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ORIGINAL RESEARCH

High-throughput sequencing of *16S rDNA* amplicons characterizes bacterial composition in bronchoalveolar lavage fluid in patients with ventilator-associated pneumonia

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Abstract: Ventilator-associated pneumonia (VAP) is a life-threatening disease that is associated with high rates of morbidity and likely mortality, placing a heavy burden on an individual and society. Currently available diagnostic and therapeutic approaches for VAP treatment are limited, and the prognosis of VAP is poor. The present study aimed to reveal and discriminate the identification of the full spectrum of the pathogens in patients with VAP using high-throughput sequencing approach and analyze the species richness and complexity via alpha and beta diversity analysis. The bronchoalveolar lavage fluid samples were collected from 27 patients with VAP in intensive care unit. The polymerase chain reaction products of the hypervariable regions of *16S rDNA* gene in these 27 samples of VAP were sequenced using the 454 GS FLX system. A total of 103,856 pyrosequencing reads and 638 operational taxonomic units were obtained from these 27 samples. There were four dominant phyla, including *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. There were 90 different genera, of which 12 genera occurred in over ten different samples. The top five dominant genera were *Streptococcus*, *Acinetobacter*, *Limnohabitans*, *Neisseria*, and *Corynebacterium,* and the most widely distributed genera were *Streptococcus*, *Limnohabitans*, and *Acinetobacter* in these 27 samples. Of note, the mixed profile of causative pathogens was observed. Taken together, the results show that the high-throughput sequencing approach facilitates the characterization of the pathogens in bronchoalveolar lavage fluid samples and the determination of the profile for bacteria in the bronchoalveolar lavage fluid samples of the patients with VAP. This study can provide useful information of pathogens in VAP and assist clinicians to make rational and effective therapeutic decisions.

Keywords: bioinformatics, drug resistance, OTU, PCR, DNA sequencing

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Introduction

Increasing evidence shows that ventilator-associated pneumonia (VAP), a type of nosocomial pneumonia, has emerged as an important challenge in the intensive care unit (ICU) that lead to a considerable morbidity and mortality, placing a substantial heavy burden on an individual and society.[1] VAP occurs in patients who receive mechanical ventilation and is usually acquired in the hospital setting ~48–72 hours after mechanical ventilation.[2] The overall rate of VAP is 13.6 per 1,000 ventilator-day, according to the International Nosocomial Infection Control Consortium (http://www.inicc.org/english/index.php). The incidence of VAP varies within a range from 13–51 per 1,000 ventilation-day according to the patient group and hospital setting; the mean duration of occurrence of VAP is ~5–7 days. The VAP mortality ranges from 24%–76%,

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and there is even higher mortality rate among critically ill patients.[1,3,4] In USA, VAP accounts for over 25% of all ICU-acquired infections, and there are over 100,000 cases annually.[5] In addition, VAP represents more than one-half of all antibiotic use in the ICU.[6,7]

The pathogenesis of VAP is complex but typically relates with colonization of the aerodigestive tract with pathogens, micro-aspiration of contaminated secretions, and formation of biofilms.[8] In particular, there are several bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter*, *Citrobacter*, *Stenotrophomonas maltophilia*, *Acinetobacter*, *Burkholderia cepacia*, and Methicillin-resistant *Staphylococcus aureus,* that can cause drug resistance in the treatment of VAP. These pathogens make the VAP treatment more challenging. Recently, the commonly used strategies to reduce the development of drug resistance in the treatment of VAP include de-escalation therapy, truncated courses of antibiotics, dosing regimens that account for patient-antibiotic pharmacokinetics and pharmacodynamics, antibiotic cycling, and surveillance cultures.[1,6,7] However, the approaches for pathogen monitoring and to avoid drug resistance are still limited, and VAP still is a life-threatening infectious disease with challenges in the diagnosis and poor prognosis. Thus, it needs advanced methods to detect the full array of pathogens in patients with VAP to avoid the compromise of therapy and achieve maximum therapeutic outcome in clinical practice.

Growing evidence shows that the accurate and comprehensive identification of the causative pathogen can facilitate the VAP therapy in clinical practice.[1] *16S rDNA* gene sequencing approach possesses the capability of fast and accurately revealing the identity of the pathogens, because it can overcome the limitations of conventional culture-based bacterial detection method.[9,10] With advances in the sequencing technology, the feasibility of *16S rDNA* analysis using 454 GS FLX system has already been proven in the research of microbiota in the oral cavity, wound, urine, and gastrointestinal tract; and the massive data generated by 454 GS FLX system make it possible to analyze the diversity of the bacterial communities.[11–17] The employment of the *16S rDNA* gene sequencing approach can provide a global spectrum of the composition of the pathogens in samples, which will have a great clinical importance in the optimization of the therapy, leading to the maximum therapeutic outcome.

In the present study, in order to reveal the full array of the pathogens of VAP and help the optimization of VAP therapy, we explored the complexity of the bacterial communities in bronchoalveolar lavage fluid samples of patients with VAP using *16S rDNA* amplicon 454 pyrosequencing.

Materials and methods

Patients

A total number of 27 patients from the department of ICU in the General Hospital of Ningxia Medical University were enrolled. All patients were diagnosed with VAP according to the clinical criteria provided by the China Health Ministry Guidelines. Clinical signs and symptoms of VAP included fever or low body temperature, new purulent sputum, and hypoxemia (Tables 1 and 2). Consent forms were obtained from all enrolled patients with VAP. The protocol was approved by the Ethics Committee of the General Hospital of Ningxia Medical University. All procedures were conducted in accordance with the criteria of the Declaration of Helsinki.

Clinical specimens and clinical laboratory work

The bronchoalveolar lavage fluid samples were collected from the enrolled patients. The collected bronchoalveolar lavage fluid samples were aliquoted into sterile Eppendorf tubes. Following the collection, the bacterial culture was performed. The remaining samples were stored at −80°C for subsequent metagenomic sequencing. The sample collection and transportation were carried out in strict accordance with the sterile operating procedures to avoid contamination.

DNA extraction from the bronchoalveolar lavage fluid samples

The bacterial DNA was isolated from bronchoalveolar lavage fluid samples using QIAamp DNA Micro Kit (Qiagen NV, Venlo, the Netherlands) according to the manufacturer's instructions. Briefly, 2 mL of bronchoalveolar lavage fluid samples was required for the DNA extraction. The samples were centrifuged at 8,000 rpm for 15 minutes, and the supernatant was removed. The pellet was resuspended and digested using 200 µL of 20 mg/mL lysozyme at 37°C for 1 hour. Following the digestion, the supernatant was collected via centrifugation, and the sample was then resuspended in 200 µL PBS buffer containing 20 µL of proteinase K. Then, a quota of 200 µL AL was added to each diluted sample and incubated at 56°C for 2 hours. The resultant bacterial and human DNA were collected and transferred to sterilized 1.5 mL EP tubes for further extraction using the QIAamp DNA Micro Kit, and the DNA purification was performed according to the manufacturer's instructions. The DNA

Table 1 The clinical data of 27 patients with severe pneumonia

Sample ID	Sex	Age (yr)	Main symptom	Peripheral blood#			APACHE II	CPIS	Hospital stay (days)	Prognosis
				WBC[a] (10^9/L)	N[b] (%)	PCT (μg/mL)				
T1	M	62	Dyspnea, wheeze	5.50	89.0	≥2 and <10	17	5	15	Upturn
T2	F	73	Dyspnea, fever	8.59	79.5	≥0.5 and <2	15	5	31	Upturn
T3	M	68	Dyspnea, fever	10.88	89.7	<0.5	14	5	9	Upturn
T4	M	73	Unconsciousness	9.15	82.8	<0.5	17	1	8	Upturn
T5	M	61	Dyspnea, fever	12.09	92.0	≥0.5 and <2	21	8	5	Upturn
T6	M	76	Dyspnea, wheeze	12.78	82.0	<0.5	29	9	10	Upturn
T7	M	72	Dyspnea, fever	13.02	94.1	<0.5	20	6	12	Upturn
T8	F	70	Dyspnea	8.83	94.1	<0.5	26	6	5	Death
T10	F	57	Unconsciousness, fever	19.38	89.7	≥2 and <10	20	7	10	Death
T12	M	70	Dyspnea, fever	2.42	94.0	≥0.5 and <2	20	7	42	Upturn
T13	M	76	Dyspnea, fever	12.54	95.6	<0.5	15	8	12	Upturn
T14	M	62	Dyspnea, fever	8.11	85.4	≥0.5 and <2	22	8	22	Upturn
T15	F	37	Dyspnea	9.66	93.2	≥10	7	5	17	Upturn
T16	F	52	Dyspnea, fever	12.45	86.5	≥2 and <10	16	6	15	Upturn
T17	F	67	Unconsciousness	7.58	92.2	≥0.5 and <2	21	5	9	Upturn
T18	M	70	Unconsciousness	2.18	83.0	≥0.5 and <2	24	6	13	Upturn
T20	M	67	Dyspnea, fever	4.15	91.3	≥0.5 and <2	17	5	10	Upturn
T22	F	65	Dyspnea, fever	20.59	89.6	<0.5	17	9	9	Death
T23	M	61	Dyspnea, fever	17.39	83.2	<0.5	12	9	16	Upturn
T24	M	61	Unconsciousness	9.78	87.0	<0.5	20	5	15	Upturn
T25	M	86	Dyspnea	9.98	93.9	≥0.5 and <2	20	7	5	Death
T26	M	81	Dyspnea	13.85	87.8	<0.5	19	7	15	Death
T27	M	47	Dyspnea, fever	17.60	86.2	≥10	25	8	24	Upturn
T28	F	47	Dyspnea	18.33	90.8		11	8	7	Upturn
T29	M	69	Dyspnea	13.41	88.8	≥0.5 and <2	14	6	2	Death
T30	M	40	Dyspnea, fever	15.58	85.4	<0.5	8	9	27	Upturn
T31	M	30	Dyspnea, fever	18.09	77.2	≥0.5 and <2	20	10	46	Upturn

Notes: #Peripheral blood test before hospitalization. [a]White blood cells count of peripheral blood; [b]neutrophils count of peripheral blood.
Abbreviation: CPIS, clinical pulmonary infection score.

Table 2 Simplified version of the clinical pulmonary infection score used in this study

Component	Value	Points
Temperature °C	≥36.5 and ≤38.4	0
	≥38.5 and ≤38.9	1
	≥39.0 and ≤36.0	2
Blood leukocytes per mm^3	≥4,000 and ≤11,000	0
	<4,000 or >11,000	1
Tracheal secretions	Few	0
	Moderate	1
	Large	2
	Purulent	1
Oxygenation Pao_2/ Fio_2 mmHg	>2,400 or presence of ARDS	0
	≤240 and absence of ARDS	2
Chest radiograph	No infiltrate	0
	Patchy or diffuse infiltrate	1
	Localized infiltrate	2

Abbreviation: ARDS, acute respiratory distress syndrome.

concentration was measured by UV spectrophotometer at 260 nm. The average DNA concentration of the samples was 22.8 ng/mL.

The design of barcoded primers

The polymerase chain reaction (PCR) enrichment of the *16S rDNA* V3-V5 hypervariable region was performed using the primers as shown in Table 3. The primers contained a ten-base oligonucleotide tag at the 5′ terminal. The sequence after the hyphen was able to pair with the sequences of the end region. The 27 pairs of primers that contained 27 different ten-base oligonucleotides and identical following sequences were used to lead the PCR enrichment (Table 4). The barcoded primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (Shanghai, People's Republic of China).

PCR enrichment of the V3–V5 region

The extracted and purified DNA from 27 bronchoalveolar lavage fluid samples was used for PCR assay. Each PCR reaction system consisted of 12.5 μL PCR master Mix (Dream Taq PCR Master Mix, Fermentas, Burlington, Canada), 0.5 μL forward primer, 0.5 μL reverse primer, 1 μL DNA, and 10.5 μL nuclease-free water. Touchdown PCR conditions were comprised of initial denaturation for 5 minutes at 95°C, denaturation for 30 seconds at 95°C, annealing for 30 seconds at 56°C, and extension for 30 seconds at 72°C. The resultant products were stored at –20°C after the reaction. The pooled tagged single-stranded pyrosequencing library underwent emPCR and pyrosequencing using a Roche 454 GS FLX Pyrosequencer (Hoffman-La Roche Ltd., Basel, Switzerland) according to the manufacturer's instructions.

Bioinformatic analysis

The high-throughput pyrosequencing reads from 27 bronchoalveolar lavage fluid samples were reassigned to samples according to barcodes. The resultant sequences were clustered into operational taxonomic units (OTUs). The OTUs that reached 97% similarity level were used for alpha diversity analysis that analyzed the species diversity in the single sample by the evaluation of Chao, abundance-based coverage estimators (ACE), Shannon, and Simpson parameters; and the rarefaction curve was also analyzed using the Mothur software v1.27.0 program.[18–21] Following the alpha diversity analysis, taxonomy-based analyses were performed through the classification of each sequence using the Naïve Bayesian classifier program in Ribosomal Database Project (RDP) at the Center for Microbial Ecology in Michigan State University (http://rdp.cme.msu.edu/; MI, USA).[20,21] The confidence level was of 95%. The sequences were assigned until the genus level in bacteria domain was collected and screened from 27 bronchoalveolar lavage fluid samples.[18–21] Each read was assigned a phylum, class, family, and genus. The taxonomic assignment was unambiguous within an 80% confidence threshold, which has been estimated to taxonomically assign reads with over 98% accuracy at genus level. Furthermore, the beta diversity analysis was performed to assess the distribution and content of bacteria and evaluate the total diversity in different samples based on the bacterial profile. Sequences were clustered at 97% nucleotide identity over 90% sequence alignment length using the Mothur software. For this analysis, sequences over 97% identical were considered to correspond to the same OTUs, representing a group of reads that likely belong to the same species.[22]

Results

High-throughput sequencing reveals 103,856 pyrosequencing reads

In order to identify the pathogens, we first performed the high-throughput sequencing to examine the possible bacterial DNA sequences in 27 bronchoalveolar lavage fluid samples from patients with VAP. There were a total of

Table 3 The sequences of the primers for *16S rDNA* V3-V5 hypervariable region

16S rDNA	Forward: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTCCGTCAATTCMTTTGAGTTT-3′
	Reverse: 5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGACTCCTACGGGAGGCTCCTACGGGAGGCAGCAG-3′

Table 4 The barcode sequences of 27 pairs of primers

Sample ID	Barcode sequence	Forward	Reverse
T1	ACGAGTGCGT	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T2	ACGCTCGACA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T3	AGACGCACTC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T4	AGCACTGTAG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T5	ATCAGACACG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T6	ATATCGCGAG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T7	CGTGTCTCTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T8	CTCGCGTGTC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T10	TCTCTATGCG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T12	TACTGAGCTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T13	CATAGTAGTG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T14	CGAGAGATAC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T15	ATACGACGTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T16	TCACGTACTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T17	CGTCTAGTAC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T18	TCTACGTAGC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T20	ACGACTACAG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T22	TACGAGTATG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T23	TACTCTCGTG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T24	TACTGAGCTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T25	CATAGTAGTG	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T26	CGAGAGATAC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T27	ATACGACGTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T28	TCACGTACTA	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T29	CGTCTAGTAC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T30	TCTACGTAGC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG
T31	TGTACTACTC	CCGTCAATTCMTTTGAGTTT	ACTCCTACGGGAGGCAGCAG

103,856 pyrosequencing reads that were identified through 454 pyrosequencing (Table 5). The sequences with insufficient quality or sequences that could not be adequately assigned were not included, such as chimera sequences and a small amount of non-target sequences. For the identified pyrosequencing reads, the average length of the sequences was 550 bp after trimming the primers. Taken together, the high-throughput sequencing approach shows a capability of identifying the bacterial DNA sequences from bronchoalveolar lavage fluid samples from patients with VAP, which may be clinically helpful for the treatment of VAP.

Classification of the bacteria found in bronchoalveolar lavage fluid samples from patients with VAP

We next classified the obtained pyrosequencing reads using the RDP classifier at a confidence level of 95% and assigned taxonomic classifications to the sequences for biological analysis. The tag sequences of the identified DNA in 27 bronchoalveolar lavage fluid samples from patients with VAP were analyzed using the RDP to annotate species. The data showed that most of the bacterial reads were assigned to genus level and a small number of bacterial reads were assigned to species level (Figures 1 and 2). There were 638 OTUs

Table 5 Sampling depth found by 454 sequencing from 27 bronchoalveolar lavage fluid samples

Sample ID	Raw tag number	Final tag number
T1	2,539	1,163
T2	2,896	1,793
T3	3,900	1,284
T4	3,122	1,094
T5	3,567	1,120
T6	3,549	1,735
T7	3,129	1,328
T8	3,203	939
T10	3,077	1,398
T12	3,304	1,019
T13	3,636	944
T14	3,017	1,599
T15	3,106	811
T16	3,230	995
T17	2,528	1,467
T18	2,671	1,257
T20	2,590	1,098
T22	3,552	967
T23	2,559	1,184
T24	7,024	3,258
T25	6,555	2,066
T26	5,764	2,155
T27	5,012	1,647
T28	5,882	2,509
T29	4,411	1,578
T30	4,838	2,745
T31	5,195	2,716

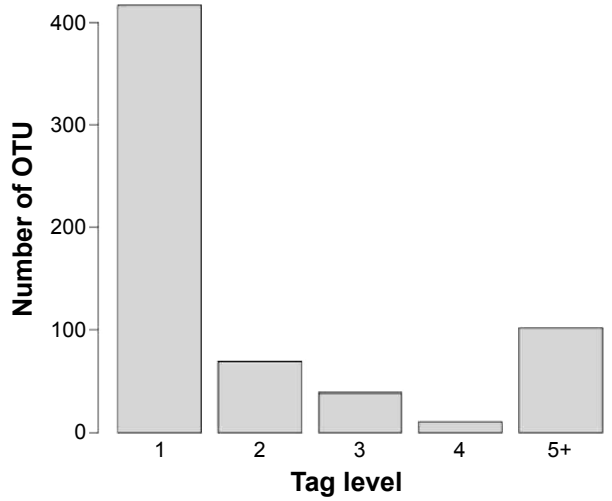

Figure 1 The OTU numbers at the different tags level.
Notes: There were 683 OTUs that were identified from 27 bronchoalveolar lavage fluid samples from patients with VAP. Horizontal axis shows the level of tags (the tags belonging to corresponding OTU) and vertical axis shows the specific tag level of the OTU numbers.
Abbreviations: OTU, operational taxonomic units; VAP, ventilator-associated pneumonia.

that were obtained from the 27 bronchoalveolar lavage fluid samples from patients with VAP using Mothur, according to the tag annotation information of species for OTU comments. The Mothur analysis results are able to comment on the classification level under each OTU number (Figure 2), the classification level of each OTU ratio was phylum, class, order, family, genus, and species at 91.4%, 84.3%, 79.0%, 72.7%, 58.2%, and 31.5%, respectively.

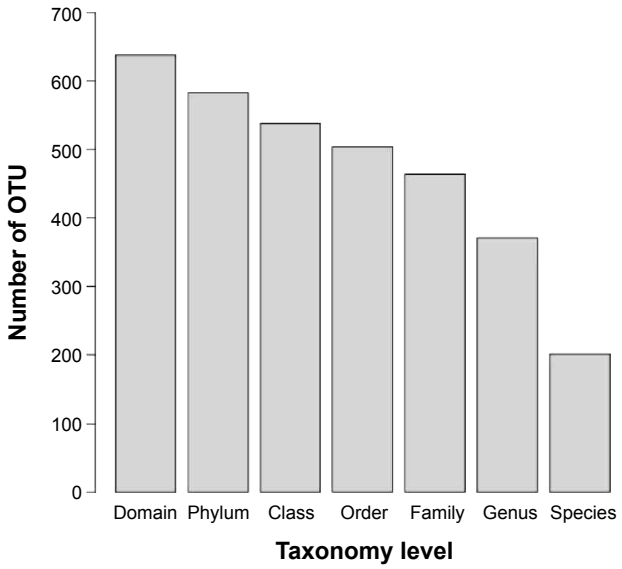

Figure 2 The OTU numbers at different taxonomy levels.
Notes: The species notation for OTU was analyzed according to notation of tags obtained from 27 bronchoalveolar lavage fluid samples from patients with VAP. The horizontal axis indicates all different taxonomy levels, and vertical axis indicates the numbers of OTU in different taxonomy levels. Different taxonomy levels of OTU proportion were 91.4%, 84.3%, 79.0%, 72.7%, 58.2%, and 31.5% for phylum, class, order, family, genus, and species, respectively.
Abbreviations: OTU, operational taxonomic units; VAP, ventilator-associated pneumonia.

Species richness and complexity estimation of microbiota in the bronchoalveolar lavage fluid samples from patients with VAP

Following the classification of the obtained pyrosequencing reads, the species richness complexity were analyzed using alpha diversity analysis. We performed bioinformatic analysis of the large number of pyrosequencing reads to evaluate the species richness and diversity of microbiota in the bronchoalveolar lavage fluid samples from patients with VAP. Based on the OTU data, we calculated alpha diversity. The indices of bacterial richness and diversity of OTUs at a 3% sequence dissimilarity level were summarized in Table 6. The bronchoalveolar lavage fluid sample containing low number of OTU indicated that it had a relatively low diversity in the cerebrospinal fluid sample compared with other environmental species (Figure 3). The Chao and ACE values and the Shannon and Simpson index were calculated, and the results showed that Chao value was ~80, ACE value was ~130, Shannon index was 1.25, and Simpson index was 0.46 (Figure 3). Moreover, the richness of bacterial communities in each bronchoalveolar lavage fluid sample was estimated as presented by the rarefaction curve. The trend of the rarefaction curves also confirmed that there was a low richness in each bronchoalveolar lavage fluid sample, and the saturated shape of the rarefaction curves indicated that bacterial richness of each bronchoalveolar lavage fluid sample was completely sampled (Figure 4).

Comparative analysis of multiple samples

Further, the comparison of the pathogens among the 27 bronchoalveolar lavage fluid samples from patients with VAP was performed. The pathogen profile at phylum and genus level was analyzed (Figures 5 and 6). The relative abundance of each pathogen was determined in the 27 bronchoalveolar lavage fluid samples (Figures 5A and 6A). At phylum level, there were four dominant phyla, including *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Figure 5B), to a lesser extent, *Fusobacteria*, *Deinococcus-Thermus*, *Spirochaetes*, *Nitrospira*, *Planctomycetes*, *Synergistetes*, and *Tenericutes*. Of note, *Proteobacteria* and *Firmicutes* were widely distributed in all of the samples (Figure 5A); *Bacteroidetes* and *Actinobacteria* occurred in half of the samples (Figure 5A). At genus level, these

Table 6 Comparison of phylotype richness and diversity estimation at 3% dissimilarity from the pyrosequencing analysis

Sample ID	OTU number	Chao	ACE	Jackknife	Shannon	Npshannon	Simpson
T1	33	96.250	175.163	126.492	1.247	1.332	0.365
T10	41	83.167	82.105	87.537	0.776	0.860	0.727
T12	9	9.333	11.074	0.000	1.115	1.127	0.374
T13	26	44.200	83.238	42.104	1.222	1.288	0.466
T14	15	15.429	16.650	0.000	0.593	0.610	0.742
T15	16	26.500	51.897	25.083	1.450	1.483	0.290
T16	40	107.667	375.474	114.976	0.489	0.636	0.863
T17	23	28.600	41.421	31.000	1.037	1.067	0.548
T18	14	21.500	37.082	20.472	0.366	0.391	0.879
T2	15	24.333	39.182	23.815	0.128	0.155	0.966
T20	58	112.375	167.734	117.955	2.292	2.386	0.166
T22	40	66.250	112.928	63.055	1.192	1.291	0.563
T23	53	65.214	77.867	72.000	2.081	2.157	0.230
T24	54	124.125	208.976	121.862	0.823	0.880	0.690
T25	75	296.000	671.875	1186.636	1.645	1.750	0.300
T26	87	392.000	707.645	367.753	1.755	1.870	0.301
T27	93	221.333	376.515	251.031	2.215	2.343	0.200
T28	69	111.500	124.030	111.733	1.517	1.588	0.359
T29	113	386.000	896.042	387.827	2.515	2.677	0.159
T3	11	14.333	30.143	16.000	0.263	0.286	0.905
T30	47	83.111	126.877	86.082	0.514	0.571	0.840
T31	48	106.000	151.567	109.196	0.981	1.037	0.518
T4	47	74.273	156.638	73.418	1.288	1.396	0.538
T5	60	153.500	259.506	152.614	1.680	1.799	0.415
T6	4	4.000	5.878	0.000	0.049	0.054	0.985
T7	25	51.250	129.015	52.288	1.028	1.081	0.471
T8	20	42.000	109.198	40.727	1.282	1.334	0.330

Abbreviations: ACE, abundance-based coverage estimators; OTU, operational taxonomic units.

sequences represented 90 different genera (Figure 6A), and there were 12 genera occurring in over ten different samples (Figure 6B). The top five dominant genera, which contained the largest number of sequences, were *Streptococcus*, *Acinetobacter*, *Limnohabitans*, *Neisseria*, and *Corynebacterium* (Figure 6A). The most widely distributed genera were *Streptococcus*, *Limnohabitans*, and *Acinetobacter* in the 27 bronchoalveolar lavage fluid samples, and *Prevotella*, *Sphingomonas*, *Aquabacterium*, *Corynebacterium*, *Klebsiella*, *Pseudomonas*, *Peptostreptococcus*, *Porphyromonas*, and *Tepidimonas* were distributed to a lesser extent (Figure 6B; ≥10 samples).

Additionally, in order to evaluate the total diversity and assess the distribution and content of bacteria in these 27 bronchoalveolar lavage fluid samples from patients with VAP, the beta analysis was performed. As shown in Figure 7A and B and Figure 8A–F, there was a substantial difference in the species distribution in the 27 bronchoalveolar lavage fluid samples. The beta diversity of 27 bronchoalveolar lavage fluid samples was indicated by Whittaker index that was used to evaluate the species difference in diversity between different samples. The higher index indicates more difference. The pathogens from number 1, 2, and 12 samples showed the most different diversity from other samples (Figure 7A). According to the Ward analysis data, there were four clusters that can be further divided into ten subclusters (Figure 7B). In addition, the total diversity and distribution of bacteria in these 27 bronchoalveolar lavage fluid samples were evaluated by weighted- and unweighted-UniFrac index (Figure 8A–F), which showed a similar results to that of Whittaker index. Collectively, the results show a comparable difference in the diversity of species distribution and evolution in the 27 bronchoalveolar lavage fluid samples from patients with VAP.

Discussion

Due to the substantial VAP-associated morbidity and mortality and the resultant considerable heavy burden on an individual and society, it is of great importance to identify the cause of VAP for the optimization of the treatment of VAP in clinical practice. The complex of pathogen-induced VAP is still a major challenge for the use of current therapeutics. Advances in the identification and profiling of the bacteria have facilitated the revealing of the global view of

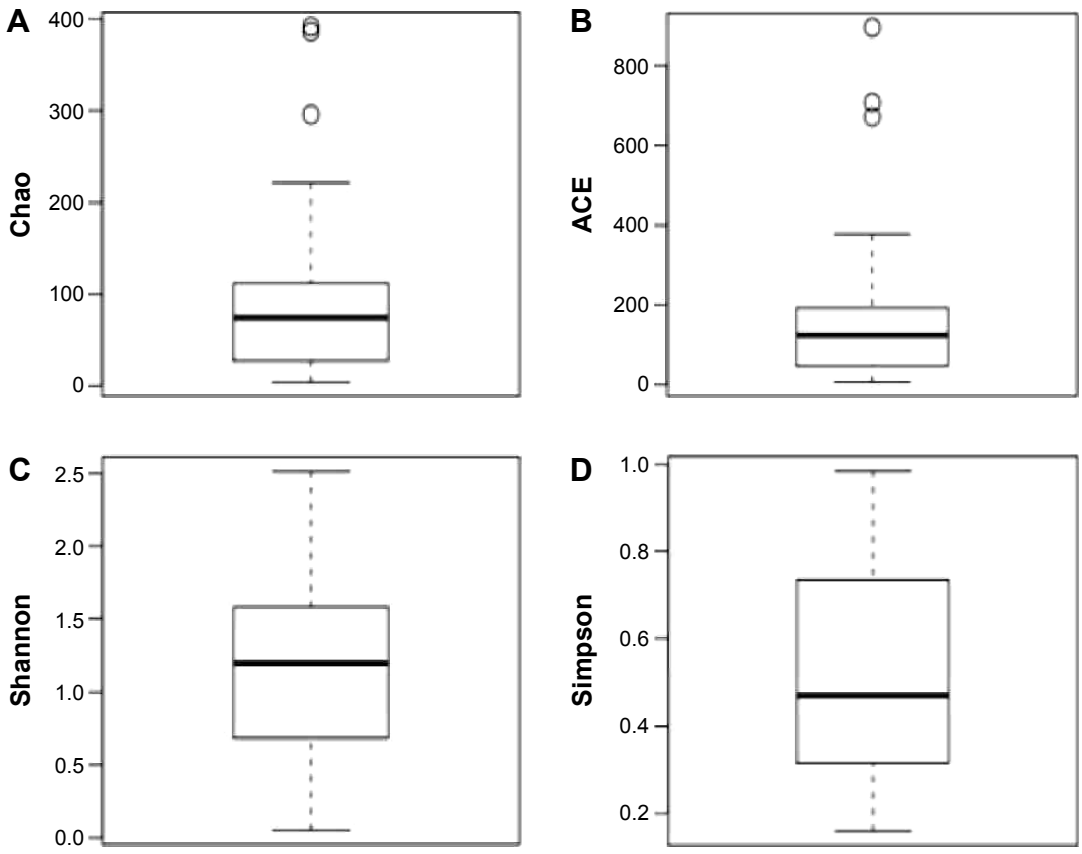

Figure 3 Alpha diversity analysis of the species distribution in 27 bronchoalveolar lavage fluid samples from patients with VAP.
Notes: Alpha diversity analysis is a species diversity of single sample, including the Chao and ACE value, Shannon and Simpson index. Chao and ACE are used to estimate species richness; Shannon index and Simpson index are a diversity index. Shannon index is larger, and Simpson index is closer to 0. It means there are abundant species in the sample. The results showed that Chao1 value was ~80 (**A**), ACE value was ~130 (**B**), Shannon index was 1.25 (**C**), and Simpson index was 0.46 (**D**).
Abbreviations: ACE, abundance-based coverage estimators; OTU, operational taxonomic units; VAP, ventilator-associated pneumonia.

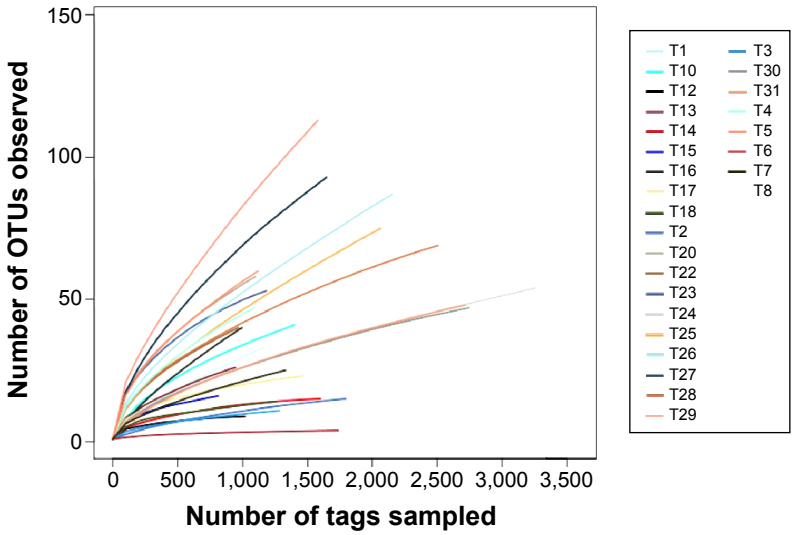

Figure 4 Rarefaction curves of the microbial communities of 27 bronchoalveolar lavage fluid samples from patients with VAP.
Notes: Rarefaction curves were used to estimate whether the number of sequence is sufficient to cover all species and estimate species richness for the 27 bronchoalveolar lavage fluid samples from patients with VAP (in this case, the number of taxonomy at a 3% dissimilarity level). The vertical axis shows the number of operational taxonomic units that would be expected to be found after sampling the numbers of tags shown on the horizontal axis. Lines of different colors represent 27 bronchoalveolar lavage fluid samples. The saturated shapes of the rarefaction curves indicate that sequencing depth has covered all species in the sample. The unsaturated shapes of the rarefaction curves indicate that bacterial richness of the sample is high and there are many undetected species.
Abbreviations: OTU, operational taxonomic units; VAP, ventilator-associated pneumonia.

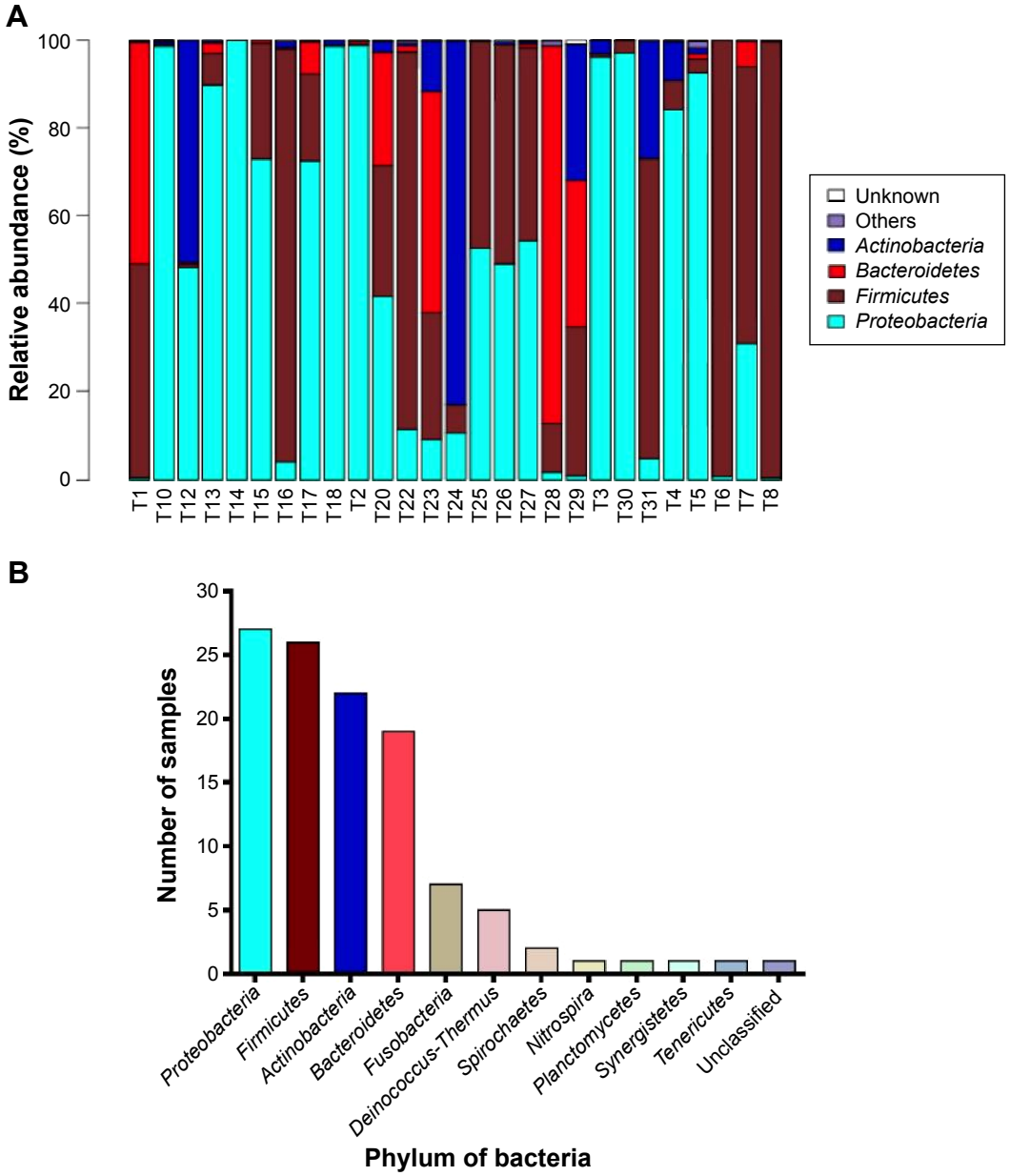

Figure 5 Bacterial community composition of 27 bronchoalveolar lavage fluid samples at phylum level.

Notes: It shows the bacterial community composition of each sample at phylum level. (**A**) The vertical axis represents relative bacterial abundance of corresponding phylum. The horizontal axis represents 27 bronchoalveolar lavage fluid samples. Different color components represent different phylum. Taxonomic name of higher abundance is shown in these charts. Those failed to meet the lower abundance is assigned the label "Others". "Unknown" indicates that the taxonomy level is unable to be defined according to the corresponding abundance. (**B**) The vertical axis represents the number of samples and the horizontal axis represents phylum of bacterial. The most abundant sequences belonged to *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*.

causative pathogens of VAP. Metagenomic studies have dramatically expanded our understanding of the microbial world without the cultivation of microorganisms and can overcome the shortages of the conventional culture-based approach.[17,18,21,23,24] In the present study, we have performed metagenomics high-throughput sequencing to analyze the pathogens that were present in 27 bronchoalveolar lavage fluid samples from patients with VAP. We have identified most of the bacterial pathogens in these 27 samples from patients with VAP. The percentage of sequences belonging to each bacterial genus has been calculated for each patient that may facilitate the optimization of the therapy for VAP treatment and the achievement of maximum therapeutic outcome in clinical practice.

Emerging evidence shows the advantage of high-throughput sequencing in the identification of the pathogens to potentially improve the specificity of the diagnosis of VAP and the consequent unnecessary antibiotic use and its

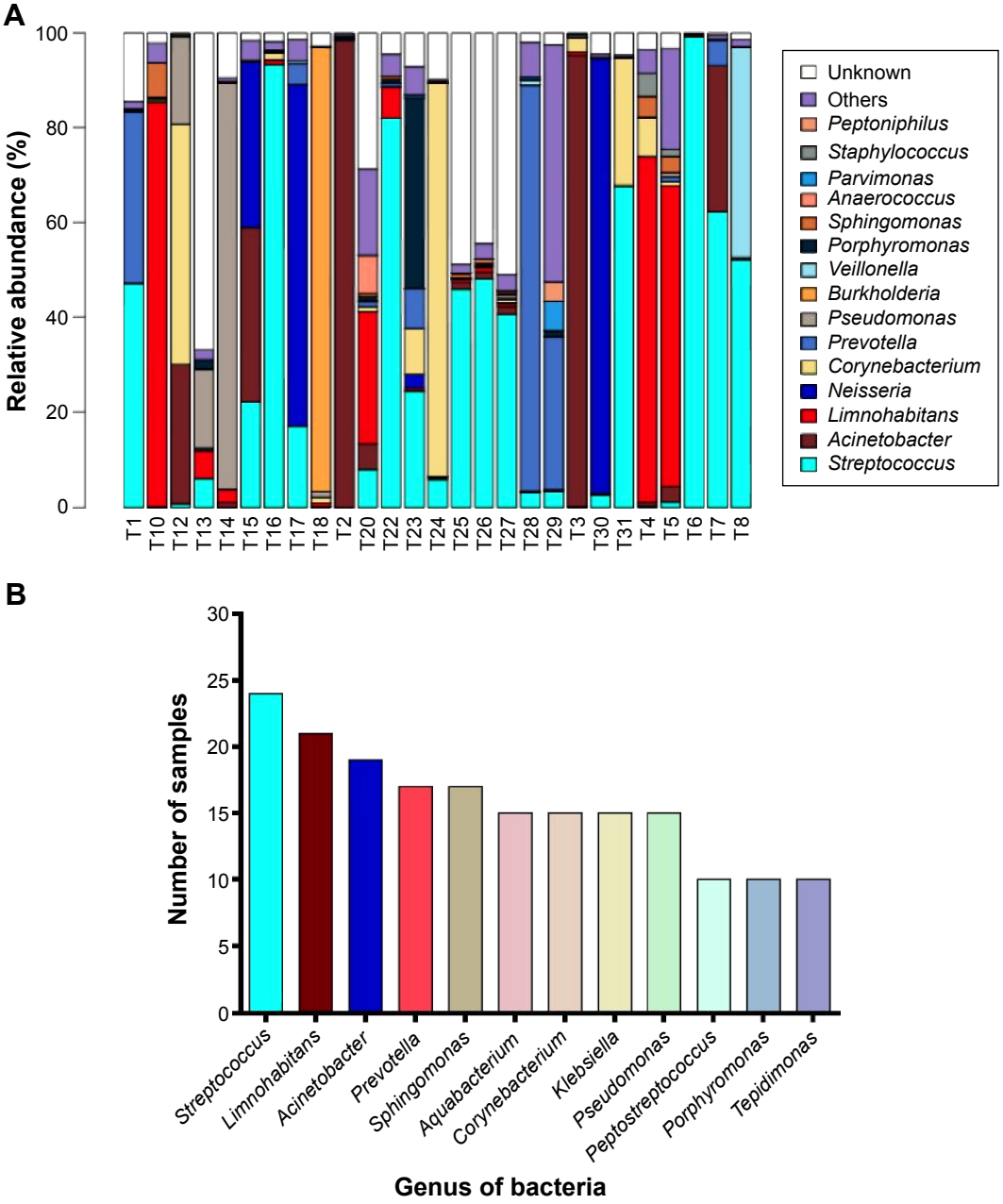

Figure 6 Bacterial community composition of 27 bronchoalveolar lavage fluid samples at genus level.

Notes: It shows bacterial community composition of each sample at genus level. (**A**) The vertical axis represents relative bacterial abundance of corresponding phylum. The horizontal axis represents 27 bronchoalveolar lavage fluid samples. Different color components represent different genus. Taxonomic name of higher abundance is shown in these charts. Those failed to meet the lower abundance was assigned the label "Others". "Unknown" indicates that the taxonomy level is unable to be defined according to the abundance. (**B**) The vertical axis represents the number of samples, and the horizontal axis represents genus of bacterial. There were 15 major genera and much mixed infection in the samples.

associated problems, such as drug resistance.[9,17,25] The advent of 454 pyrosequencing technology has greatly accelerated studies on the profiling and deciphering the complexity of the human microbiota composition,[26,27] leading to an improved understanding in the causative pathogens in the pathogenesis of infectious diseases. The utilization of barcoded primers and 454 pyrosequencing in metagenomics generates a global and comprehensive profiling of the microbiota.[28,29] In our study, the findings showed that the high-throughput sequencing approach provided a fast, accurate, and global pyrosequencing reads, which can expand our understanding of the diversity of bacteria present in the respiratory tract of patients with VAP. On the other hand, we detected part of the sequences that belong to unknown species, and there are completely unknown species in 22 of 27 samples in the present study. The observation of these results may be due to the

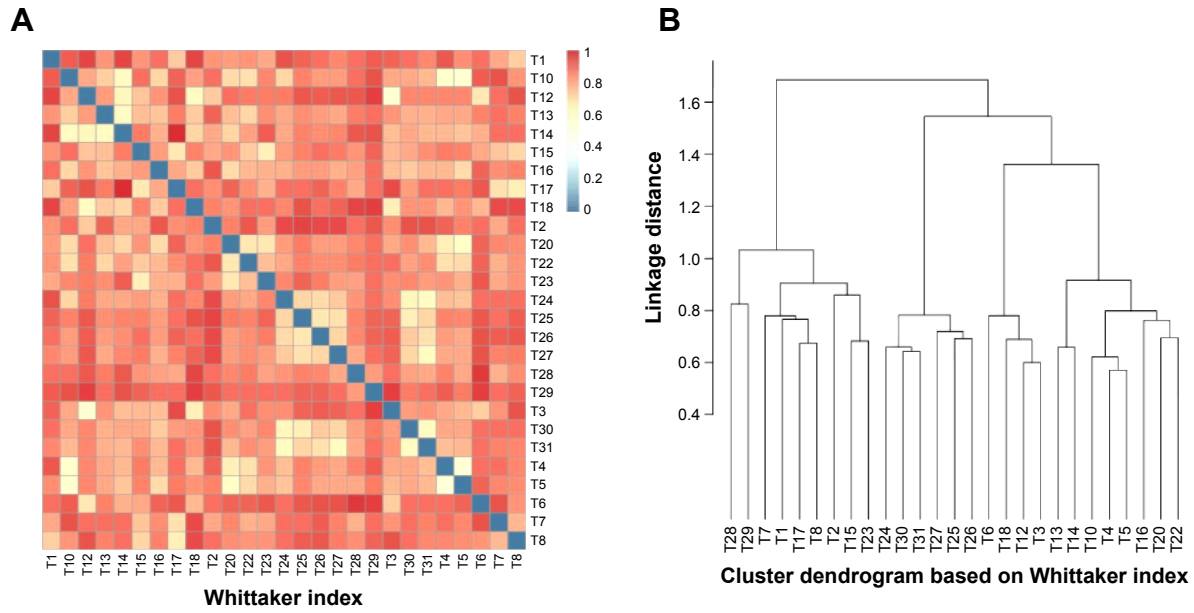

Figure 7 Beta diversity analysis of 27 bronchoalveolar lavage fluid samples from patients with VAP indicated by Whittaker index.
Notes: Whittaker index was used to evaluate the difference in species diversity among different samples. The higher index indicates more difference. (**A**) The Whittaker index of species distribution of 27 bronchoalveolar lavage fluid samples from patients with pneumonia. (**B**) The cluster dendrogram based on Whittaker index analyzed by Ward of hclust in R program.
Abbreviation: VAP, ventilator-associated pneumonia.

non-homogenous lysis of bacterial cells, primer mismatches, and the presence of mixed bases in PCR process.[30–32] These reasons can lead to the failure of the species functional annotation. Therefore, it is critical to eliminate these factors.

Due to the complicated composition of pathogens in the development of VAP, it is of great importance to discriminate the composition of the pathogens at different levels. This will be clinically helpful for the proper therapeutics selection and therapy optimization in the treatment of VAP. The causative bacteria for an early-onset VAP include *Streptococcus*, *Hemophilus influenzae*, methicillin-sensitive *S. aureus*, antibiotic-sensitive enteric Gram-negative bacilli, *Escherichia coli*, *K. pneumoniae*, *Enterobacter* species, *Proteus* species, and *S. marcescens*, and the bacteria causing late VAP are typically multiple drug-resistant bacteria, such as methicillin-resistant *S. aureus*, *Acinetobacter*, *P. aeruginosa*, and extended-spectrum beta-lactamase producing bacteria.[33,34] Our findings showed that the most common isolates were *Streptococcus*, *Acinetobacter*, and *Limnohabitans* at genus level. In addition, the most prevalently representative sequence belonged to *Streptococcus*. They belong to opportunistic pathogens, which are widely distributed in nature. They exist in the form of carrier state, generally not pathogenic, but can cause disease when immunity decreased.[35–37] The *Streptococcus* encompasses both commensal and pathogenic Gram-positive bacteria that inhabit various human body sites, such as *Streptococcus pneumoniae*, a well-known pathogen associated with pneumonia.[38] In recent years, *Acinetobacter baumannii* has become increasingly prevalent. It has been the main pathogenic bacteria in hospital-acquired infection, particularly in VAP[39,40] Thus, the global identification of the causative pathogens in VAP is important for therapeutics selection.

In the treatment of VAP in the absence of risk factors for multidrug-resistant bacteria, the empirical therapy can be selected for the causative pathogens of *Streptococcus pneumoniae*, *Haemophilus influenzae*, methicillin-sensitive *S. aureus*, and antibiotic-sensitive gram-negative enteric organisms. The antibiotics used to treat drug-sensitive VAP include ceftriaxone, quinolones (levofloxacin, moxifloxacin, or ciprofloxacin), ampicillin/sulbactam, or ertapenem. However, the mixed infections with drug-resistant bacteria represent a major challenge in the treatment of VAP, which often occurs. Therefore, in the treatment of VAP in the presence of risk factors for multidrug-resistant organisms, the clinician must consider not only empirical therapies but also the therapies for the drug-resistance causative pathogens that include *P. aeruginosa*, *Klebsiella*, *Enterobacter*, *Serratia*, *Acinetobacter*, *Stenotrophomonas maltophilia*, *B. cepacia*, and methicillin-resistant *S. aureus*. The therapeutics should be broadened to achieve the therapeutic effect. This includes 1) either an antipseudomonal cephalosporin (cefepime or ceftazidime), an antipseudomonal

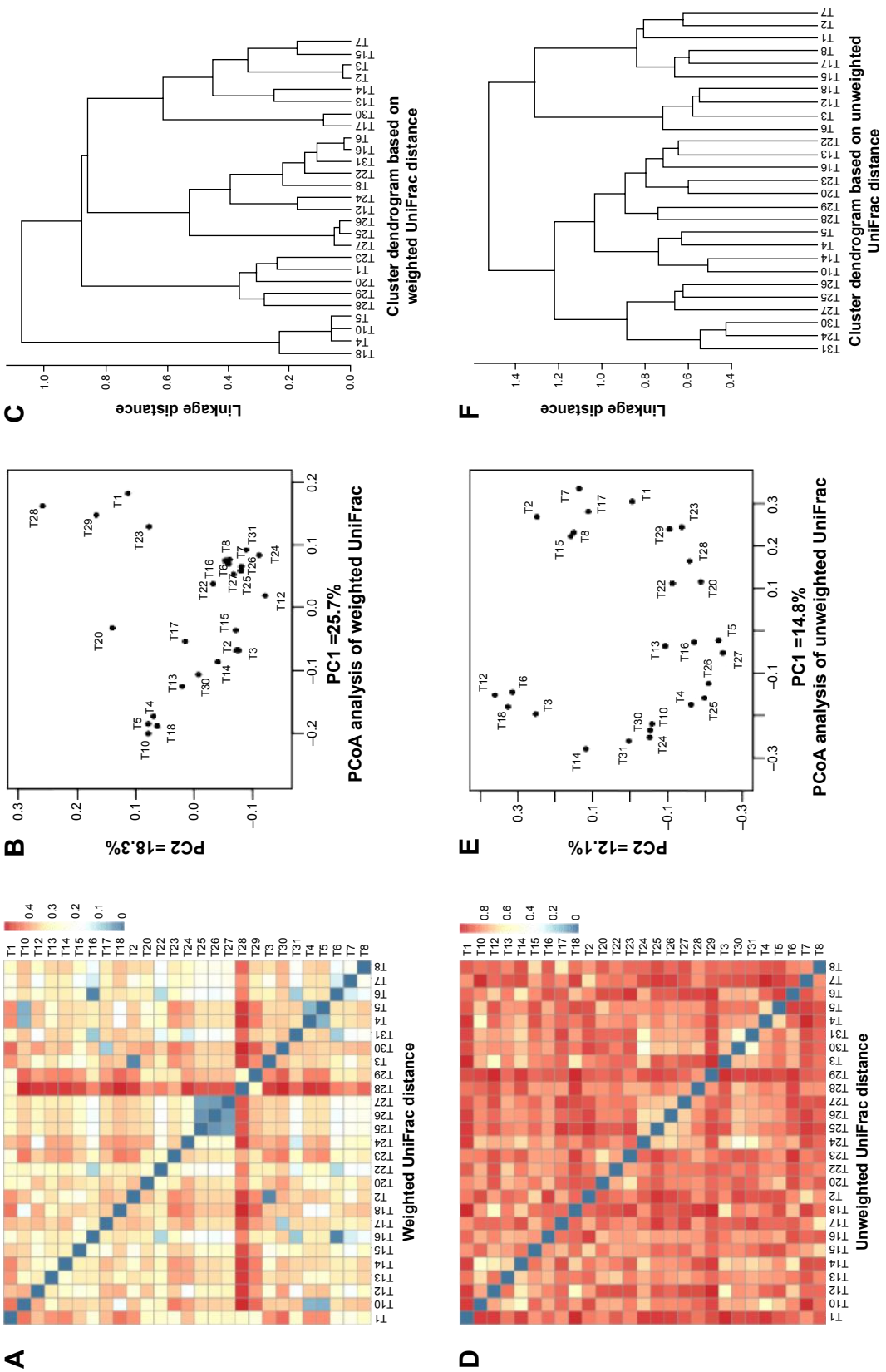

Figure 8 Beta diversity analysis of 27 bronchoalveolar lavage fluid samples from patients with VAP indicated by UniFrac index.
Notes: UniFrac index was used to evaluate the difference in species evolution among different samples. The higher index indicates more difference. (**A**) The weighted UniFrac index of species distribution of 27 bronchoalveolar lavage fluid samples from patients with VAP. (**B**) The PCoA analysis of weighted UniFrac for 27 bronchoalveolar lavage fluid samples from patients with VAP. (**C**) The cluster dendrogram based on weighted-UniFrac distance analyzed by Ward of hclust in R program. (**D**) The unweighted UniFrac index of species distribution of 27 bronchoalveolar lavage fluid samples from patients with VAP. (**E**) The PCoA analysis of unweighted UniFrac for 27 bronchoalveolar lavage fluid samples from patients with VAP. (**F**) The cluster dendrogram based on unweighted-UniFrac distance analyzed by Ward of hclust in R program.
Abbreviation: VAP, ventilator-associated pneumonia.

carbapenem (imipenem or meropenem), or a β-lactam/β-lactamase inhibitor (piperacillin-tazobactam), and 2) an antipseudomonal fluoroquinolone (ciprofloxacin or levofloxacin) or an aminoglycoside (amikacin, gentamicin, or tobramycin) plus linezolid or vancomycin.

Conclusion

In summary, the present study shows that the high-throughput sequencing is a clinically valuable approach to analyze the pathogens of VAP with the advantage of overcoming the limitations of convention approaches. The global view of the bacterial composition can provide a better understanding of pathogens in VAP, which can assist clinicians to make rational and effective therapeutic decisions to treat drug-sensitive or resistant bacteria, and achieve maximum therapeutic effect in the treatment of VAP in clinical practice.

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Disclosure

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

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