Metagenomic Next-Generation Sequencing of Bronchoalveolar Lavage Fluid From Children With Severe Pneumonia in Pediatric Intensive Care Unit

Caiyan Zhang
Children's Hospital of Fudan University
Tingyan Liu
Children's Hospital of Fudan University
Yixue Wang
Children's Hospital of Fudan University
Weimin Chen
Children's Hospital of Fudan University
Jing Liu
Children's Hospital of Fudan University
Jinhao Tao
Children's Hospital of Fudan University
Xuemei Zhu
Children's Hospital of Fudan University
Zhenyu Zhang
Children's Hospital of Fudan University
Meixiu Ming
Children's Hospital of Fudan University
Mingbang Wang
South China Hospital, Shenzhen University
Guoping Lu
Children's Hospital of Fudan University
Gangfeng Yan (gangfeng_yan@fudan.edu.cn)
Children's Hospital of Fudan University

Research Article

Keywords: metagenomic next-generation sequencing, severe pneumonia, bronchoalveolar lavage fluid, Epstein–Barr virus, Pneumocystis jiroveciimetagenomic next-generation sequencing, severe pneumonia, bronchoalveolar lavage fluid, Epstein–Barr virus, Pneumocystis jirovecii

Posted Date: June 30th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1781582/v1

Abstract

Background: Severe pneumonia due to lower respiratory tract infections (LRTIs) is a significant cause of morbidity and mortality in children. Noninfectious respiratory syndromes resembling LRTIs can complicate the diagnosis and may also make targeted therapy difficult because of the difficulty of identifying LRTI pathogens. In the present study, a highly sensitive metagenomic next-generation sequencing (mNGS) approach was used to characterize the microbiome of bronchoalveolar lavage fluid (BALF) in children with severe lower pneumonia and identify pathogenic microorganisms that may cause severe pneumonia.

Methods: In total, 126 BALF samples were collected from children admitted to the pediatric intensive care unit (PICU) with severe pneumonia, and mNGS was performed at the DNA and/or RNA level. The pathogenic microorganisms in BALF were identified and correlated with serological inflammatory indicators, lymphocyte subtypes, and clinical symptoms.

Results: mNGS of BALF identified potentially pathogenic bacteria in children with severe pneumonia in the PICU. An increased BALF bacterial diversity index was positively correlated with serum inflammatory indicators and lymphocyte subtypes. Children with severe pneumonia in the PICU had the potential for coinfection with viruses including Epstein–Barr virus, *Cytomegalovirus*, and *Human betaherpesvirus 6B*, the abundance of which was positively correlated with immunodeficiency and pneumonia severity, suggesting that the virus may be reactivated in children in the PICU. There was also the potential for coinfection with fungal pathogens including *Pneumocystis jirovecii* and *Aspergillus fumigatus* in children with severe pneumonia in the PICU, and an increase in potentially pathogenic eukaryotic diversity in BALF was positively associated with the occurrence of death and sepsis.

Conclusions: mNGS can be used for clinical microbiological testing of BALF samples from children in the PICU. Bacterial combined with viral or fungal infections may be present in the BALF of patients with severe pneumonia in the PICU. Viral or fungal infections are associated with greater disease severity and death.

Background

The global COVID-19 pandemic is now in its third year. Even without COVID-19, however, pneumonia causes more deaths each year than any other type of infectious disease [1]. Accurate detection of pathogens and differentiation from background commensal organisms is essential to guide optimal antimicrobial therapy. Bronchoalveolar lavage fluid (BALF) is considered a sterile specimen suitable for the detection of pathogens involved in respiratory tract infections [2–5]. Metagenomic next-generation sequencing (mNGS) can be used to assess the microbial composition of clinical samples without culture, improving the sensitivity of pathogen detection and providing guidance for clinical practice [6]. mNGS of BALF samples can identify pathogenic agents and improve treatment precision. Leo *et al.* [7] found that a protocol based on sequential lysis of human and bacterial cells or mechanical disruption of all cells

could be used to extract DNA from BALF samples and perform mNGS assays. Fang *et al.* [8] performed mNGS on blood, BALF, and cerebrospinal fluid samples from an adult patient with severe pneumonia with unexplained fever and subsequent encephalitis, and they detected high loads of human adenovirus B55. The patient was treated with intravenous ribavirin and cleared the virus after 26 days, and mNGS testing was performed to diagnose unexpected herpes simplex virus 1 encephalitis during hospitalization, leading to timely treatment [8]. Qi *et al.* [9] used mNGS on BALF samples from hospitalized patients with suspected ventilator-associated pneumonia. mNGS detected new pathogens in addition to those obtained by culture methods, while potential pathogens, including bacterial, fungal, and viral organisms, were detected by mNGS in 89% (40 of 45) culture-negative samples [9]. Li *et al.* [10] performed mNGS on 35 BALF samples from 32 adults with respiratory failure. They found that the diagnostic sensitivity of mNGS was 88.89% and specificity was 74.07% compared with the culture method, with a concordance rate of 77.78% between the two methods; additionally, the diagnostic sensitivity of mNGS was 77.78% and specificity was 70.00% compared with the smear method and polymerase chain reaction. The diagnostic sensitivity of the mNGS findings led to a change in the treatment strategy in 11 of 32 (34.4%) patients [10].

The results of BALF mNGS testing in patients with severe respiratory infections have been shown to be similar to those of transbronchial lung biopsy (TBLB) and transtracheal aspiration. Liu *et al.* [11] compared mNGS testing on BALF samples and TBLB tissue samples from patients with peripheral lung infections. Whereas the specificity of mNGS was lower for BALF than for TBLB tissue, the sensitivity of mNGS was higher for BALF than for TBLB tissue, and the most common infectious agents of the lung were *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* [11]. Kalantar *et al.* [12] performed mNGS on 52 mini-BALF samples from adults with severe pneumonia and compared them with mNGS results from tracheal aspirate samples. They found that although there were significant differences between sample types in patients with non-infectious acute respiratory disease, there were significant similarities in the composition of samples from patients with bacterial pneumonia, whose microbial community was characterized as the main pathogen [12].

Studies of mNGS on BALF samples from patients with severe respiratory infections have generally been limited to adults; few reports have focused on children. Takeuchi *et al.* [13] performed mNGS on BALF samples from 10 children with respiratory failure and detected significant bacterial or viral sequencing reads in 8 of the 10 patients. In addition, candidate pathogens were detected in three patients in whom no pathogens were identified by conventional methods. Moreover, the complete genome of enterovirus D68 was identified in two patients, and phylogenetic analysis showed that both strains belonged to subtype B3, a common strain that has spread worldwide in recent years [13]. These findings indicate that mNGS can be used for comprehensive molecular diagnosis as well as for pathogen surveillance in BALF in patients with respiratory tract infections.

Methods

Participant enrollment

The participants in this study were children treated in the pediatric intensive care unit (PICU) of the Children's Hospital of Fudan University from February 2018 to February 2020. The inclusion criterion was a diagnosis of severe pneumonia according to the diagnostic criteria established by the World Health Organization [14–16]. The primary diagnostic criteria were invasive mechanical ventilation, fluid-refractory shock, an urgent need for noninvasive positive-pressure ventilation, and hypoxemia requiring an FiO_2 greater than the inhalation concentration or flow rate feasible within general care. The secondary criteria were an increased respiratory rate, PaO_2/FiO_2 ratio of < 250, multilobar infiltration, Pediatric Early Warning Score of > 6, altered mental status, hypotension, presence of effusion, comorbidities (e.g. immunosuppression, immunodeficiency), and unexplained metabolic acidosis. The exclusion criteria for this study were an age of < 28 days or > 18 years; noninfectious factors such as congenital heart disease, pulmonary edema, asthma, upper airway obstruction, or pulmonary cystic fibrosis; contraindications to fiberoptic bronchoscopy; severe cardiopulmonary dysfunction; and coagulopathy.

BALF collection

With reference to the recommendations of the European Respiratory Society [17], sedation and topical anesthesia were administered, and the patient's lungs were lavaged using an age-appropriate pediatric flexible fiberoptic bronchoscope. The more severely diseased region in patients with diffuse lung disease or the right middle lobe was selected based on radiological findings or evidence from bronchoscopy. Warm saline (1 mL/kg body weight, maximum 20 mL per fraction) was dripped into the selected lung lobes, and at least 40% of the fluid was recovered by mechanical suction using a pressure of approximately 50 to 100 mmHg.

Serum inflammatory markers

Venous blood (2 mL) was collected and stored at room temperature with heparin anticoagulation to complete the assay for serum lipopolysaccharide (LPS), procalcitonin (PCT), C-reactive protein (CRP), interleukin 6 (IL-6), and other indices. The specific process is detailed in the **Supplementary Appendix**.

Lymphocyte subsets

Venous blood (2 mL) was collected, and the peripheral blood lymphocyte subsets were first labeled with fluorescently labeled monoclonal antibodies, including anti-CD19 antibodies for B cells, anti-CD3 and anti-CD8 antibodies for CD8 + T cells, anti-CD3 and anti-CD4 antibodies for CD4 + T cells, and anti-CD16 and anti-CD56 antibodies for natural killer cells. Flow cytometry (BD Biosciences, San Jose, CA, USA) was then performed to complete the lymphocyte subpopulation analysis.

Clinical microbiology testing

Microbiological culture

BALF was inoculated in blood agar, chocolate agar, and MacConkey agar plates; placed in a 5–10% carbon dioxide environment; and incubated at 35°C for 24 to 48 hours. Fungal culture required two temperatures of 28°C and 35°C with a 5- to 7-day incubation period, during which bacterial or fungal

growth on the plate was observed daily. Initial determination of specific microorganisms was performed based on the different colony morphologies and staining on the medium.

Mass spectrometry

Suspicious colonies on the medium were selected for further identification of bacterial or fungal taxa by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Drug sensitivity testing

For suspected growth of *Streptococcus pneumoniae*, suspicious colonies on the blood agar plate were selected for further purification. The optochin test was performed for identification and drug sensitivity testing of microorganisms such as *Escherichia coli* strains *ATCC25922*, *ATCC35218*, *ATCC25923*, *ATCC27853*, *ATCC29213*, *ATCC29212*, *ATCC49619*, *ATCC66027*, and *ATCC49766*. The drug sensitivity test was performed at Shanghai Clinical Laboratory Centre.

Detection of common respiratory viruses

Common respiratory viruses such as respiratory syncytial virus, adenoviruses, influenza A and B, parainfluenza virus, and human metapneumovirus were detected using an immunofluorescence test kit (Diagnostic Hybrids, Athens, OH, USA). The procedure is detailed in the **Supplementary Appendix**.

mNGS

We performed metagenomic sequencing with reference to our previously published studies [18–23], as described in the **Supplementary Appendix.**

Metagenomics analysis

Microbial ecological diversity

The sequencing data from each sample were first normalized to obtain the number of sequenced reads of individual microorganisms out of 1 million total sequenced reads [i.e., reads per million (rpm)]. The microbial ecological diversity index of the sample was then calculated based on the rpm value of each microorganism in the sample, including the abundance (i.e. the sum of rpm of all microorganisms), number of species, and alpha diversity index (Chao1 diversity index and Shannon diversity index). The Chao1 diversity index and Shannon diversity index were calculated using the vegan package of R software (version 3.6.1).

Determination of pathogenic microorganisms

We referred to a previously published article [23] for the determination of pathogenic microorganisms in BALF, as described in the **Supplementary Appendix.**

Correlation/regression analysis

Pearson correlation coefficients and Spearman correlation coefficients for each microbial ecological diversity index and clinical phenotype were first calculated by the cor.test() function of R software (version 3.6.5). Next, the lm() function of R software (version 3.6.5) was used to perform a linear fit of the microbial ecological diversity index to the clinical phenotypes, was then the ggplot2 package of R software was used to draw the scatterplot. Statistically significant p-values were obtained by the Wilcoxon rank sum test function (wilcox.test) of R software.

Results

Clinical information

This study involved 126 children in the PICU, including 84 with community-acquired pneumonia (CAP) and 42 with hospital-acquired pneumonia (HAP). Their mean maximum body temperature was 39ºC ± 0.95ºC, mean heart rate was 160 ± 19 beats per minute, mean total duration of hospitalization was 37 ± 29 days, and mean number of days in the PICU was 30 ± 29 days. The 30-day mortality analysis revealed 24 deaths and 16 cases of abandoned treatment, accounting for 29.4% of all patients. In total, 86.5% (109/126) of patients had combined respiratory failure, 24.6% (31/126) had combined severe pneumonia, 24.6% (31/126) had combined sepsis, 5.5% (7/126) had septic shock, one had encephalitis, and four were being hospitalized after cord blood stem cell transplantation.

The mean serum CRP concentration was 67 ± 56 mg/L, PCT concentration was 8.9 ± 19 ng/mL, LPS concentration was 1.0 ± 7.6 pg/mL, and IL-6 concentration was 390 ± 850 pg/mL. The white blood cell (WBC) count was 18 ± 12 × 10^9 cells/L, CD3 + lymphocyte count was 1.2 ± 1.3 × 10^9 cells/L, CD4 + lymphocyte count was 6.8 ± 8.7 × 10^8 cells/L, CD56 + lymphocyte count was 1.4 ± 1.8 × 10^8 cells/L, and CD8 + lymphocyte count was 5.0 ± 5.9 × 10^8 cells/L. Pathogenic microorganisms were detected in 81 patients based on conventional clinical microbiological testing methods such as blood and other cultures.

Use of mNGS of BALF samples to identify pathogenic microorganisms associated with clinical symptoms

mNGS was performed on 126 samples [at the DNA level in most (101/126, 80.1%) and at the RNA level in a smaller number (25/126, 19.8%)]. Each sample sequenced yielded 2.6 ± 1.4 × 10^7 clean sequences. The potentially pathogenic microorganisms in the samples were further identified by considering both the relative abundance of the microorganisms in the samples to be tested and the outlier levels (z-score) in all samples, following the method used in our previous study [23]. The relative abundance and z-score distribution of the pathogenic microorganisms obtained are shown in Fig. 1.

Nucleic acid sequences of 88 pathogenic bacteria were detected, including 9 species of *Streptococcus* (*Streptococcus agalactiae*, *Streptococcus australis*, *Streptococcus milleri*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus parasanguinis*, *Streptococcus pneumoniae*, *Streptococcus*

pseudopneumoniae, and *Streptococcus salivarius*), 7 species of *Staphylococcus* (*Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, and *Staphylococcus warneri*), and 3 species of *Acinetobacter* (*Acinetobacter baumannii*, *Acinetobacter johnsonii*, and *Acinetobacter nosocomialis*). The top 25 bacteria are listed in Fig. 2. In total, 13 viral nucleic acid sequences were detected, including 4 human herpesviruses [*Human alphaherpesvirus 1*, *Human betaherpesvirus 5* (also known as human cytomegalovirus), *Human betaherpesvirus 6B*, and *Human gammaherpesvirus 4* (also known as Epstein–Barr virus)], *Human mastadenovirus B*, and *Human polyomavirus 4*. All viruses are shown in **Supplementary Fig. 1**. Nucleic acid sequences were detected for 39 pathogenic fungi, including 4 *Aspergillus* species (*Aspergillus fischeri*, *Aspergillus fumigatus*, *Aspergillus mulundensis*, and *Aspergillus nidulans*) and *Pneumocystis jirovecii*; the other fungi are shown in **Supplementary Fig. 2**. Nucleic acid sequences were detected for 27 protozoans, including 10 protozoa of the genus *Plasmodium* (*Plasmodium falciparum*, *Plasmodium gaboni*, *Plasmodium gallinaceum*, *Plasmodium gonderi*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium reichenowi*, *Plasmodium relictum*, *Plasmodium sp*, and *Plasmodium yoelii*) and 2 protozoa of the genus *Trypanosoma* (*Trypanosoma cruzi* and *Trypanosoma theileri*). The others are specified in **Supplementary Fig. 3**.

Correlation of pathogenic microbial abundance in BALF with serum inflammatory indicators

We found that the serum LPS concentration was correlated with the pathogenic bacterial abundance in BALF. Notably, most of the 12 serum LPS-associated bacteria identified were positively correlated with the serum LPS concentration, including *Acinetobacter baumannii*, *Neisseria meningitidis*, *Neisseria subflava*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Mycoplasma salivarium*, and several others; the full results are shown in Fig. 3a. We also found that the serum CRP concentration was correlated with the pathogenic bacterial abundance in BALF, with *Pseudomonas aeruginosa* and *Corynebacterium striatum* positively correlated with the serum CRP concentration, as shown in Fig. 3b. Moreover, the serum PCT concentration was correlated with the abundance of pathogenic bacteria in BALF; the abundance of *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Mycoplasma salivarium* was positively correlated with the serum PCT concentrations, as shown in Fig. 3c. Likewise, we found that serum IL-6 concentration was correlated with the abundance of pathogenic bacteria in BALF, with positive correlations noted for *Acinetobacter baumannii* and *Acinetobacter nosocomialis*. Notably, we found that certain *Streptococcus spp.* Pathogenic bacteria in BALF, such as *Streptococcus pseudopneumoniae*, *Streptococcus agalactiae*, *Streptococcus oralis*, and *Streptococcus mitis*, were negatively correlated with the serum IL-6 concentration, as shown in Fig. 3d.

Correlation of pathogenic microbial abundance in BALF with total WBC count and T-lymphocyte count

We found that the total WBC count was negatively correlated with the abundance of pathogenic bacteria in BALF, such as *Pseudomonas aeruginosa*, *Prevotella jejuni*, *Streptococcus australis*, *Achromobacter xylosoxidans*, *Sphingopyxis fribergensis*, *Actinomyces pacaensis*, *Actinomyces naeslundii*, *Herbaspirillum huttiense*, and others (**Supplementary Fig. 4a**). We also found that the total WBC count was negatively correlated with the abundance of *Human betaherpesvirus 6B* and *Human gammaherpesvirus 4* in BALF (**Supplementary Fig. 4b**) and with the abundance of fungi in BALF such as *Pneumocystis jirovecii* and *Aspergillus terreus* (**Supplementary Fig. 4c**).

We found that the CD3 + T-cell count was correlated with the abundance of pathogenic bacteria in BALF such as *Staphylococcus aureus*, *Staphylococcus haemolyticus*, and *Staphylococcus warneri* (**Supplementary Fig. 5a**). Notably, we found that the abundance of *Staphylococcus aureus, Staphylococcus haemolyticus*, and *Staphylococcus warneri* in BALF was negatively correlated with the blood CD4+, CD8+, and CD56 + T-cell counts (**Supplementary Fig. 5b–d**).

Differences in microbiological composition of BALF between patients with CAP and HAP

We performed a differential analysis of the pathogenic microbial composition of BALF in patients with CAP and HAP and identified 11 significantly different pathogenic bacteria. Notably, we found significantly greater amounts of *Acinetobacter baumannii* and *Acinetobacter nosocomialis* in the BALF of patients with HAP than of patients with CAP, while the amounts of *Streptococcus spp.*, *Streptococcus salivarius*, and *Streptococcus mitis* were significantly lower in the patients with HAP than in the patients with CAP. We also found a significantly higher amount of the potentially pathogenic fungus *Diplodia corticola* and the potentially pathogenic parasite *Plasmopara halstedii* in the BALF of patients with HAP than of patients with CAP (Fig. 4).

Correlation of mortality and oxygenation index (OI) of severe pneumonia with pathogenic microbial composition of BALF

We assessed the correlation between lethal phenotypes (death or abandonment of treatment) and the ecological diversity of pathogenic microorganisms in BALF. The results showed that the fungal and protozoa richness (Chao1 index) was significantly higher in BALF from deceased than non-deceased patients (Fig. 5a); the fungal and protozoal diversity (Shannon index) was also significantly higher in BALF from deceased than non-deceased patients (Fig. 5b and **c**). We also assessed the correlation between lethal phenotypes (death or abandonment of treatment) and the pathogenic microbial composition of the BALF. The results showed that *Escherichia coli* abundance was significantly higher in the BALF from deceased than non-deceased patients. Notably, we found significantly higher abundance of *Klebsiella pneumoniae* and *Corynebacterium segmentosum* in the BALF of patients in the abandoned treatment group than in the deceased/non-deceased group (Fig. 5d). We also found significantly higher abundance of *Pneumocystis jirovecii* in the BALF of deceased patients than in both the non-deceased and abandoned treatment groups (Fig. 5e).

We found that the OI, which reflects the severity of disease in patients with severe pneumonia, was associated with increased abundance of pathogenic bacteria in BALF, including *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, and *Staphylococcus aureus* (Fig. 6a). The OI was also positively correlated with an increased abundance of the fungi *Aspergillus fumigatus* and *Rhizopus microspores* in BALF (Fig. 6b) and with an increased abundance of the viruses *Human mastadenovirus B* and *Torque teno virus 29* in BALF (Fig. 6c). The OI was also correlated with the overall viral abundance in BALF (Fig. 6d).

Sepsis and immunodeficiency in patients with severe pneumonia associated with BALF microorganisms

Among patients with severe pneumonia, the BALF fungal and parasite species richness (Chao1 index), Shannon diversity, and number of species were significantly higher in patients with than without sepsis (**Supplementary Fig. 6**). Additionally, the total number of bacterial species in BALF was significantly higher in patients with than without immunodeficiency (**Supplementary Fig. 7a**). Moreover, the abundance of *Elizabethkingia anophelis* in BALF was significantly higher in patients with than without immunodeficiency (**Supplementary Fig. 7b**). Notably, we found that the abundance of *Human betaherpesvirus 5* (i.e., human cytomegalovirus) in BALF was significantly higher in patients with than without immunodeficiency (**Supplementary Fig. 7c**).

Discussion

Correlation of abundance of potentially pathogenic bacteria in BALF with serum inflammatory markers and lymphocyte subtypes

In the present study, we found that the BALF bacterial diversity indices, including the Chao1 diversity index and overall abundance, were positively correlated with the serum LPS concentration; the bacterial species count was positively correlated with the serum PCT concentration; and that the *Pseudomonas aeruginosa* abundance in BALF was positively correlated with the serum LPS, CRP, and PCT concentrations and negatively correlated with the whole blood WBC count and CD4 + T-cell count.

Pseudomonas aeruginosa is the most common multidrug-resistant Gram-negative bacterium and a common cause of ventilator-associated pneumonia in patients in ICUs. Ventilator-associated pneumonia caused by *Pseudomonas aeruginosa* infection is characterized by high morbidity and mortality [24, 25]. Experimental models suggest that ventilator-induced lung injury is associated with increased bronchial vascular permeability in BALF, higher cell counts and protein concentrations, and increased infiltration of inflammatory cells into lung tissue [26]. Cytokines are small proteins that communicate through intercellular signaling and can be considered immunomodulators of immune and inflammatory responses [27]. Human studies have shown that cytokine/chemokine release and leukocyte recruitment contribute to ventilator-associated lung injury [28] and that IL-6, an inflammatory marker of ventilator-associated lung injury, may contribute to alveolar barrier dysfunction in patients with acute respiratory

distress syndrome [26]. Tsay *et al.* [29] found that intranasal drip of *Pseudomonas aeruginosa* induced IL-6 expression in the lungs of mice.

Acinetobacter baumannii, a Gram-negative aerobic bacterium, is listed by the World Health Organization as posing the greatest threat to human health and as being in urgent need of new antibiotics [30, 31]. In recent years, isolates of *Acinetobacter baumannii* have been recovered from a variety of extra-hospital sources (e.g., vegetables, water treatment plants, and fish and shrimp farms) in addition to known natural habitats (soil and moist environments), and the wide range of sources of this bacterium might explain the occurrence of community-acquired infections [32]. *Acinetobacter baumannii* is a serious pathogen involved in hospital-acquired and community-acquired infections [33]. Cases of CAP caused by *Acinetobacter baumannii* are rare in Europe and the United States [34]; instead, most cases of CAP caused by *Acinetobacter baumannii* occur in countries with tropical or subtropical climates. *Acinetobacter baumannii* is an emerging pathogen in the Asia-Pacific region, with a high prevalence in Hong Kong, Singapore, Taiwan, Korea, and Australia [35]. In the present study, we found that the abundance of *Acinetobacter baumannii* in BALF of children in the PICU was positively correlated with the serum IL-6 and LPS concentrations. Notably, the abundance of *Acinetobacter baumannii* in BALF was significantly higher in patients with HAP than CAP; this may be related to the fact that *Acinetobacter baumannii* is more common in hospitals than in the community. The primary target of *Acinetobacter baumannii* is patients in ICUs, and the use of antibiotics has led to outbreaks and epidemics of infections caused by multidrug-resistant *Acinetobacter baumannii* in hospitals [33, 36].

Correlation of potentially pathogenic viral infections in BALF of children in PICU with immunodeficiency and pneumonia severity

Respiratory viral infections are an important cause of pneumonia in children. Viral bronchiolitis is the most common cause of respiratory failure in young children requiring invasive ventilation, and coinfections such as bacterial and fungal infections may prolong and complicate hospital stays in the PICU [37, 38]. We performed mNGS on BALF from children in the PICU and found that viral nucleic acid sequences were present; in addition, the Chao1 diversity index of viruses was positively correlated with the maximum body temperature on admission and negatively correlated with the CD3 + lymphocyte count. Moreover, the Shannon diversity index of viruses was negatively correlated with the CD3+, CD4+, CD8+, and CD56 + lymphocyte counts.

The number of ICU admissions for viral infections is rising. The Herpesviridae family, including *Human gammaherpesvirus 4* (i.e., Epstein–Barr virus) and *Cytomegalovirus*, can be reactivated in ICU patients, and such viral activation is associated with morbidity and mortality [39]. Consistent with this, we found that the abundance of *Human betaherpesvirus 6B* and *Human gammaherpesvirus 4* was negatively correlated with the total WBC count. Additionally, the abundance of *Human betaherpesvirus 5* (i.e., human cytomegalovirus) in the BALF of patients with severe pneumonia was significantly higher in those with than without immunodeficiency, suggesting a possible correlation between immune system dysregulation and viral activation. We also found that the severity of pneumonia (i.e., the OI) was positively correlated

with the overall viral abundance in the BALF; notably, we found that the OI was positively correlated with an increase in the abundance of the viruses *Human mastadenovirus B* and *Torque teno virus 29* in BALF. In summary, these findings suggest a correlation between the viral load in BALF and the severity of pneumonia.

Detection of potentially pathogenic eukaryotes in BALF of children with severe pneumonia in the PICU and their association with death and sepsis

Fungi are another important cause of respiratory infections [40]. We found significantly higher species richness (Chao1 index), Shannon diversity, and number of species of pathogenic fungi in the BALF of patients with than without sepsis in the present study. *Aspergillus*, *Pneumocystis*, and *Cryptococcus* are the key fungal pathogens known to cause respiratory infections [41]. *Pneumocystis jirovecii* causes pneumocystis pneumonia, a life-threatening disease in the ICU with a high mortality rate, particularly in immunocompromised patients [40, 42]. Schmidt *et al.* [40] found that the overall hospital mortality rate was 25.4% and increased to 58.0% if ICU admission was required. In line with this, we found a significantly higher abundance of *Pneumocystis jirovecii* in the BALF of deceased patients than in both non-deceased patients and patients for whom treatment was abandoned.

Patients in the ICU are more likely to have a history of coinfection with bacteria and fungi than are patients in the general ward [43]. Beumer *et al.* [44] found that the incidence of coinfection with bacteria and fungi in the lungs of patients with influenza was 55.6% among ICU inpatients, which was significantly higher than in patients in the normal ward (20.1%), and *Aspergillus fumigatus* and *Pneumocystis jirovecii* were the predominant coinfecting fungi [44]. In line with this, we found that an increase in the abundance of the fungal pathogen *Aspergillus fumigatus* in BALF was positively correlated with the severity (i.e., the OI) of patients with severe pneumonia, while an increase in the abundance of the bacterial pathogens *Klebsiella pneumoniae*, *Streptococcus agalactiae*, and *Staphylococcus aureus* in BALF of children in the PICU was also positively correlated with the severity (i.e., the OI) of patients with severe pneumonia.

Protozoan coinfection increases the risk of pneumonia in children [45, 46]. We found significantly higher protozoan species richness (Chao1 index), Shannon diversity, and number of species in the BALF of patients with than without sepsis in the present study; we also detected nucleic acid sequences of *Plasmodium* and *Trypanosoma* in the BALF of children in the PICU. Notably, protozoa have a large genome, and the mNGS results only cover a very small part of their genome. Although in-depth validation is needed, our results suggest the possibility of protozoan coinfection.

This study had some limitations. The pathogenic nucleic acid sequences we identified in BALF in the PICU require in-depth validation, including completion by clinical microbiological methods such as BALF culture and antibody testing. Further, multicenter scale-up validation is also needed for an in-depth assessment of mNGS results relevance to clinical symptoms in children with severe pneumonia in the PICU.

Conclusions

mNGS of BALF from children with severe pneumonia in the PICU revealed potentially pathogenic bacterial infections. The increased bacterial Chao1 diversity index and overall abundance in the BALF were positively correlated with serum inflammatory indicators, and altered abundance of potentially pathogenic bacteria was correlated with serum inflammatory indicators and altered lymphocyte subtypes. In addition, there was the potential for coinfection with viruses in these children as shown by the correlation of the abundance of potentially pathogenic viruses in BALF with the presence of immunodeficiency and the severity of pneumonia. We also found the potential for fungal coinfections in these children as indicated by the positive association of increased diversity of potentially pathogenic fungi in BALF with the occurrence of death and sepsis.

Declarations

Ethics approval and consent to participate

This study was completed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Children's Hospital of Fudan University with an IRB of 2019-312. Permission was obtained from the patients' parents, and written informed consent was obtained prior to the bronchoalveolar lavage fluid collection.

Consent for publication

Written informed consent for the publication of this article was obtained from guardians of the children.

Availability of data and materials

The unbiased metagenomic next generation sequencing data in the current study cannot be made publicly available because it is not allowed according to the national requirements on human genetic resource of research: The Regulations of the People's Republic of China on the Management of Human Genetic Resources (http://www.moj.gov.cn/government_public/content/2019-06/10/593_236557.html). The data that support the findings of this study are available upon request from the corresponding author.

Competing interests

The authors state that they have no conflicts of interest related to this study.

Funding

The study was supported by the National Key Research and Development Program of China (2021YFC2701800–2021YFC2701803) and the Scientific Research Project of Shanghai Municipal Health Commission (No. 202140442).

Authors' contributions

TL, YW, WC and JT recruited and characterized the human participants. CZ, MW and TL designed and analyzed mNGS data, and were major contributors in writing the manuscript. GY and GL planned, designed, supervised, and coordinated the overall research efforts. All authors read and approved the final manuscript.

Acknowledgment

We thank Angela Morben, DVM, ELS, from Liwen Bianji (Edanz) (www.liwenbianji.cn), for editing the English text of a draft of this manuscript.

References

1. Hansen, V., et al., *Infectious Disease Mortality Trends in the United States, 1980-2014.* JAMA, 2016. **316**(20): p. 2149-2151.
2. Friaza, V., et al., *Metagenomic analysis of bronchoalveolar lavage samples from patients with idiopathic interstitial pneumonia and its antagonic relation with Pneumocystis jirovecii colonization.* J Microbiol Methods, 2010. **82**(1): p. 98-101.
3. Charlson, E.S., et al., *Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant.* Am J Respir Crit Care Med, 2012. **186**(6): p. 536-45.
4. Willner, D.L., et al., *Reestablishment of recipient-associated microbiota in the lung allograft is linked to reduced risk of bronchiolitis obliterans syndrome.* Am J Respir Crit Care Med, 2013. **187**(6): p. 640-7.
5. Seo, S., et al., *Idiopathic pneumonia syndrome after hematopoietic cell transplantation: evidence of occult infectious etiologies.* Blood, 2015. **125**(24): p. 3789-97.
6. Gu, W., S. Miller, and C.Y. Chiu, *Clinical Metagenomic Next-Generation Sequencing for Pathogen Detection.* Annu Rev Pathol, 2019. **14**: p. 319-338.
7. Leo, S., et al., *Detection of Bacterial Pathogens from Broncho-Alveolar Lavage by Next-Generation Sequencing.* Int J Mol Sci, 2017. **18**(9).
8. Fang, X., et al., *Real-time Utilization of Metagenomic Sequencing in the Diagnosis and Treatment Monitoring of an Invasive Adenovirus B55 Infection and Subsequent Herpes Simplex Virus Encephalitis in an Immunocompetent Young Adult.* Open Forum Infect Dis, 2018. **5**(6): p. ofy114.
9. Qi, C., et al., *Detection of respiratory pathogens in clinical samples using metagenomic shotgun sequencing.* J Med Microbiol, 2019. **68**(7): p. 996-1002.
10. Li, Y., et al., *Application of metagenomic next-generation sequencing for bronchoalveolar lavage diagnostics in critically ill patients.* Eur J Clin Microbiol Infect Dis, 2020. **39**(2): p. 369-374.
11. Liu, N., et al., *Metagenomic next-generation sequencing diagnosis of peripheral pulmonary infectious lesions through virtual navigation, radial EBUS, ultrathin bronchoscopy, and ROSE.* J Int Med Res,

2019. **47**(10): p. 4878-4885.

12. Kalantar, K.L., et al., *Metagenomic comparison of tracheal aspirate and mini-bronchial alveolar lavage for assessment of respiratory microbiota.* Am J Physiol Lung Cell Mol Physiol, 2019. **316**(3): p. L578-L584.
13. Takeuchi, S., et al., *Metagenomic analysis using next-generation sequencing of pathogens in bronchoalveolar lavage fluid from pediatric patients with respiratory failure.* Sci Rep, 2019. **9**(1): p. 12909.
14. Bradley, J.S., et al., *The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America.* Clin Infect Dis, 2011. **53**(7): p. e25-76.
15. Chisti, M.J., et al., *Bubble continuous positive airway pressure for children with severe pneumonia and hypoxaemia in Bangladesh: an open, randomised controlled trial.* Lancet, 2015. **386**(9998): p. 1057-65.
16. Williams, D.J., et al., *Predicting Severe Pneumonia Outcomes in Children.* Pediatrics, 2016. **138**(4).
17. de Blic, J., et al., *Bronchoalveolar lavage in children. ERS Task Force on bronchoalveolar lavage in children. European Respiratory Society.* Eur Respir J, 2000. **15**(1): p. 217-31.
18. Zhou, S., et al., *Diversity of Gut Microbiota Metabolic Pathways in 10 Pairs of Chinese Infant Twins.* PLoS One, 2016. **11**(9): p. e0161627.
19. Zhou, S., et al., *Association of serum bilirubin in newborns affected by jaundice with gut microbiota dysbiosis.* J Nutr Biochem, 2019. **63**: p. 54-61.
20. Wang, M., et al., *Alteration of gut microbiota-associated epitopes in children with autism spectrum disorders.* Brain Behav Immun, 2019. **75**: p. 192-199.
21. Xu, R., et al., *Altered gut microbiota and mucosal immunity in patients with schizophrenia.* Brain Behav Immun, 2020. **85**: p. 120-127.
22. Wang, M., et al., *Virulence factor-related gut microbiota genes and immunoglobulin A levels as novel markers for machine learning-based classification of autism spectrum disorder.* Comput Struct Biotechnol J, 2021. **19**: p. 545-554.
23. Yan, G., et al., *Metagenomic Next-Generation Sequencing of Bloodstream Microbial Cell-Free Nucleic Acid in Children With Suspected Sepsis in Pediatric Intensive Care Unit.* Front Cell Infect Microbiol, 2021. **11**: p. 665226.
24. Zhuo, H., et al., *Increased mortality of ventilated patients with endotracheal Pseudomonas aeruginosa without clinical signs of infection.* Crit Care Med, 2008. **36**(9): p. 2495-503.
25. Valencia, M. and A. Torres, *Ventilator-associated pneumonia.* Curr Opin Crit Care, 2009. **15**(1): p. 30-5.
26. Wolters, P.J., et al., *Neutrophil-derived IL-6 limits alveolar barrier disruption in experimental ventilator-induced lung injury.* J Immunol, 2009. **182**(12): p. 8056-62.
27. Spelman, K., et al., *Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators.* Altern Med Rev, 2006. **11**(2): p. 128-50.

28. Halbertsma, F.J., et al., *Cytokines and biotrauma in ventilator-induced lung injury: a critical review of the literature.* Neth J Med, 2005. **63**(10): p. 382-92.
29. Tsay, T.B., et al., *Pseudomonas aeruginosa colonization enhances ventilator-associated pneumonia-induced lung injury.* Respir Res, 2016. **17**(1): p. 101.
30. Lee, C.R., et al., *Biology of Acinetobacter baumannii: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options.* Front Cell Infect Microbiol, 2017. **7**: p. 55.
31. in *Guidelines for the Prevention and Control of Carbapenem-Resistant Enterobacteriaceae, Acinetobacter baumannii and Pseudomonas aeruginosa in Health Care Facilities*. 2017: Geneva.
32. Eveillard, M., et al., *Reservoirs of Acinetobacter baumannii outside the hospital and potential involvement in emerging human community-acquired infections.* Int J Infect Dis, 2013. **17**(10): p. e802-5.
33. Dijkshoorn, L., A. Nemec, and H. Seifert, *An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii.* Nat Rev Microbiol, 2007. **5**(12): p. 939-51.
34. Serota, D.P., et al., *Severe Community-Acquired Pneumonia due to Acinetobacter baumannii in North America: Case Report and Review of the Literature.* Open Forum Infect Dis, 2018. **5**(3): p. ofy044.
35. Kim, Y.A., et al., *Seasonal and Temperature-Associated Increase in Community-Onset Acinetobacter baumannii Complex Colonization or Infection.* Ann Lab Med, 2018. **38**(3): p. 266-270.
36. Ramirez, M.S., R.A. Bonomo, and M.E. Tolmasky, *Carbapenemases: Transforming Acinetobacter baumannii into a Yet More Dangerous Menace.* Biomolecules, 2020. **10**(5).
37. Wiegers, H.M.G., et al., *Bacterial co-infection of the respiratory tract in ventilated children with bronchiolitis; a retrospective cohort study.* BMC Infect Dis, 2019. **19**(1): p. 938.
38. Li, Y., et al., *The role of viral co-infections in the severity of acute respiratory infections among children infected with respiratory syncytial virus (RSV): A systematic review and meta-analysis.* J Glob Health, 2020. **10**(1): p. 010426.
39. Cantan, B., C.E. Luyt, and I. Martin-Loeches, *Influenza Infections and Emergent Viral Infections in Intensive Care Unit.* Semin Respir Crit Care Med, 2019. **40**(4): p. 488-497.
40. Schmidt, J.J., et al., *Clinical course, treatment and outcome of Pneumocystis pneumonia in immunocompromised adults: a retrospective analysis over 17 years.* Crit Care, 2018. **22**(1): p. 307.
41. Hayes, G.E. and D.W. Denning, *Frequency, diagnosis and management of fungal respiratory infections.* Curr Opin Pulm Med, 2013. **19**(3): p. 259-65.
42. Salzer, H.J.F., et al., *Clinical, Diagnostic, and Treatment Disparities between HIV-Infected and Non-HIV-Infected Immunocompromised Patients with Pneumocystis jirovecii Pneumonia.* Respiration, 2018. **96**(1): p. 52-65.
43. Schauwvlieghe, A., et al., *Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza: a retrospective cohort study.* Lancet Respir Med, 2018. **6**(10): p. 782-792.
44. Beumer, M.C., et al., *Influenza virus and factors that are associated with ICU admission, pulmonary co-infections and ICU mortality.* J Crit Care, 2019. **50**: p. 59-65.

45. D'Acremont, V., et al., *Beyond malaria--causes of fever in outpatient Tanzanian children.* N Engl J Med, 2014. **370**(9): p. 809-17.
46. Edwards, C.L., et al., *Coinfection with Blood-Stage Plasmodium Promotes Systemic Type I Interferon Production during Pneumovirus Infection but Impairs Inflammation and Viral Control in the Lung.* Clin Vaccine Immunol, 2015. **22**(5): p. 477-83.

Figures

Figure 1

Distribution of microbial abundance and z-score detected by mNGS of BALF samples. (a) Bacteria. (b) Viruses. (c) Fungi. (d) Protozoa. An rpm value of <0.01 abundance defaults to a log10 (rpm) of −2 for that microorganism in that sample, and a z-score pf >6 defaults to 6 for all samples. The red dots represent potential pathogens in our selected samples.

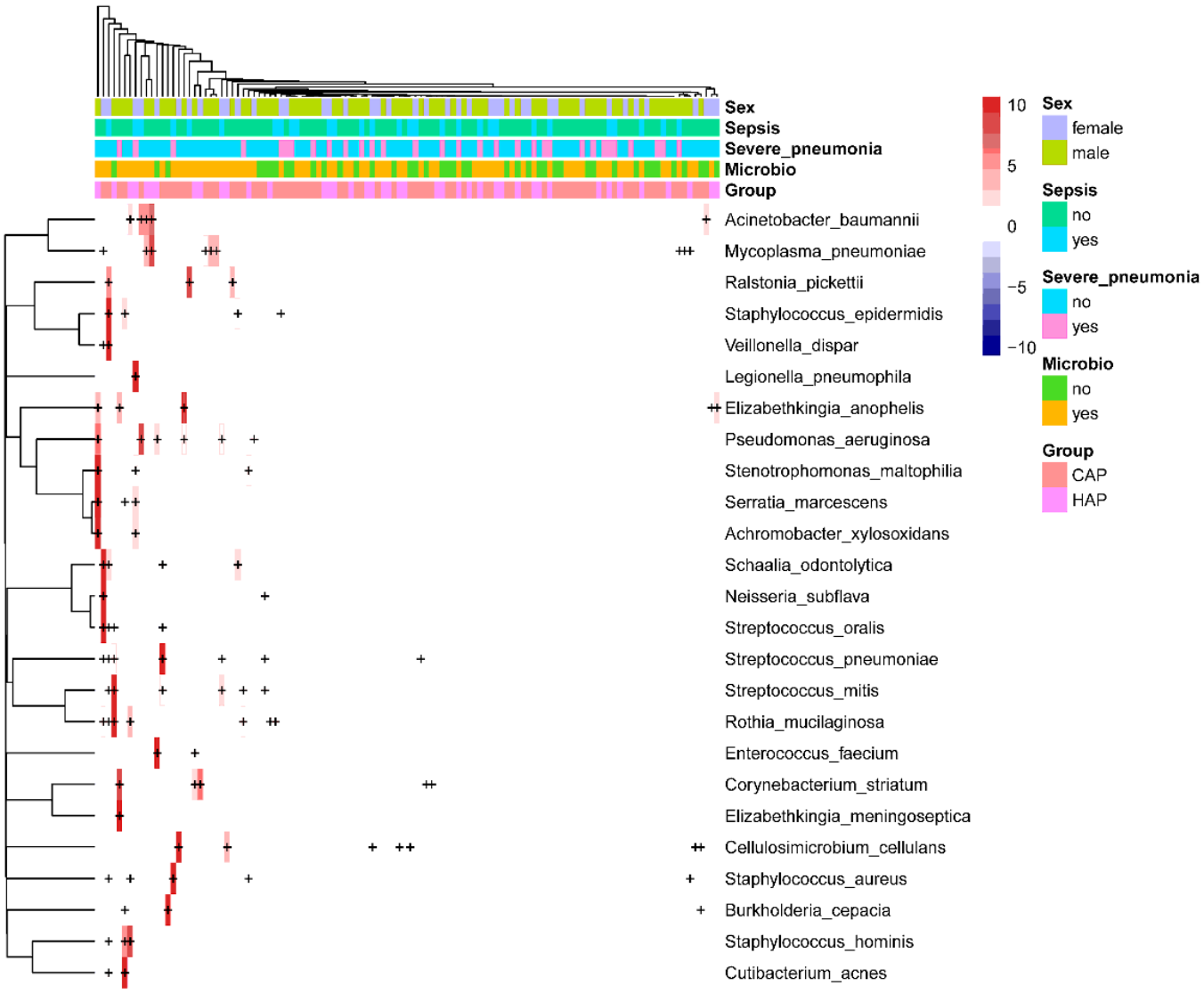

Figure 2

Heat map of potentially pathogenic bacteria detected by mNGS of BALF samples. For the top 25 bacteria, each horizontal row represents a sample, and each vertical column represents potentially pathogenic bacteria. The plus and minus signs in the graph represent significant positive and negative correlations, respectively. The clinical phenotype is at the top, and the color block on the right represents the value of the specific clinical phenotype.

Figure 3

Correlations of potentially pathogenic bacteria in BALF with serum inflammatory indicators. The heat map displays the correlations of the potentially pathogenic bacteria in BALF with the serum (a) LPS, (b) CRP, (c) PCT, and (d) IL-6 concentrations. The horizontal coordinate represents the Spearman correlation coefficient, and the vertical coordinate represents the negative logarithm of the p-value of the correlation between the clinical phenotype and potential pathogen; i.e., −log10 (p value). Each point in the graph represents a potentially pathogenic microorganism, and those screened for significant differences have been marked in red.

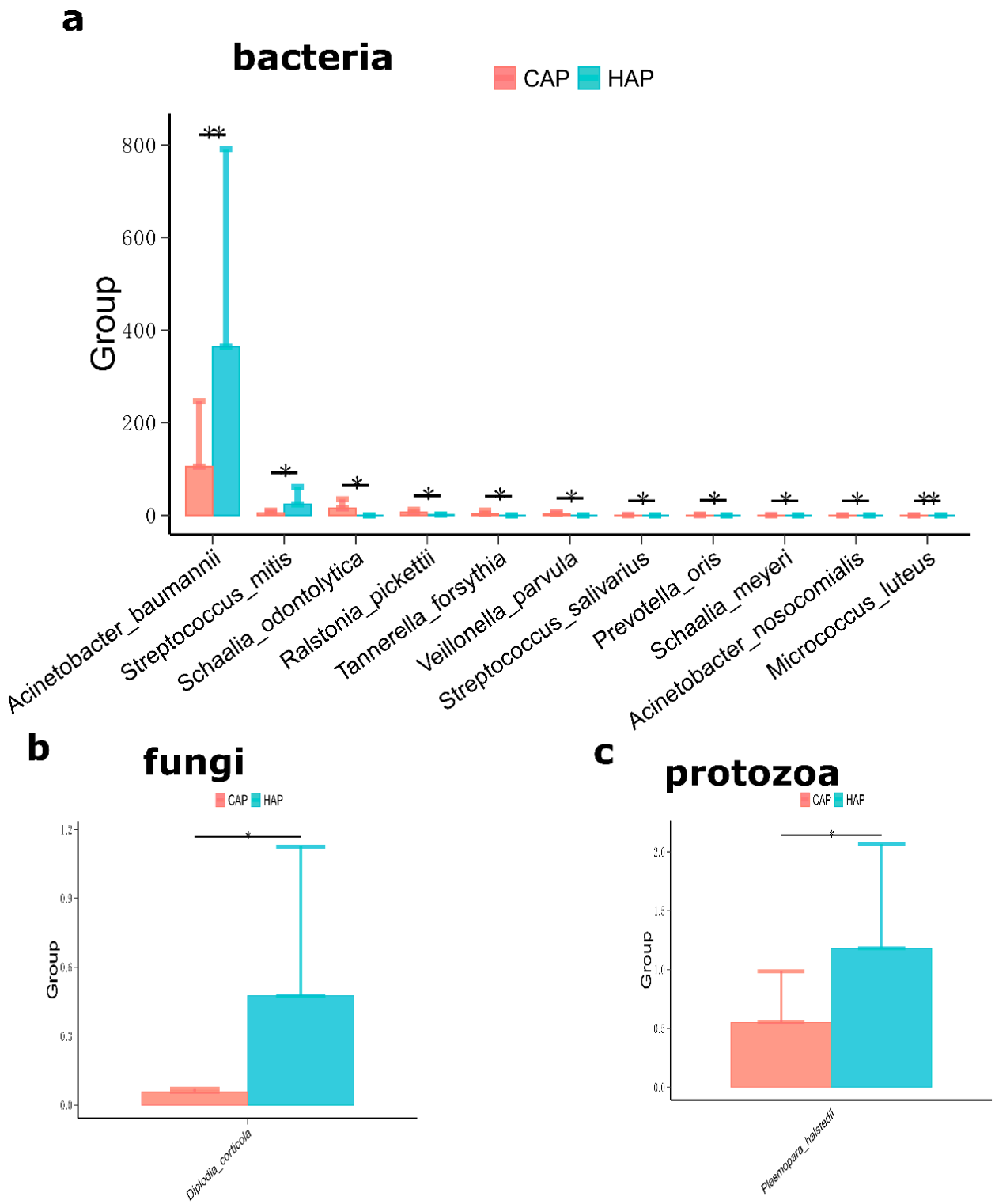

Figure 4

Significant differences in pathogenic microbial composition of bronchoalveolar lavage fluid in the CAP and HAP groups. Significant differences were noted in potentially pathogenic (a) bacteria, (b) fungi, and (c) protozoans between patients with CAP versus HAP.

Figure 5

Ecological diversity and composition of pathogenic microorganisms in BALF associated with mortality. (a) Mortality associated with Chao1. (b) Mortality associated with Shannon index. (c) Mortality associated with species count. (d) Mortality associated bacteria. (e) Mortality associated with fungi.

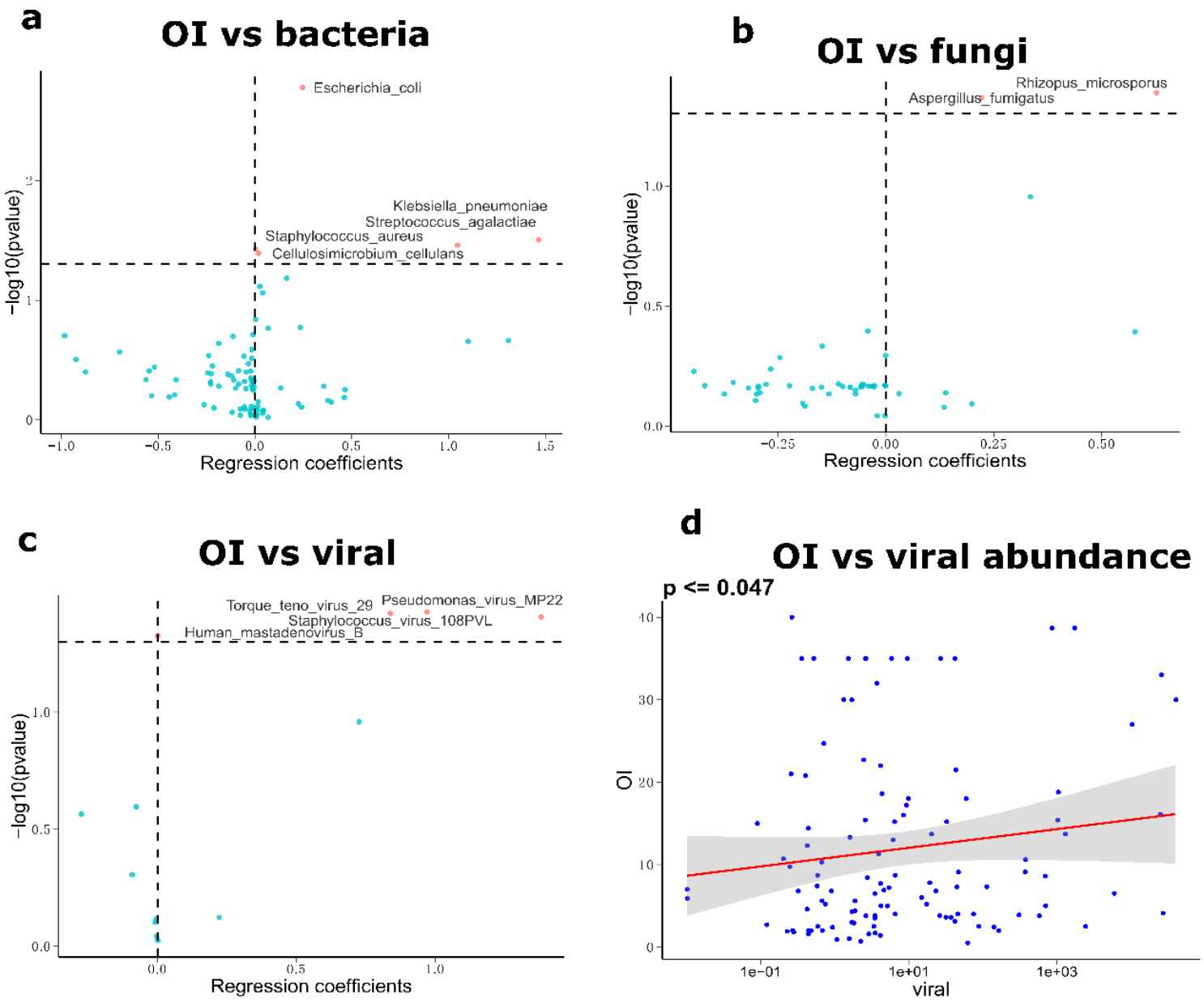

Figure 6

Correlation of oxygenation index (OI) with pathogenic microorganisms in BALF of patients with severe pneumonia. (a) The OI was positively correlated with the abundance of pathogenic bacteria in BALF. (b) The OI was positively correlated with the abundance of pathogenic fungi in BALF. (c) The OI was positively correlated with the abundance of viruses in BALF. (d) The OI was correlated with the overall abundance of viruses (viral abundance) in BALF. Fitted linear model, p = 0.047.