Letter to the Editor

Rapid Identification of Bacterial Species Associated with Bronchiectasis via Metagenomic Approach[*]

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Bronchiectasis is a chronic lung disorder and a number of bacterial pathogens are involved. However, 30%-40% of sputum and purulent samples in good quality failed to grow any pathogenic bacteria, making it difficult to confirm the pathogen. In this study, we collected bronchoalveolar lavage fluid from a bronchiectasis patient undergoing acute exacerbation, and sent for *16S rDNA* pyrosequencing by a 454 GS Junior machine. Metagenomic analysis showed the composition of bacterial community in sample was complex. More than a half of reads (51.3%) were from *Pseudomonas aeruginosa*. This result was corresponding with the culture result but came out 2 d earlier, which is meaningful for early diagnosis and treatment. The detection with *16S rDNA* pyrosequencing technology is more sensitive and rapid than routine culture, and can detect the co-infection or symbiosis in airway, giving us a novel and convenient approach to perform rapid diagnosis.

Bronchiectasis is a chronic lung disorder defined by permanent and abnormal widening of the bronchi and characterized by recurrent cough, sputum production, and recurrent respiratory infections[1]. The clinical course usually involves chronic and progressive bronchial infection and inflammation which should always be investigated, particularly when it can be treated[2]. A number of bacterial pathogens are related to this disease including *Staphylococcus aureus*, *β-Hemolytic streptococci*, *Nocardia*, and *Mycobacteria*, et al., specially *Pseudomonas aeruginosa* (PA), whose chronic colonization causes an accelerated decline in lung function[3-4]. *Haemophilus influenzae* is the most common pathogen (29%-70%) followed by PA (12%-31%), meanwhile 30%-40% of sputum and purulent samples in good quality failed to grow any pathogenic bacteria[2]. Although from patients with the most severe disease the usual pathogen isolated is PA, the microbiology of bronchiectasis is complex and varies significantly between different studies and there appears to be a change in microbial flora with severity of disease[5], which could not be well studied by traditional methods, such as culture and ordinary polymerase chain reaction (PCR). Gene of 16S ribosome RNA (*16S rDNA*) is considered the best marker in research of the microbial community, and since the rapid development of the sequencing technology, it is very efficient to sequence both cultivable and uncultivable microbes directly from the sample, which provides a novel approach for pathogen detection[6].

In this study, we performed rapid identification of microbial composition in the low respiratory tract from a bronchiectasis patient undergoing acute exacerbation using *16S rDNA* sequencing approach and compared its time cost with routine culture. The patient is a 55-year-old woman with a history of chronic bronchitis presented cough, chest distress and green-yellow expectoration, and was diagnosed bronchiectasis according to the diagnostic criteria of British Thoracic Society guideline for non-cystic fibrosis bronchiectasis in 2010[7]. Microscopic examination of sputum smear revealed a few gram-negative bacilli, but nothing was obtained by sputum culture in 4 d after admission. The acute exacerbation and poor condition made it urgent to

confirm the pathogen and give her specific treatment as soon as possible. Therefore, bronchoalveolar lavage (BAL) was carried out in the 5th day after admission and the fluid was collected, and then sent to the laboratory for culture and *16S rDNA* sequencing immediately.

To perform pathogen identification by *16S rDNA* sequencing and metagemomic analysis, hypervariable region 3 and region 4 (V3-V4, -460 bps) were amplified. DNA templates were extracted from the BAL fluid using Qiagen Stool mini DNA Kit (Qiagen Corp., Chatsworth, CA) and PCR was carried out under following parameters: denaturation 94 °C, annealing 51 °C, extension 72 °C for 45 s over 25 amplification cycles, and the primer set following: forward, 341F 5'-CCTACGGGNGGCWGCAG-3', reverse: 805R 5'-GACTACHVGGGTATCTAATCC-3'[8]. After running on a 1% agarose gel and being retrieved using Qiagen Gel Extraction Kit (Qiagen Corp., Chatsworth, CA), amplicons were linked to sequencing barcode to construct the library and then to be sequenced on a 454 GS Junior (Roche). The data was submitted to GenBank database (Accession number: ERP005554). Reads data was first trimmed to filter out the with low quality reads, including the ones with short length (<50 bps), low quality (average base quality <20), and the ones with unknown bases. Then the high quality reads were first put into operation taxonomic units (OTUs) using Mothur software under the similarity of 97%, then annotated using QIIME, finally mapped to complete bacterial genome using BLAST software for more details. In total, 33,796 high quality reads were remained with average length 390 bps and average base quality 34.2, which are perfect for further analysis. The experiments were completed in less than a half and one day, and the analysis was done within one hour using our automatic pipeline.

OTU analysis showed the composition of the bacteria community in sample was complex but the bacteria of *Pseudomonadaceae* family took the main part (Figure 1, Supplementary Table 1). Reads were put into 3256 OTUs and the taxonomy of 1868 OTUs covered 30,880 reads were confirmed at phylum level at least. Most reads were identified to belong to the family *Pseudomonadaceae* (58.1%), then *Carnobacteriaceae* (9.2%) and *Enterobacteriaceae* (7.3%) (Supplementary Table 1). By mapping to the genome sequence (Table 1), 24,974 reads mapped to 35 strains of 18 genus and the similarities are over 97%, including PA (51.3%), *Carnobacterium sp.* (9.3%), *Serratia proteamaculans* (1.5%), and *Enterobacter cloacae* (1.2%), et al. Except PA which is known to relate with bronchiectasis and was confirmed by culture, there is a sign of high proportion of *Carnobacterium sp.*.
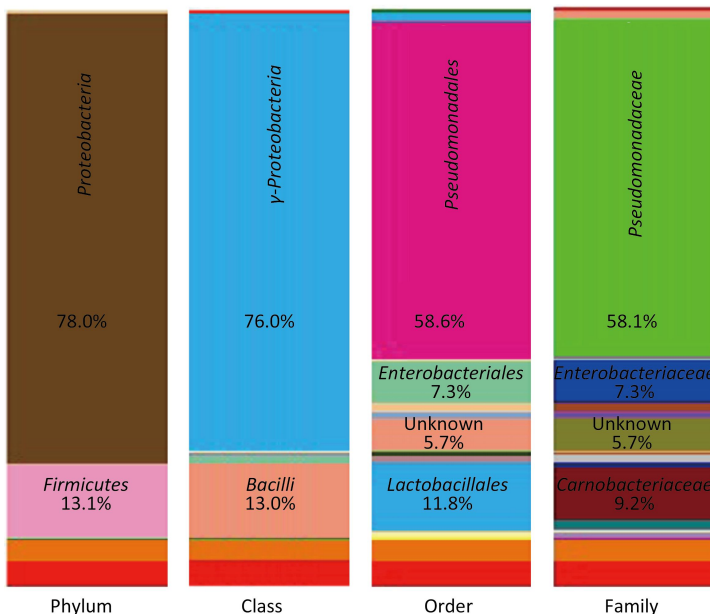

Figure 1. Bacterial compositions on different taxonomic levels. The bars from the left to right represent the compositions on phylum, class, order, and family, respectively. The unit name and OTU proportions of taxonomic units which took the main part are marked using white characters.

To perform routine culture and isolation in the same time, the sample was grown on 5% blood agar and incubated at 37 °C in the presence of 5% CO_2 for 12-15 h. Then the isolate was tested by VITEK 2 identification cards and antibiotic susceptibility card for gram-negative bacteria (BioMerieux) according to guidelines of the Clinical and Laboratory Standards Institute (CLSI). The culture result was obtained 3 d after BAL sampling and PA was identified with the colony counting of '+++', which is corresponding with the result of *16S rDNA* sequencing and analysis.

The rapid detection by *16S rDNA* pyrosequencing supplied meaningful information for clinic treatment and is 2 d earlier than routine culture. Cefoperazone/sulbactam sodium (3 g, twice daily) and etimicin (0.3 g, once daily) was used to deal with the PA infection immediately. After 2 d treatment, the symptoms of this patient were ameliorated. The body temperature was normal, the cough was eased, and then the expectoration turned

Table 1. Reads Distribution of Best Matches with Bacterial Genomes

Species	Reads Number	
	n	%
Pseudomonas aeruginosa	17,324	51.26
Pseudomonas syringae	595	1.76
Pseudomonas fluorescens	552	1.63
Pseudomonas putida	308	0.91
Pseudomonas stutzeri	122	0.36
Pseudomonas mendocina	13	0.04
Carnobacterium sp.	3,156	9.34
Serratia proteamaculans	512	1.51
Enterobacter cloacae	422	1.25
Shewanella baltica	205	0.61
Shewanella sp.	195	0.58
Klebsiella variicola	200	0.59
Klebsiella pneumoniae	83	0.25
Vibrio vulnificus	61	0.18
Vibrio sp.	28	0.08
Vibrio cholerae	15	0.04
Aeromonas veronii	14	0.04
Pectobacterium wasabiae	131	0.39
Streptococcus suis	60	0.18
Cronobacter turicensis	49	0.14
Nitrobacter winogradskyi	38	0.11
Salmonella enterica	12	0.04
Erwinia billingiae	17	0.05
Rhizobium sp.	13	0.04
Leuconostoc gasicomitatum	11	0.03
Total	24,136	71.42

to white gradually. Culture and antibiotic susceptibility test helped to adjust the treatment. Thirteen days after admission, most of the symptoms were disappeared or improved and then the patient was discharged, which is much earlier than expected.

Although we could not obtain accurate quantitative result via *16S rDNA* sequencing approach, the relative propositions showed acceptable result for early diagnosis. In our study, PA took the major part in sample, which is confirmed by culture but more rapidly, and the relative proposition could also give us a half-quantitative signature of PA amount in the sample. For one hand, it is important in treating with critically ill patients because of the lack of time. For the other hand, the results help to set a specific therapy and are very meaningful for the treatment, because it could help to estimate the main pathogen, use the antibiotics more targeted and control the dosage better. The accurate microbiological background made the clinic doctors more confident. At the time of fast emerging of drug resistance and frequently occurring of mixed infections, it is more valuable to apply in the clinic, and the cost would be decreased significantly when multiple samples could be detected in a run.

In addition, the microbiology of bronchiectasis is also changing continuously. This *16S rDNA* sequencing approach helps us investigate the complex composition of bacterial communities better. In this study, several other species of bacteria were observed except PA, in which the existence of *Carnobacterium sp.* is interesting and worth for further research. Only two cases of human pus and one case of human blood were reported[9-10]. And, the taxonomy of 8.7% reads has not been identified based on the available reference database now. Infection plays important roles in the development of bronchiectasis, but the etiology is complex and still not well defined. Etiology research using *16S rDNA* pyrosequencing may provide new insights into pathogenesis of bronchiectasis.

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