High-throughput sequencing of 16S rDNA amplicons characterizes bacterial composition in cerebrospinal fluid samples from patients with purulent meningitis

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Abstract: Purulent meningitis (PM) is a severe infectious disease that is associated with high rates of morbidity and mortality. It has been recognized that bacterial infection is a major contributing factor to the pathogenesis of PM. However, there is a lack of information on the bacterial composition in PM, due to the low positive rate of cerebrospinal fluid bacterial culture. Herein, we aimed to discriminate and identify the main pathogens and bacterial composition in cerebrospinal fluid sample from PM patients using high-throughput sequencing approach. The cerebrospinal fluid samples were collected from 26 PM patients, and were determined as culture-negative samples. The polymerase chain reaction products of the hypervariable regions of 16S rDNA gene in these 26 samples of PM were sequenced using the 454 GS FLX system. The results showed that there were 71,440 pyrosequencing reads, of which, the predominant phyla were Proteobacteria and Firmicutes; and the predominant genera were Streptococcus, Acinetobacter, Pseudomonas, and Neisseria. The bacterial species in the cerebrospinal fluid were complex, with 61.5% of the samples presenting with mixed pathogens. A significant number of bacteria belonging to a known pathogenic potential was observed. The number of operational taxonomic units for individual samples ranged from six to 75 and there was a comparable difference in the species diversity that was calculated through alpha and beta diversity analysis. Collectively, the data show that high-throughput sequencing approach facilitates the characterization of the pathogens in cerebrospinal fluid and determine the abundance and the composition of bacteria in the cerebrospinal fluid samples of the PM patients, which may provide a better understanding of pathogens in PM and assist clinicians to make rational and effective therapeutic decisions.

Keywords: Bioinformatics, cerebrospinal fluid sample, OTU

Introduction

Meningitis is a clinical syndrome featured with inflammation of the meninges that cover the brain and spinal cord, resulting in high morbidity and mortality rates.1,2 It was estimated that meningitis resulted in 420,000 deaths in 2010 and this disease killed 303,000 people in 2013 globally.3 The causes of meningitis include bacteria, viruses, fungi, parasites, drugs, chemical irritation, subarachnoid hemorrhage, cancer and other conditions. Viral meningitis is the most common type of meningitis and is often less severe than bacterial meningitis. Bacterial meningitis is often severe that represents the most significant contributor to the mortality and morbidity.4,5 Some of the leading causes of bacterial meningitis include Haemophilus influenzae (most often caused by type b, Hib), Streptococcus pneumoniae, group B Streptococcus, Listeria.
and Neisseria meningitidis. The incidence of bacterial meningitis significantly varies worldwide, due to differences in the specific etiologic agents and access to medical resources. In US, an estimated 4,100 new cases (ie, 1.33/100,000) and 500 deaths from bacterial meningitis occurred annually during 2003–2007.6 There are about 3,200 people suffering from bacterial meningitis in the UK each year. The estimated annual incidence (per 100,000 population) of probable bacterial meningitis ranged from 1.84 to 2.93 for the entire population and from 6.95 to 22.30 for children aged <5 years in People’s Republic of China.7 It is speculated that the incidence of bacterial meningitis is ten times higher in the developing countries than that in developed countries, in particular, in Niger, Nigeria, Burkina Faso, Chad, and Mali. Purulent meningitis (PM) is the most serious infectious diseases caused by bacteria in the central nervous system. It has been reported that the average mortality due to PM was higher than 20%.8,9 Particularly, the mortality reaches to 35%–36% in some African countries, and approximately 30%–50% of the PM survivors experience permanent neurological sequelae including epilepsy, hydrocephalus, hearing loss, learning and behavioral difficulties, as well as decreased intelligence.10–12 Thus, it is important to develop new and more efficacious therapy for PM treatment.

Given the complexity of the pathogens of PM, identification of pathogenic microbes is critical for selecting optimal therapy to reach maximum therapeutic effect in the treatment of PM in clinical practice. It has been shown that H. influenzae, L. monocytogenes, N. meningitidis, Streptococcus agalactiae (group B Streptococcus), and S. pneumoniae are the most comment etiologic bacteria.1,13 Currently, the cerebrospinal fluid bacterial culture is the golden standard for the diagnosis of PM and the culture-based methods do provide a good reference for clinicians; however, the current diagnostic approaches are incapable of unveiling the full spectrum of the causative pathogens of PM due to the limitations,14,15 which include false negative results in the culture specimens because of application of antibiotics, infections with slow-growing, fastidious, and inert microorganisms, and suppression of non-competitive bacteria growth by the secretions from the competitive bacteria when they are co-cultured.16,17 Moreover, pathogens like Tropheryma whippelii, Bartonella henselae, and Bayesian Rickettsia are considerably difficult to culture, thus, they may escape from the screening using culture-based method.18,19 Due to the limitations of the current cerebrospinal fluid bacterial culture-based method, the full spectrum of the pathogens in cerebrospinal fluid specimens of PM patients cannot be presented, which will jeopardize the therapeutic outcome in the treatment of PM in clinical practice. Thus, it requires advanced methods to detect the full array of pathogens in cerebrospinal fluid specimens of PM patients to avoid the compromise of therapy and achieve maximum therapeutic outcome in clinical practice.

Increasing evidence shows that 16S rDNA gene sequencing approach is able to identify fast and accurately the bacteria because it can overcome the limitations of culture-based bacterial detection method.20,21 With advances in 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 sufficient data generated by 454 GS FLX system make it possible to analyze the diversity of the bacterial communities.22–28 Such a sequencing approach can provide a global view of the bacterial flora; however, 16S rDNA gene sequencing has not been used to identify the bacteria in cerebrospinal fluid samples of PM patients. In this study, we collected the cerebrospinal fluid samples from the PM patients, which were the most valuable sample reflecting the main part of the bacterial communities in the central nervous system, and we aimed to investigate the complex of bacterial communities in the cerebrospinal fluid in patients with culture-negative PM using 16S rDNA Amplicon 454 pyrosequencing.

Materials and methods
Patients
Twenty-six patients from the General Hospital of Ningxia Medical University were enrolled in the present study. All patients were confirmed as PM according to the clinical criteria provided by the China Health Ministry Guidelines. Clinical signs and symptoms of PM included fever, severe headache, neck stiffness, vomit, seizures, and/or rash (Table 1). All patients were tested for lumbar puncture according to the diagnosis standard for PM and were fully informed of the risks and potential benefits of the cerebrospinal fluid examination. Consent forms were obtained from all enrolled PM patients. 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.

Cerebrospinal fluid sampling
The cerebrospinal fluid was collected by a lumbar puncture through the L3/L4 or L4/L5 intervertebral space. All the cerebrospinal fluid samples were immediately stored in a sterile container. Then, the cerebrospinal fluid samples were aliquot
<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age (years)</th>
<th>Main symptom</th>
<th>WBC&lt;sup&gt;a&lt;/sup&gt; (10&lt;sup&gt;9&lt;/sup&gt;/L)</th>
<th>N&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Time&lt;sup&gt;c&lt;/sup&gt; (days)</th>
<th>WBC&lt;sup&gt;d&lt;/sup&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>N&lt;sup&gt;e&lt;/sup&gt; (%)</th>
<th>Pro&lt;sup&gt;f&lt;/sup&gt; (g/L)</th>
<th>Glu&lt;sup&gt;g&lt;/sup&gt; (mmol/L)</th>
<th>Cl&lt;sup&gt;h&lt;/sup&gt; (mmol/L)</th>
<th>Hospital stay (days)</th>
<th>Prognosis</th>
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<tbody>
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<td>Fever, headache, nausea</td>
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<td>80.1</td>
<td>4</td>
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<td>79</td>
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<td>3.0</td>
<td>115.1</td>
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</tr>
<tr>
<td>4</td>
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<td>1,100</td>
<td>81</td>
<td>1.680</td>
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</tr>
<tr>
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<td>31</td>
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<td>610</td>
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<td>113.9</td>
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<td>96</td>
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<td>9</td>
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<td>2</td>
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<td>5.780</td>
<td>1.1</td>
<td>112.6</td>
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<td>Unconsciousness</td>
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<td>71.6</td>
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<td>96</td>
<td>1.970</td>
<td>1.1</td>
<td>120.2</td>
<td>14</td>
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</tr>
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<td>87.4</td>
<td>7</td>
<td>1,100</td>
<td>80</td>
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<td>82.4</td>
<td>2</td>
<td>880</td>
<td>88</td>
<td>2.760</td>
<td>1.1</td>
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<td>550</td>
<td>74</td>
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<td>2.01</td>
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<td>45</td>
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<td>2.340</td>
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</tr>
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<td>Y4</td>
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<td>82.3</td>
<td>5</td>
<td>850</td>
<td>67</td>
<td>5.910</td>
<td>6.1</td>
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<td>12</td>
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</tr>
<tr>
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<td>Y5</td>
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<td>79.6</td>
<td>3</td>
<td>1,080</td>
<td>70</td>
<td>0.750</td>
<td>3.4</td>
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<tr>
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<td>Y6</td>
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<td>13.93</td>
<td>84.1</td>
<td>1</td>
<td>510</td>
<td>78</td>
<td>0.950</td>
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<td>1.270</td>
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<tr>
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<td>Y8</td>
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<td>12.70</td>
<td>84.6</td>
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<td>1.570</td>
<td>1.07</td>
<td>122.0</td>
<td>35</td>
<td>Disease cured</td>
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</tbody>
</table>
| Y7        | Y9          | Headache                      | 10.78                             | 87.7              | 1                      | 1,800               | 81                | 6.140             | 2.2                  | 101.2               | 3                 | Symptoms not relieved/ 
|            | Y10         | Fever, headache               | 9.86                              | 88.8              | 5                      | 1,150               | 87                | 0.870             | 3.7                  | 125.5               | 23                | Disease cured    |
| Y11       | Y12         | Fever, headache               | 7.34                              | 79.0              | 1                      | 750                 | 74                | 2.390             | 1.2                  | 112.1               | 22                | Symptoms relieved|
| Y12       | Y13         | Fever, headache               | 12.86                             | 73.0              | 1                      | 980                 | 77                | 1.070             | 1.6                  | 120.3               | 34                | Symptoms relieved|
| Y14       | Y15         | Headache                      | 12.52                             | 82.7              | 1                      | 8,000               | 88                | 2.530             | 6.0                  | 133.5               | 4                 | Symptoms not relieved/ 
|            | Y16         | Fever, headache               | 11.64                             | 75.5              | 1                      | 890                 | 85                | 1.330             | 2.14                 | 110.0               | 18                | Symptoms relieved|

**Notes:** *Peripheral blood test before hospitalization.* *White blood cells count of peripheral blood; *neutrophils count of peripheral blood; *time of sampling first cerebral spinal fluid after hospitalization; *white blood cells count of cerebral spinal fluid; *neutrophils count of cerebral spinal fluid; *protein contents of cerebral spinal fluid; *glucose contents of cerebral spinal fluid; *chloride contents of cerebral spinal fluid.

**Abbreviations:** PCR, polymerase chain reaction; WBC, white blood count; Pro, protein; Glu, glucose.
into 1.5 mL sterile Eppendorf tubes and stored at −80°C for subsequential assays. All specimens were culture-negative, which were examined in the clinical laboratory in General Hospital of Ningxia Medical University. Collection and transportation of specimens were strictly in accordance with the sterile operating procedures.

**DNA extraction and quantity detection**

DNA was isolated from cerebrospinal fluid using a QIAamp DNA Micro Kit (Qiagen NV, Venlo, the Netherlands). A quota of 1 mL of cerebrospinal fluid was transferred into a 1.5 mL microcentrifuge tube and centrifuged at 8,000×g for 15 minutes. The supernatant was decanted and the pellet was resuspended in the remaining solution. Then the samples were resuspended in phosphate-buffered saline containing lysozyme and lysostaphin at concentrations of 5 mg/mL and 32 U/mL, respectively. The mixture solution was incubated for 2 hours at 37°C. Following that, a volume of 20 μL proteinase K and 200 μL Buffer AL that were provided in the QIAamp DNA Micro Kit were added and incubated for 1 hour at 56°C. After the incubation, the DNA purification was performed according to the manufacturer’s instructions. The DNA concentration was measured by UV spectrophotometer at 260 nm. The average DNA concentration of the samples was 22.8 ng/mL.

**Barcoded primer design**

The polymerase chain reaction (PCR) enrichment of the V3–V5 hypervariable region was performed using the forward primer: 5′-CCATCTCATCCCTGGCGTG TCTCCGACTCAGAGTCCGTCCATATTCTMT TTGAGTTT-3′ and the reverse primer: 5′-CTATCCCC TGTTCCTGGCCGAGTCCTCAGTCTCTACCGAG GCTCTACGGGACGCGACGAC-3′. For aforementioned primers, the 5′-terminal of each primer contained a ten-base-oligonucleotide tag, while the sequence after the hyphen was able to pair with the sequences of the end region. The 26 pairs of primers that contained 26 different ten-base-oligonucleotides and identical following sequences were used to perform the subsequential PCR enrichment. The barcoded primers were synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (Shanghai, People’s Republic of China).

**PCR enrichment of the V3–V5 region and pyrosequencing with the 454 platform**

The extracted DNA from 26 cerebrospinal fluid samples was used as a template for PCR amplification by the V3–V5 primers. Each PCR reaction system was comprised 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 template DNA and 10.5 μL nuclease-free water. Touchdown PCR conditions were as follows: 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. Samples 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 (Roche Life Sciences Inc., Basel, Switzerland) according to the manufacturer’s instructions.

**Bioinformatic analysis**

The high-throughput pyrosequencing reads were reassigned to samples according to barcodes. 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 abundance-based coverage estimators, Chao1, Shannon, and Simpson parameters; and the rarefaction curve was also analyzed using the Mothur software v1.27.0 program.29-32 Following this, taxonomy-based analyses were performed through the classification of each sequence using the Naïve Bayesian classifier program in Ribosomal Database Project (RDP) database at the Center for Microbial Ecology in Michigan State University (http://rdp.cme.msu.edu; East Lansing, MI, USA).31,32 The confidence level was of 95%.

The sequences were assigned until the genus level in bacteria domain was collected and screened.29-32 Each of the reads 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 which likely belong to the same species.33

**Results**

**High-throughput sequencing reveals 71,440 pyrosequencing reads**

First, we performed the high-throughput sequencing to examine the possible bacterial DNA sequences in 26 culture-negative cerebrospinal fluid samples. As shown in Table 2,
Table 2 Sampling depth found by 454 sequencing from 26 culture-negative cerebrospinal fluid samples

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Raw tag number</th>
<th>Final tag number</th>
<th>OTU number</th>
<th>Phyla (6)</th>
<th>Genera (62)</th>
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<td>16</td>
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Notes: a The total number of reads per dataset before removing sequences of insufficient quality; b the number of reads per dataset after removing sequences of insufficient quality and used for the actual analysis; c OTUs, operational taxonomic units at 3% nucleotide difference; d the number of phyla detected in every sample and the total number of phyla detected in parenthesis; e the number of genera detected in every sample and the total number of genera detected in parenthesis.

Abbreviation: OTU, operational taxonomic unit.

there were a total of 71,440 pyrosequencing reads which were obtained from the 26 culture-negative cerebrospinal fluid samples. 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 nontarget sequences. For the identified the pyrosequencing reads, the average length of the sequences was 550 bp after trimming the primers. Taken together, the high-throughput sequencing approach shows an ability for identifying the bacterial DNA sequences from culture-negative cerebrospinal fluids of patients with PM, which may be clinically helpful for the treatment of PM.

Identification of the bacteria found in culture-negative cerebrospinal fluid samples from patients with PM

Following the sequencing, these pyrosequencing reads were classified 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 26 culture-negative cerebrospinal fluid samples were compared using the RDP database to annotate species. The data showed that most of bacterial reads were assigned to genus level and a small number of bacterial reads were assigned to species level.

As shown in Figures 1 and 2, the sequences provided an overview of pathogens in the 26 cerebrospinal fluid samples. At phylum level, there were six known phyla which were identified in the cerebrospinal fluid samples, including Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Deinococcus-Thermus, and Actinobacteria (Figure 1A and B). Proteobacteria and Firmicutes were widely distributed and predominated among the pathogens in the 26 cerebrospinal fluid samples from patients with PM (Figure 1A and B). At genus level, these sequences represented 62 different genera, of which, there was a high abundance of 16 genera (Figure 2). The top five genera with the most abundance of sequences were Streptococcus, Acinetobacter, Pseudomonas, Neisseria, and Sphingomonas (Figure 2A); and the top five most widely distributed genera were Streptococcus, Acinetobacter, Limnohabitans, Sphingomonas, and Pseudomonas (Figure 2B). The data were comparable to those previously
reported (Table 3). Of note, the data showed that the dominant pathogenic microorganism was *Streptococcus* which presented in 84.6% single infection or mixed infection samples. Moreover, 16 out of 26 (61.5%) samples presented mixed pathogens (Figure 2) and *Acinetobacter* and *Pseudomonas* were the most frequent combinational pathogens that were present in the 26 cerebrospinal fluid samples from patients with PM. In addition, a part of the sequences represented uncultured bacteria and many of them belonged to unknown pathogenic potential (Figure 2). Taken together, there are a variety of pathogens that were present in the culture-negative cerebrospinal fluid, which might be involved in the pathogenesis of PM. The identification and classification of pathogens in patients with PM can facilitate the optimization of the therapy and improve clinical outcomes.

**Species richness and diversity estimation of microbiota in the culture-negative cerebrospinal fluid samples from PM patients**

Furthermore, we performed a bioinformatic analysis of the large number of pyrosequencing reads to evaluate the species richness and diversity of microbiota in the culture-negative cerebrospinal fluid samples. These reads clustered into 413 OTU at a 97% similarity level. Based on the OTU data, we further calculated alpha diversity. The indices of bacterial richness and diversity of OTUs at a 3% sequence dissimilarity level are summarized in Table 4. Alpha diversity indicated that each culture-negative cerebrospinal fluid sample contained low number of OTU, which means it has relatively low diversity in the cerebrospinal fluid sample compared with other environmental species (Table 4). Moreover, the richness of bacterial communities in culture-negative cerebrospinal fluid sample was estimated as presented by the rarefaction curve. The trend of the rarefaction curves also confirmed that there was low richness in culture-negative cerebrospinal fluid sample and the saturated shape of the rarefaction curves indicated that bacterial richness of cerebrospinal fluid sample was completely sampled (Figure 3).

Furthermore, in order to evaluate the total diversity and assess the distribution and content of bacteria in 26 cerebrospinal fluid samples from patients with PM, the beta analysis was performed. As shown in Figure 4A and B and Figure 5A–F, there was substantial difference in the species distribution in the 26 cerebrospinal fluid samples from patients with PM. The beta diversity of 26 culture-negative cerebrospinal 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 3 sample showed the most different diversity from other samples (Figure 4A). According to the Ward analysis data, there were two clusters which can be further divided into seven subclusters (Figure 4B). In addition, the total diversity and distribution of bacteria in 26 cerebrospinal fluid samples from patients with PM were evaluated by weighted- and unweighted-UniFrac index (Figure 5A–F).
Figure 2 Bacterial community composition of 26 culture-negative cerebrospinal fluid samples from patients with PM at genus level.

Notes: This figure 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 26 culture-negative cerebrospinal 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 16 major genera and much mixed infection in 26 culture-negative cerebrospinal fluid samples. The top five most widely distributed genera were Streptococcus, Acinetobacter, Limnohabitans, Sphingomonas, and Pseudomonas.

Abbreviation: PM, purulent meningitis.
which showed similar results to that of Whittaker index. Collectively, the results show a comparable difference in the diversity of species distribution and evolution in the 26 cerebrospinal fluid samples from patients with PM.

**Discussion**

Due to the significant morbidity and mortality resulting from PM, it is important to characterize the cause of PM for the optimization of the treatment of PM in clinical practice. The complex of the pathogen-induced PM remains a major challenge for the use of current therapeutics. Advances in the characterization and identification of the bacteria have facilitated the uncovering of the global view of causative pathogens of PM. Metagenomic studies have dramatically expanded our knowledge of the microbial world without the cultivation of microorganisms and can overcome the shortage of the culture-based approach. In the present study, we have performed metagenomics high-throughput sequencing to analyze the pathogens present in 26 cerebrospinal fluid samples from
PM patients based on complete quantitative analysis. We have identified most of the bacterial pathogens in 26 culture-negative cerebrospinal samples from patients with PM. The percentage of sequences belonging to each bacterial genus has been calculated for every patient, which can facilitate the choice of therapy for the PM treatment, in order to achieve the optimal therapeutic outcome in clinical practice.
Figure 5 Beta diversity of 26 culture-negative cerebrospinal fluid samples from patients with PM indicated by UniFrac index.

Notes: UniFrac index was used to evaluate the difference in species evolution between different samples. The higher index indicates more difference. (A) The weighted-UniFrac index of species distribution of 26 cerebrospinal fluid samples from patients with PM. (B) The PCoA analysis of weighted-UniFrac for 26 cerebrospinal fluid samples from patients with PM. (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 26 cerebrospinal fluid samples from patients with PM. (E) The PCoA analysis of unweighted-UniFrac for 26 cerebrospinal fluid samples from patients with PM. (F) The cluster dendrogram based on unweighted-UniFrac distance analyzed by Ward of hclust in R program.

Abbreviations: PCoA, principal coordinates analysis; PM, purulent meningitis; hclust, hierarchical clustering.
Given the complex of pathogens in the development of PM, it is of great importance to identify the composition of the causative pathogens at different levels which is clinically helpful for the proper therapeutics selection and optimization of therapy in the treatment of PM. *Streptococcus* is proposed to be the most dominant causative pathogen of PM. *S. pneumoniae* is commensal colonizer of the nasopharyngeal cavity and a damaged mucosal barrier allows direct invasion from the pharynx into the meninges, which in turn can cause PM. In agreement with previous studies, our findings have shown that the most prevalent representative sequence belonged to *Streptococcus* and that *Streptococcus* was most widely distributed with a proportion of 84.6%. At phylum level, our observations have shown six known phyla, including Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Deinococcus-Thermus, and Actinobacteria using 16S rDNA gene sequencing approach. Of note, the present study has shown the advantage of 16S rDNA gene sequencing approach over the conventional culture-based method, as evidenced by the numerous pathogens that had been identified from culture-negative cerebrospinal fluid samples.

Increasing evidence shows that multiple pathogens have been implicated in the pathogenesis of infectious diseases, including PM. It has been reported that meningitis was caused by multiple microorganisms. In the present study, the data have shown that 16 out of 26 (61.5%) samples had mixed infection. The combination of *Acinetobacter* and *Pseudomonas* was found most frequently. Notably, this is the first study to report this combination in patients with PM. There is a clear evidence of mixed infectious pathogens in patients with PM that may compromise the therapeutic effect in the treatment of PM in clinical practice. Thus, the mixed infections should be considered by clinicians when choosing the therapy. Although the present study was not able to determine which bacteria species was dominant in mixed infection, it did provide new insight into the treatment of PM in clinical practice.

In addition, we detected sequence of unknown species in 15.4% samples, which might be attributable to the a variety of factors, such as non-homogenous lysis of bacterial cells, primer mismatches, and the presence of mixed bases in PCR process. These reasons can lead to the failure of the species functional annotation. However, PCR-generated errors are very low. Therefore, these unknown species may be a new phylotype of unknown genus. In the present study, it provides information of rank order of causative pathogens from the present study and others. It clearly shows that the first three causative pathogens of PM are different. This discrepancy may due to the following reasons. First, the microbial diversity is different due to the geographical location and living condition. The microbial distribution depends on the environment. Second, the age of the patient is different. Lastly, the methodology used to characterize the pathogens of the various studies is different.

Strikingly, evidence indicates that environmental factors, such as allergic and pollution agents, can affect or damage the function or integrity of pharyngeal mucosal barrier and bacteria can directly enter into the meninges from the compromised pharyngeal mucosal barrier. Consequently, the penetrated bacteria through the impaired pharyngeal mucosal barrier can induce the development of PM. Therefore, the causal role of environmental factors in the development of PM cannot be excluded and there might be an interplay between the environmental factors and pathogens contributing to the development of PM. Notably, all the enrolled PM patients in the present study originated from Ningxia, the inland of northwest in People’s Republic of China, which is away from the ocean with the semi-arid or arid, windy, and sandy climate. This might be a contributing factor to high morbidity of streptococcal meningitis that was found in the present study.

Of importance, the diversity of bacterial species is crucial for PM therapy selection. The high diversity of the bacteria presents a challenge for PM treatment and it requires individualized therapy to achieve maximum therapeutic effect in clinical practice. In the present study, there is a substantial difference in the pathogen distribution, evolution, and content in the 26 cerebrospinal fluid samples from patients with PM, which indicates a comparable diversity. The high diversity also reflects that the multiple infections are an important contributing factor for the development of PM.

In summary, metagenomics high-throughput sequencing is a clinical practical approach to analyzing the pathogens of PM in culture-negative cerebrospinal fluid, which can overcome the limitations of the regular culture-based method. Also, the mixed infection has been observed in PM. The dominant genus is *Streptococcus*, while some unknown bacteria can also cause the intracranial infection. The global view of the bacterial composition can provide a better understanding of pathogens in PM, which can assist clinicians to make rational and effective therapeutic decisions to achieve maximum therapeutic effect in the treatment of PM in clinical practice.
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Disclosure

The authors report no conflicts of interest in this work.

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