella pneumophila* (*L. pneumophila*) with APH(9)-Ia were detected in BALF samples of three different patients. It was worth noting that one of the *M. tuberculosis* (*efpA*) was detected from BALF of a newborn girl, and *A. baumannii* (*adeJ, adeF*), *E. faecium* (*ermB*), *Streptococcus pneumoniae* (*S. pneumoniae*) (*patB*) were detected simultaneously.

The most prevalent bacteria with ARGs detected in male patients were *C. striatum* (41/254), *A. baumannii* (39/254) and *E. faecium* (34/254), while *A. baumannii* (13/75), *C. striatum* (10/75) and *S. pneumonia* (9/75) in female patients; In patients

### Table 1 Demographic Characteristics of ICU Admission Patients with Pulmonary Infection

|                                | N (%) | Bacteria with ARGs Detected | P    |
|--------------------------------|-------|-----------------------------|------|
|                                |       | Yes                         | No   |
| **Gender**                     |       |                             |      |
| Male                           | 312 (71.89%) | 144 (46.15%)     | 168 (53.85%) | 0.237 |
| Female                         | 122 (28.11%)  | 48 (39.34%)         | 74 (60.66%)  |
| **Age**                        |       |                             |      |
| <18                            | 61.03±15.18 | 9 (40.91%)         | 13 (59.09%)   |
| 18–30                          | 22 (5.07%)   | 4 (26.67%)          | 11 (73.34%)   |
| 31–60                          | 15 (3.46%)    | 63 (44.68%)        | 78 (55.32%)   |
| 61–96                          | 256 (58.99%)  | 116 (45.31%)       | 140 (54.69%)  | 0.5504 |
| **Antibiotic history**         |       |                             |      |
| Yes                            | 376 (86.64%)  | 167 (44.41%)        | 209 (55.59%)  |
| No                             | 58 (13.36%)   | 25 (43.10%)         | 33 (56.90%)   | 0.8878 |
| **Department**                 |       |                             |      |
| GICU                           | 306 (70.51%)  | 148 (48.37%)        | 158 (51.63%)  |
| PICU                           | 16 (3.69%)    | 7 (43.75%)          | 9 (56.25%)    |
| RICU                           | 89 (20.51%)   | 28 (31.46%)         | 61 (68.54%)   |
| SICU                           | 7 (1.61%)     | 5 (71.43%)          | 2 (28.57%)    |
| EICU                           | 16 (3.69%)    | 4 (25.00%)          | 12 (75.00%)   | 0.014  |
| **Comorbidities**              |       |                             |      |
| None                           | 172 (39.63%)  | 72 (41.86%)         | 100 (58.14%)  |
| Respiratory disorders          | 36 (8.29%)    | 13 (36.12%)         | 23 (63.88%)   |
| Malignancies                   | 51 (11.75%)   | 19 (37.26%)         | 32 (62.74%)   |
| Brain disease                  | 37 (8.53%)    | 24 (64.87%)         | 13 (35.13%)   |
| Cardiovascular disease         | 37 (8.53%)    | 17 (45.95%)         | 20 (54.05%)   |
| Renal+ liver disease           | 21 (4.84%)    | 8 (38.10%)          | 13 (61.90%)   |
| Diabetes/diabetes +others      | 20 (4.61%)    | 10 (50.0%)          | 10 (50.0%)    |
| Autoimmune diseases            | 18 (4.15%)    | 8 (44.45%)          | 10 (55.55%)   |
| Trauma                         | 8 (1.84%)     | 5 (62.50%)          | 3 (37.50%)    |
| Organ transplantation          | 11 (2.53%)    | 8(72.73%)           | 3 (27.27%)    |
| Others                         | 23 (5.30%)    | 8 (34.79%)          | 15 (65.21%)   | 0.1351 |

**Frequency of Bacteria with ARGs in Clinical Samples**

A total of 329 bacteria with ARGs of 30 species were identified in 485 clinical samples with mNGS results from 434 patients, one or more bacteria with ARGs were detected in samples from 192 patients (44.24%, 192/434). Among those, poly bacteria with ARGs were detected in samples from 85 (19.59%, 85/434) patients. The bacteria with ARGs positive result was associated with the ICU department ($P < 0.014$) but gender, age, antibiotic history and comorbidities (Table 1). But All 30 bacteria species with ARGs were detected in the patients of male, age ≥61, with antibiotic therapy history, GICU and with comorbidities (Figure 1A). The predominant detected bacteria with ARGs were *Corynebacterium striatum* (*C. striatum*) (11.76%, 51/434), *A. baumannii* (11.52%, 50/434) and *E. faecium* (8.99%, 39/434) (Figure 2). Two *Mycobacterium tuberculosis* (*M. tuberculosis*), one with *efpA* and *mtrA*, another with *efpA* alone, and one *Legionella pneumophila* (*L. pneumophila*) with APH(9)-Ia were detected in BALF samples of three different patients. It was worth noting that one of the *M. tuberculosis* (*efpA*) was detected from BALF of a newborn girl, and *A. baumannii* (*adeJ, adeF*), *E. faecium* (*ermB*), *Streptococcus pneumoniae* (*S. pneumoniae*) (*patB*) were detected simultaneously.
Figure 1 Spectrum and absolute number of bacteria with ARGs detected in samples from ICU admission patients with pulmonary infection. (A) Spectrum and absolute number of bacteria with ARGs detected in samples from different patients groups. (B) Spectrum and absolute number of bacteria with ARGs detected in different types of samples.
with age <18, the most frequent detected bacteria with ARGs was S. pneumoniae (8/18), while C. striatum (18/106, 33/205), A. baumannii (15/106, 33/205) in age 18–60 and ≥61, respectively. Among the patients with previous antibiotic histories, the predominant detected bacteria with ARGs were C. striatum (47/293), A. baumannii (45/293) and E. faecium (35/293) and S. pneumoniae (7/36) in patients without antibiotic history. In GICU, A. baumannii (42/256), C. striatum (41/256) and E. faecium (29/256) were the most prevalent bacteria with ARGs, while E. faecium (9/45) in RICU and S. pneumoniae (8/28) in others; C. striatum (19/138, 32/191), A. baumannii (21/138, 29/191) and E. faecium (19/138, 20/191) in patients with none and other comorbidities respectively (Figure 1A).

Characteristics of Bacteria with ARGs in Different Types of Samples

An average of 13.5 M reads were generated for each sample of PB and CSF samples, similar with the sequencing data of BALF and sputum (average 14.3M for each sample). The difference in the sensitivity of bacteria with ARGs detected by mNGS between BALF (50%, 140/280) and sputum (50.5%, 48/95) was not statistically significant. However, the sensitivity was significantly higher for BALF, sputum samples than for PB, CSF samples (Figure 3 and Table S1). We did not analyze the differences in other types of sample for few cases, which would undermine the reliability of the results. In 88 PB samples with mNGS results, only one sample was detected K. pneumoniae (OmpK37, acrA). Bacteria with ARGs were detected from two CSF samples, Escherichia coli (acrB, msbA, mdtB, evgS) in one sample, Staphylococcus haemolyticus (ermC) and C. striatum (ermX) in another. In total, 241 bacteria with ARGs were detected from 280 BLAF samples, and A. baumannii (38/241), C. striatum (34/241) and S. pneumoniae (28/241) were the most common bacteria with ARGs. While in 81 bacteria with ARGs detected from 95 sputum samples, the most frequency bacteria were C. striatum (17/81) and E. faecium (16/81) (Figure 1B).

In 31 BALF+PB paired samples, K. pneumoniae (OmpK37, acrA) was detected in one BALF-PB paired sample simultaneously. There was no bacteria with ARGs detected simultaneously in paired sputum-PB samples. Bacteria with ARGs were observed simultaneously in one BALF-sputum paired sample, K pneumoniae (OmpK37, acrA), A. baumannii (adeJ, adeB) were detected in BALF, while A. baumannii (adeJ, adeB), C. striatum (ermX, cmx), E. faecium (efmA) in sputum.
Identification of Antibiotic Resistance Genes (ARGs)

In total, 538 ARGs were detected in all 485 clinical specimens. By antibiotic class, these ARGs comprised multi-drug resistance (n = 220), Macrolide-Lincosamide-Streptogramin B (MLS\(_B\)) (n = 123), tetracycline (n = 53), aminoglycoside (n = 42), β-lactam (n = 39), phenicol (n = 23), unclassified (n = 17), fluoroquinolone (n = 11) and others (n = 10) genes (Table S2). Most of the multidrug resistance genes were efflux-associated genes. \(\text{ermX}\) conferred resistance to MLS\(_B\) and \(\text{cmx}\) to phenicol were the two resistance genes detected in \(C.\) \textit{striatum}; In \(A.\) \textit{baumannii}, the most frequently detected ARGs were \(\text{adeJ}\) and \(\text{adeB}\), which conferred resistance to multi-drug; while \(\text{ermB}\) conferred resistance to MLS\(_B\) and \(\text{efmA}\) to multi-drug were the most prevalent ARGs detected in \(E.\) \textit{faecium}. \(\text{mel}\) conferred resistance to multi-drug was most detected in \(S.\) \textit{pneumoniae}, while \(\text{smeB}\) and \(\text{smeE}\) in \(\text{Stenotrophomonas maltophilia}\); In \(K.\) \textit{pneumonia}, \(\text{ompK37}\) conferred resistance to β-lactam and \(\text{acrA}\) to multi-drug were the most common ARGs, while \(\text{MexB}\) and \(\text{TriC}\) in \(P.\) \textit{aeruginosa} (Table S3).

Discussion

In this retrospective study, we have reported the mNGS analysis of 504 clinical specimens from 452 patients, and 3.77% (19/504) specimens were failed to get final mNGS result for variable reasons as described in results. Similar to other routine test in clinical laboratory, a wide range of pre-analytical variables may impact mNGS analysis, including specimen quality, storage and transit time, temperature, pre-analytical handling steps, etc. Studies showed that sputum quality affected the yield of \(M.\) \textit{tuberculosis} diagnostic tests using smear and/or culture.\(^6\) The number of cells and red blood cells rarely interfered with \(M.\) \textit{tuberculosis} detection by PCR in sputum samples, but a high concentration of cells and erythrocytes interfered with \textit{mycobacterial} detection in BLAF and PF samples.\(^6\) There are no studies assessing the impact of specimen quality on mNGS assay till now. In this study, we did not perform sample quality evaluation before sample processing. Pre-analytical handling of clinical specimens is vital for the successful implementation of mNGS analysis. However, there is still no published standard operating procedure for sampling despite increasing studies on mNGS. A single-spin low-speed plasma separation, which is used in our study, is a feasible and inexpensive pre-analytical step for the recovery of pathogen DNA from blood.\(^6\) Although there was a study describing that freezing and thawing of plasma after storage at −80°C for up to 6 months did not influence the abundance of pathogen, it should not undergo >3 freeze-thaw cycles.\(^6\) Induced sputum samples from patients need to be dispersed before analysis, and among several methods, the method utilizing DTT is widely used for its ability to split...
disulfide bonds and disperse sputum samples. We used the same method to proceed sputum samples before DNA extraction in our study.

Although it is still not a routine identification tool, mNGS presents great potential in resistance prediction by sequencing the ARGs. In our study, we explored the prevalence and distribution of bacteria with ARGs in clinical samples from real-world ICU admission patients with pulmonary infection in Mainland, China, using mNGS. In our study, bacteria with ARGs were detected in 44.24% patients and one or more bacteria with ARGs were detected in 19.54% cases. The predominant detected bacteria with ARGs were *C. striatum*, *A. baumannii* and, *E. faecium*. Of the top 11 most frequently bacteria with ARGs in our study, 5 (*A. baumannii*, *Stenotrophomonas maltophilia*, *K. pneumonia*, *P. aeruginosa*, *Escherichia coli*) were Gram-negative and 6 (*C. striatum*, *E. faecium*, *S. pneumoniae*, *Staphylococcus epidermidis*, *S. aureus*, *Staphylococcus haemolyticus*) were Gram-positive pathogens. But only one Gram-positive pathogen *S. aureus* was among the top 11 most frequently isolated organism from the patients with ventilator-associated pneumonia in the United States and European medical centers from January 2009 to December 2012, and *P. aeruginosa* was the most frequently antibiotic resistance bacteria in both regions, followed by *Klebsiella spp*, *Escherichia coli* ranked fifth in the United States and third in Europe. In a study of 726 patients with nosocomial pneumonia, the most frequently isolated ceftazidime-nonsusceptible pathogens were *Enterobacteriaceae*, *S. aureus* and *P. aeruginosa*. The difference was caused by many factors, e.g. geographic areas, time, hospital unit, patients’ specific characteristic, etc. According World Health Organization (WHO) global tuberculosis (TB) report (2020), China is still one of three highest drug-resistant TB burden countries worldwide. In our study, 2 drug-resistant *M. tuberculosis* were detected in samples from two different patients, and one was from BALF of a newborn girl.

*C. striatum* is non-sporing gram-positive bacterium, traditionally deemed to be a part of the normal flora of skin and mucous membranes, including the respiratory tract. It was first reported in 1980 as a pathogen and has been reported as an emerging multi-drug resistant nosocomial pathogen in recent studies. It has the potential to cause nosocomial infection in many parts of the body, and the respiratory tract is one of the main infection sites. Antibiotic-resistant *C. striatum* was detected in 11.73% patient samples and was the top one detected bacterium with ARGs in our study. High-level resistance to many antibiotics with different mechanisms such as the β-lactam resistance, gyrase subunit A gene mutation for fluoroquinolones resistance and tetA, tetB genes for tetracycline resistance in *Corynebacterium* have been reported. And high level resistance to macrolides, lincosamides, and streptogramin B (MLSb) in *Corynebacterium* is noticeable recently, especially in the multi-drug resistance clinical isolates. The *ermX* gene (erythromycin ribosome methylation) which may be found on plasmid, chromosomes and within transposon, encoding the rRNA methylase enzyme is responsible for MLSb resistance.

The *cmx* gene encoding the efflux protein is responsible for phenicol resistance, and has been reported in several reports. In this study, only *ermX* and *cmx* genes were found in the *C. striatum*, and *ermX* was found in 98.04% (50/51) antibiotic resistant *C. striatum*.

ESKAPE represented 65.6% of all ventilator-associated pneumonia (VAP) isolates, and almost 20% being resistant. However, pulmonary infection due to Enterococcus, included *E. faecium* are distinctly unusual. A prospective and observational study showed that 4% infections located in the respiratory tract in patients with serious infection due to Enterococcus. Another study reported that 8 cases of pneumonia were due to *E. faecium* in 24 reported Enterococcus pneumonia, and 3 were antibiotic resistance. In our study, all the most serious ESKAPE with ARGs were detected in the clinical samples. *A. baumannii* in 11.50% patient samples, *E. faecium* in 8.97% patient samples, followed by *K. pneumonia*, *P. aeruginosa*, *S. aureus* and *Escherichia coli* (Figure 2).

*A. baumannii* is the most common clinical species responsible for ICU infection, accounting for high rates of various infections in ICU, especially pneumonia worldwide. *A. baumannii* is highly resistant to number of antibiotics in clinical practice because of its ability to acquire antimicrobial resistance. Efflux-mediated resistance, particularly the resistance-nodulation-division (RND)-type efflux pumps, is associated with many different classes of antibiotic resistance. In this study, All ARGs detected in *A. baumannii* were the RND-type efflux genes, including adeA, adeB, adeC, adeH, adeJ, adeK, adeF, adel, and adel, except one with aminoglycoside resistance gene ANT(3”)-Ia. AdeJ and adeB were the most frequently ARGs among these genes, similar with the previous study in China. *E. faecium* is resistant to most clinical available antibiotics because of its intrinsic and acquired resistance mechanisms, including low-affinity PBPs to β-lactams, enzymatic inactivation to aminoglycosides, ribosomal methylation to aminoglycosides, ATP-
binding cassette (ABC)-efflux pumps to MLS₉₅, precursor modification (VanA, VanB) to glycopeptides, ribosomal methylation (erm) to macrolides, etc. In our study, the most prevalent resistance determinants in E. faecium were ermB conferring to MLS₉₅ and emfA conferring to macrolides and fluoroquinolone. Porins and efflux pumps are major contributors to antibiotic intrinsic resistance in gram-negative bacteria. OmpK35, OmpK36 are two major porins in multidrug-resistant K. pneumonia. In this study, OmpK37 was the most frequent porin gene in K. pneumonia. AcrAB of K. pneumonia, MexAB-OprM of P. aeruginosa, smeDEF and smeABC of S. maltophilia are the well-characterized efflux pumps with a demonstrated role in antibiotic resistance. ArcA, mexB and smeB were the most prevalent efflux genes in K. pneumonia, P. aeruginosa and S. maltophilia respectively in our study. Estimated prevalence of methicillin-resistant S. aureus (MRSA) ranges in ICU admission patients is between 3% and 20%. The prevalence of MRSA in our study was 1.15% (5/435). The variation is more likely due to the geographic location and type of testing used. S. pneumoniae is the most common pathogen in community-acquired pneumonia (CAP), children with bacteremic pneumonia and ICU-admitted patients with pulmonary infection. Efflux-mediated resistance due to mefE and mel is a major mechanism of macrolide resistance in S. pneumoniae. In our study, the most common resistant determine in S. pneumoniae was mel (24/32), followed by lnuC (7/32).

For mNGS, the diagnostic sensitivity may vary in different types of samples. One study demonstrated that mNGS had higher sensitivity for detecting pathogen in BALF for pulmonary infection. But another study showed that there was no difference among pus, swab and tissue samples for skin and soft tissue infections. In our study, the bacteria with ARGs diagnostic sensitivity was higher in BALF, sputum than blood and CSF. Bacteria with ARGs were detected from 17/31 BALF specimens but only in 1/31 corresponding blood specimens, while 6/15 from sputum specimens and none from corresponding specimens (Figure 3B). The prevalence of bacteria with ARGs is associated with the specimen types. Recent studies demonstrated that IMP-R P. aeruginosa were most frequently isolated from blood and sputum, MSRA from respiratory samples. In this study, the most common bacteria with ARGs in BALF were A. baumannii, C. striatum and S. pneumoniae, but C. striatum (17/81) and E. faecium (16/81) in sputum. And all the 30 species of bacteria with ARGs were detected from BALF specimens (Figure 1B). Considering sensitivity and spectrum of bacteria with ARGs detected, our results demonstrate that BALF, sputum specimens had advantages over PB, CSF specimens for mNGS analysis used to detect the bacteria with ARGs in ICU patients with pulmonary infection.

Some limitations exist in this study. The application of mNGS remains controversial due to the limitations of mNGS technologies. The precision of mNGS is affected by many factors, such as the depth of mNGS, contamination of microbial nucleic acid in reaction kit, the microbial-human ratio in the original samples, the sample processing procedures, bioinformatics analysis and interpretation of sequencing result, etc. We used a validated mNGS-based approach according guideline and the ongoing monitoring, such as sample internal controls, swipe tests for contamination and periodic proficiency testing, etc., to minimize potential assay and patient selection bias, and it is an available assay for clinical reference testing of patients, but its clinical utility has not yet to be conducted in a large-scale prospective clinical trial. For small sample size, we still need more data to reflect the spectrum of antibiotic resistant determinants in the samples from ICU-admission patients with lung infection.

Conclusion
In this study, high level of bacteria with ARGs were observed in clinical samples, especially BALF, sputum samples from ICU admission patients with pulmonary infection. C. striatum with ermX conferred resistance to MSL₉₅ and cmx to phenicol, A. baumannii with adeJ and adeB conferred resistance to multi-drug, and E. faecium with ermB to MSL₉₅, efmA to multi-drug were the most prevalent bacteria identified. The proportions of each bacteria with ARGs differed according the patients specific characteristic and sample types. BALF and sputum maybe were the optimal specimen type for mNGS to detect bacteria with ARGs for ICU patients with pulmonary infection. Identifying pathogen antibiotic resistance in ICU patients with pulmonary infection will support drug resistance monitoring, strengthen infection control in ICU in future research, and thus may contribute to improve antibiotic therapy.

Data Sharing Statement
All relevant data are provided in the manuscript, its figure, its table, its Supplementary Materials.
Ethical Approval and Informed Consent
This study was approved by the Ethic Committee of CapitalBio Medlab. Written informed consent was obtained from each patient.

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Disclosure
The authors declare that there are no conflicts of interest in this work.

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