Short Communication

Sputum purulence-associated microbial community compositions in adults with bronchiectasis

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Introduction

Bronchiectasis is a debilitating chronic inflammatory airway disease which arises from multiple etiologies. The interplay among the etiology, altered microenvironment and defective host-defense may have predisposed to recurrent chest infections of pathogens (1,2) that correlate with bronchiectasis exacerbations and increased mortality. Sputum production, which results from mucus hypersecretion and exaggerated airway inflammation, is one of the major complaints among symptomatic bronchiectasis patients (3) whose sputum bacterial compositions are heterogeneous (4,5). Clinically, sputum purulence has been an important parameter reflecting the magnitude of airway inflammation and infection. Sputum purulence, which is commonly rated by using color charts in clinical settings, has been correlated with sputum myeloperoxidase and neutrophil elastase activity and pathogen (i.e., pathogenic bacteria) infections (6,7).

Conventionally, physicians prescribed antibiotics based on sputum purulence in conjunction with assessment of clinical symptoms such as sputum volume and cough frequency. Nonetheless, antibiotics are frequently prescribed to bronchiectasis patients (particularly those with purulent sputum) regardless of culture findings in many developing countries (such as China). Some patients with purulent sputum tested negative for bacterial culture, suggesting that culture techniques have limited value for identifying pathogenic bacteria. Little is known regarding bacterial compositions associated with purulent sputum in bronchiectasis. Studies that comprehensively characterize microbial compositions according to purulence are needed to inform physicians in which subgroup should antibiotic prescription be prioritized.

We hypothesized that sputum purulence partially correlated with the sputum microbial compositions and that sputum purulence was not solely responsible for interpreting the variations in microbial compositions in patients with clinically stable bronchiectasis and during bronchiectasis exacerbations. In this study, we sought to compare bacterial compositions between purulent and non-purulent sputum, and between culture-positive and -negative purulent sputum, followed by comparison of changes in bacterial diversity during exacerbations with 16srRNA sequencing (8,9).

Methods

Study participants

Adults with bronchiectasis confirmed with high-resolution computed tomography (effective within 12 months, reviewed by an experienced radiologist) were consecutively recruited between March 2014 and November 2015. The criteria for diagnosing bronchiectasis have been published previously (3). Patients were free from exacerbations for >4 weeks (4). We excluded patients with malignancy, upper airway bacterial/viral infection, or antibiotic use (except for the low-dose

maintenance therapy with macrolides) within 4 weeks. Our local ethics committee gave approval Medical Ethics Year 2012 (The 33rd), and patients signed informed consent.

Study design

Patients underwent baseline visits when stable. For those with exacerbations who contacted investigators, exacerbation visits were scheduled, followed by 14-day antibiotic therapy as recommended by the international guidelines with minor changes (3). In this study, bronchiectasis exacerbations denoted continuous (48 h or greater) significant worsening of 3 or more symptoms or signs: significantly increased cough frequency, sputum volume, sputum purulence, dyspnea, fever, fatigue/malaise, and new-onset or worsening of haemoptysis (3,4). Convalescence visits were scheduled 1 week after antibiotic therapy. At each visit, assessment included history inquiry, spirometry and sputum sampling. Spontaneous sputum plugs (the most purulent portion of the sample) which met quality control (white blood cell: epithelial cell ratio >2.5:1) were split for culture (blood/chocolate agar for overnight incubation, targeting at *Pseudomonas aeruginosa*, *Haemophilus spp*., and other pathogenic bacteria) (4) and sequencing. Purulence was evaluated by an investigator masked to patient's profiles, with purulent sputum denoting purulence scores (scale: 0–8) of 6 or greater (0: absence of sputum; 1: completely transparent; 2: almost transparent; 3: translucent but colorless; 4: opaque and milky white; 5: grey; 6: pale green; 7: moderately green; 8: dark green sputum) (10).

16srRNA sequencing

Nucleic acids were extracted using physical disruption (vortex) and centrifugal absorption column (HiPure DNA Kit B, Magen Inc., Guangzhou, China). Quality control of the samples was achieved via agarose gel electrophoresis and ND-100 Nanodrop system (Thermo Fisher Inc., USA). Qualified samples were subject to library construction with TruSeq^{TM} Custom Amplicon Sample Prep Kit (Illumina Inc., USA), and barcoded and mixed before pooling. The quality of the library was assessed with Agilent 2200 TapeStation (Agilent Inc., USA) and Qubit 2.0 (ThermoFisher Inc., USA). 2.1nM Phix Control (19:1) and sodium hydroxide were added for incubation of the sample, followed by dilution with HT1 solution (1:100). We performed DNA sequencing with Miseq System (Illumina Inc., USA). Raw reads were denoised, followed by chimera removal. High-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity with UCLUST software. The sequences for individual OTU were aligned with BLASTn, and the taxonomic identities were assigned by using Ribosomal Database Project classifier (version 2.2). The QIMME software was employed for rarefaction of the denoised files, which enabled subsequent calculation of the relative abundance and the diversity metrics. Sequences were deposited in GenBank under accession number SAMN06768146-SAMN06768292.

Statistical analysis

We performed analyses with R package (www.r-project.org) and Graphpad Prism 5.0 (Graphpad Inc., San Diego, USA). We classified patients into the group with mucoid or mucopurulent sputum (M+MP, n=16), culture-negative (no pathogenic bacteria) purulent sputum (C–P+, n=16), and culture-positive purulent sputum (C+P+, n=73). Group M+MP was not stratified because of limited sample sizes. One-way analysis-of-variance or Kruskal-Wallis test was applied for the comparison of three groups for continuous variables, followed by least-square difference test for two-group comparisons. We used independent *t*-tests or Wilcoxon signed-rank tests to compare the continuous variables between the two groups. Paired *t*-tests were conducted for the pairwise sputum samples. Categorical variables were compared with chi-square test. We compared the relative abundance and Shannon-Wiener diversity index (an alpha-metric reflecting the number of types of sequences within any individual sample) to reflect the magnitude of dysbiosis (altered microbial compositions in response to external stimuli such as disease). We ran similarity of percentage analysis with Community Analysis Package version 5.3.3.472 (Hampshire, UK).

Results

Of 207 patients screened, 105 with spontaneous sputa had available purulence scores (64 patients declined and 38 produced insufficient sputum). Median purulence score was 6.0 (range, 1.0–8.0). 85 patients cultured positive to pathogenic bacteria (42.9% isolated *Pseudomonas aeruginosa*). Idiopathic (32.3%) and post-infectious (37.1%) were the most common etiologies. Twenty five percent, 25.0% and 12.3% of the patients in M+MP, C–P+, and C+P+ group had been prescribed with inhaled corticosteroids within 6 months (P>0.05), respectively. No patients were receiving

Table 1 Demographic and baseline levels of the three groups of patients

Category of parameters	Parameters	M+MP (n=16)	C–P+ (n=16)	C+P+ (n=73)	P value
Anthropometry	Age (years)	41.9±11.5	46.7±14.1	44.5±14.5	0.62
	BMI (kg/m^2)	22.0±3.6	20.6±3.3	20.3±3.3	0.15
	Female (n, %)	7 (43.8)	11 (68.8)	39 (53.4)	0.35
Smoking status	Never smokers (n, %)	14 (87.5)	15 (93.8)	67 (91.8)	0.68
Disease-related parameters	No. of bronchiectatic lobes	3.4±1.9	3.8±1.6	5.0 (2.0)	0.01
	Exacerbation frequency (per year)	1.0 (2.0)	1.0 (1.8)	1.0 (2.0)	0.88
	FEV_1 predicted %	70.4±24.3	70.0±22.3	54.9±22.2	0.01
Disease severity	Bronchiectasis Severity Index	3.8±3.5	3.5 (3.8)	6.8±3.9	<0.01
Medications within 6 months	Inhaled corticosteroids (n, %)	4 (25.0)	4 (25.0)	9 (12.3)	0.27
	Macrolides (n, %)	7 (43.8)	9 (56.3)	34 (46.6)	0.74
Etiology	Post-infectious (n, %)	4 (25.0)	4 (25.0)	25 (34.2)	0.64
	Other known causes (n, %)*	5 (31.3)	4 (25.0)	24 (32.8)	0.83
	Idiopathic (n, %)	7 (43.8)	8 (50.0)	24 (32.8)	0.37
Bacteria isolated at initial visits	*Pseudomonas aeruginosa* (n, %)	3 (18.8)	0 (0.0)	42 (57.5)	<0.01
	Other PPMs (n, %)**	9 (56.3)	0 (0.0)	31 (42.5)	
	Commensals (n, %)	4 (25.0)	16 (100.0)	0 (0.0)	

*, of all bronchiectasis patients (N=105), the other known etiologies consisted of immunodeficiency (n=19, 17.9%), gastroesophageal reflux (n=4, 3.8%), asthma (n=3, 2.8%), Kartagener's syndrome (n=2, 1.9%), diffuse panbronchiolitis (n=2, 1.9%), lung maldevelopment (n=2, 1.9%), aspergillosis (n=1, 0.9%), rheumatoid arthritis (n=1, 0.9%), and cystic fibrosis transmembrane regulator-related disorder (n=1, 0.9%). **, among all patients with clinically stable bronchiectasis (n=105), other potentially pathogenic bacteria comprised *Haemophilus influenzae* (n=13, 12.4%), *Haemophilus parainfluenzae* (n=8, 7.6%), *Escherichia coli* (n=5, 4.8%), *Klebsiella pneumoniae* (n=4, 3.8%), *Rothia mucilaginosa* (n=2, 1.8%), *Streptococcus pneumoniae* (n=1, 0.9%), *Moraxella catarrhalis* (n=1, 0.9%), *Proteus mirabilis* (n=1, 0.9%), *Acinetobacter haemolyticus* (n=1, 0.9%), *Achromobacter xylosoxidans* (n=1, 0.9%), *Haemophilus haemolyticus* (n=1, 0.9%), *Stenotrophomonas maltophillia* (n=1, 0.9%), and *Bordetella bronchiseptica* (n=1, 0.9%). Sputum culture was performed in all bronchiectasis patients when clinically stable and during exacerbations. All bronchiectasis patients could spontaneously produce sputum for high-throughput sequencing because this was deemed to be one of the inclusion criteria. None of the samples simultaneously grew *Pseudomonas aeruginosa* and *Haemophilus influenzae*. The total proportion of individual etiology was greater than 1 because a minority of patients had dual etiologies. Numerical data were presented as mean ± standard deviation for normal distribution or otherwise median (interquartile range). Categorical data were expressed as number (percentage) and compared with chi-square test. Analysis-of-variance or Kruskal-Wallis test was applied for among-group comparisons of the continuous variables, whereas chi-square test was adopted for among-group comparison of the categorical variables. No patients were receiving oral or inhaled antibiotics during the study. Group M+MP, bronchiectasis patients with mucoid or mucopurulent sputum (n=16); group C-P+, bronchiectasis patients with culture-negative purulent sputum (n=16); group C+P+, bronchiectasis patients with culture-positive purulent sputum (n=73). The P value denoted the comparison of the three groups. BMI, body-mass index; PPMs, potentially pathogenic microorganisms; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; HRCT, high-resolution computed tomography.

oral/inhaled antibiotics. Bronchiectasis Severity Index was significantly higher in group C+P+ (mean: 6.8) than in groups M+MP and C–P+ (mean: 3.8 and 4.8, P=0.005) (*Table 1*). Twenty-two patients attended exacerbation visits (5, 4 and 13 in group M+MP, C–P+, and C+P+, respectively), of whom 20 participated in convalescence visits.

We detected a mean of 42, 43 and 36 unique OTUs in groups M+MP, C–P+ and C+P+, respectively. Overall, *Proteobacteria* was the dominant phylum among all patients, with greater sputum purulence score correlating with higher relative abundance (*Figure 1A*). At genera levels, *Pseudomonas* dominated among all patients, with greater sputum purulence score correlating with higher relative abundance (*Figure 1B*). Group M+MP demonstrated

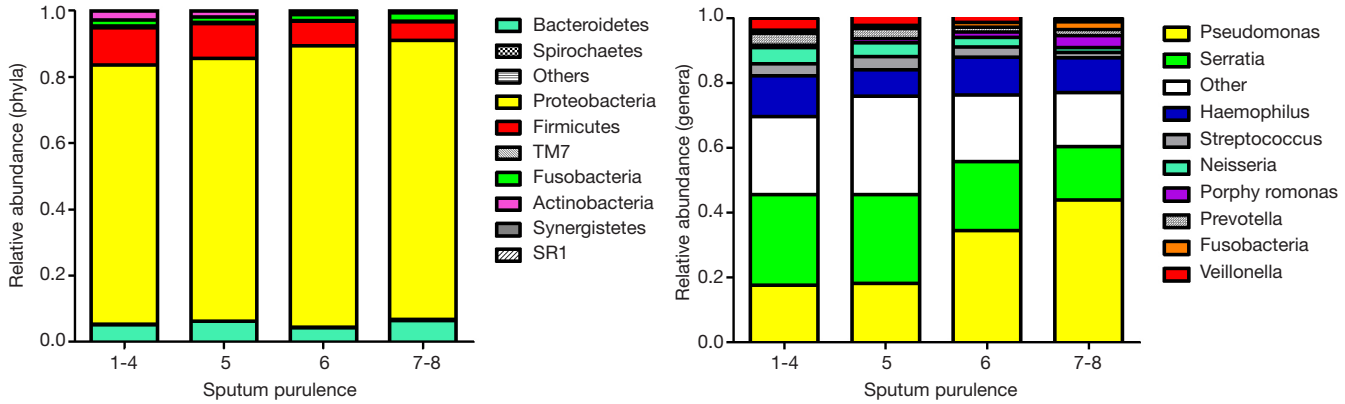

Figure 1 Microbial compositions when stratified by sputum purulence score. (A) The relative abundance of bacterial phyla in bronchiectasis patients with different sputum purulence score [1–4, 5, 6, 7–8]; (B) the relative abundance of bacterial genera in bronchiectasis patients with different sputum purulence score [1–4, 5, 6, 7–8]. Shown in these figures are the results obtained when clinically stable. In this study, 10, 6, 53 and 36 patients had a sputum purulence score of 1–4, 5, 6, 7–8, respectively. Patients with a purulence score of 1–4 were combined because of the limited sample sizes, as did those with a sputum purulence score of 7–8.

significantly lower abundance of *Proteobacteria* (78.6% *vs.* 84.6%) and higher abundance of *Firmicutes* (10.9% *vs.* 6.7%) than the rest combined (both P<0.05). Group C–P+ had significantly lower abundance of *Proteobacteria* (64.2% *vs.* 89.1%) and higher abundance of *Firmicutes* (11.5% *vs.* 5.7%) than group C+P+ (both P<0.01). Group C–P+ had significantly higher Shannon-Wiener diversity index (0.902 *vs.* 0.393) compared with group C+P+ (P<0.05) (*Figure 2A,B*). *Proteobacteria* consistently contributed most to community similarity in groups M+MP, C–P+ and C+P+ (85.8%, 74.6% and 94.9%). However, Firmicutes contributed considerably to community similarity in groups M+MP (8.4%) and C–P+ (11.7%).

Pseudomonas spp. predominated in group C+P+, whereas *Serratia spp*. predominated in groups M+MP and C–P+. Group M+MP demonstrated significantly lower abundance of *Pseudomonas* (17.8% *vs.* 38.3%) and higher abundance of *Serratia spp.* (27.8% *vs.* 19.3%) than the rest combined (both P<0.05). Group C-P+ yielded markedly lower abundance of *Pseudomonas spp.* (19.3% *vs.* 42.5%) than group C+P+ (P<0.01). However, the abundance of *Serratia spp*. was comparable (25.4% *vs.* 18.0%). Group C–P+ demonstrated significantly higher Shannon-Wiener diversity index (2.120 *vs.* 1.381) than group C+P+ (P<0.05) (*Figure 2C,D*). *Pseudomonas spp*. contributed most to community similarity in group C+P+ (49.7%), but not in groups M+MP (19.2%) and C–P+ (19.9%). *Serratia spp*. (21.6–31.8%) contributed considerably to community similarity in bronchiectasis.

Although groups C–P+ plus C+P+ differed considerably from group M+MP in microbial compositions, sputum purulence scores correlated with the abundance of *Proteobacteria* and most genera (including *Pseudomonas spp*.) and Shannon-Wiener diversity index, at phyla and genera levels (data not shown).

We compared microbial compositions of sputum samples when clinically stable and during exacerbation, and from exacerbation to convalescence. A trend towards greater differences in Shannon-Wiener diversity index was observed in groups M+MP and C–P+ at phyla and genera levels (*Figure 2E,F*). No significant differences were found in the abundance of *Proteobacteria* and *Pseudomonas spp.* when comparing paired samples collected when clinically stable and during exacerbations (P>0.05).

Discussion

In this study, we have demonstrated that bronchiectasis patients with purulent sputum and isolation with pathogenic bacteria had considerably greater magnitude of airway dysbiosis, particularly when assessed in terms of the relative abundance of *Proteobacteria* and the alpha-diversity index. We have confirmed some of the findings from a previous study that sputum purulence was a strong predictor of potentially pathogenic microorganisms (PPM) infection (11). Furthermore, our study partially echoed the findings that sputum purulence correlated with pathogenic bacterial infection in hospitalized patients with COPD exacerbations (12). Nonetheless, our study has

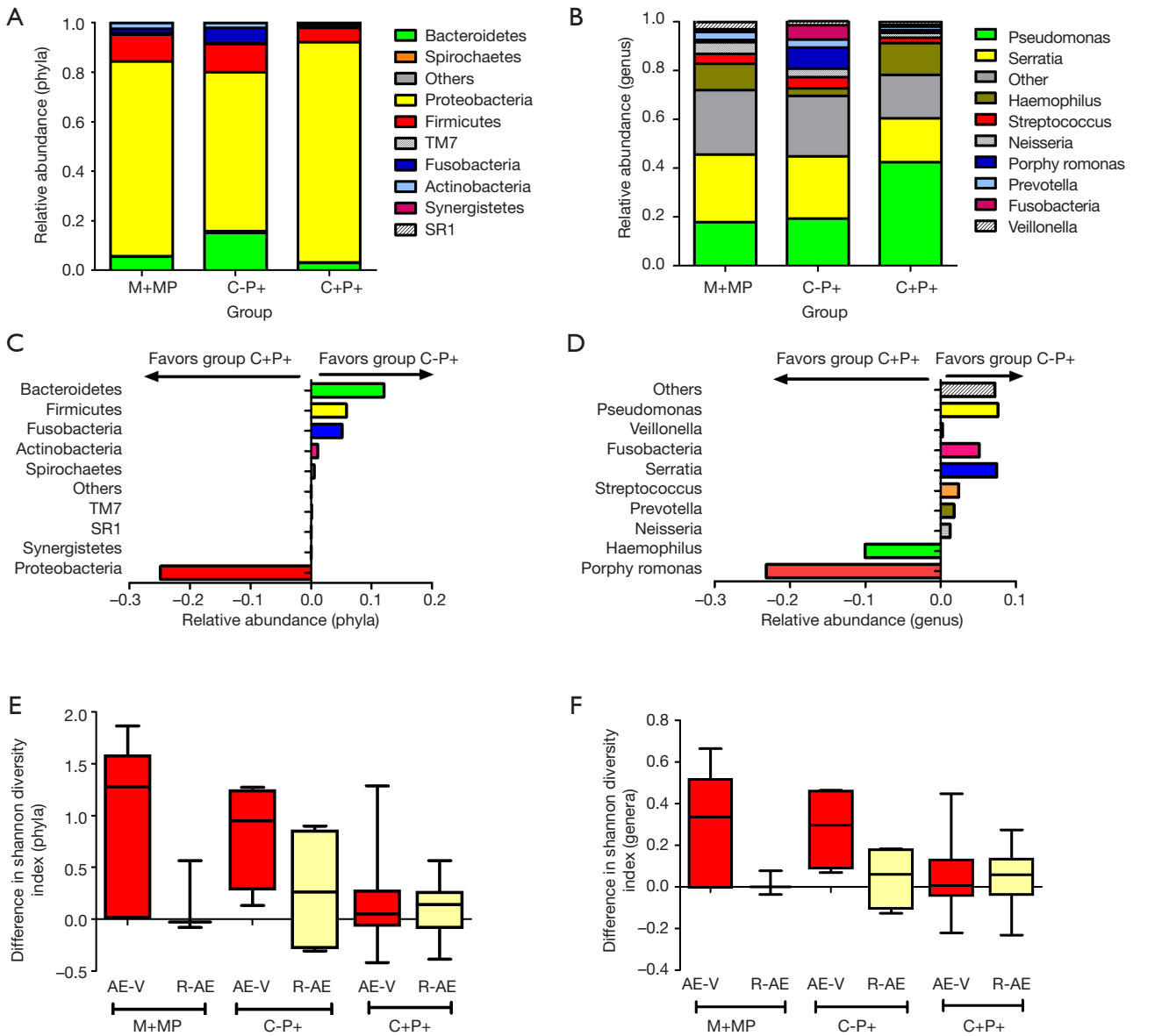

Figure 2 Microbial compositions at phyla and genera levels in bronchiectasis patients stratified by sputum purulence score and culture findings. (A) The relative abundance of bacterial phyla in bronchiectasis patients; (B) the relative abundance of bacterial genera in bronchiectasis patients; (C) the difference in the relative abundance of airway microbial compositions at phyla levels among all bronchiectasis patients; (D) the difference in the relative abundance of airway microbial compositions at genera levels among all bronchiectasis patients; (E) differences in Shannon-Wiener diversity index at phyla levels among groups M+MP, C–P+ and C+P+; (F) differences in Shannon-Wiener diversity index at genera levels among groups M+MP, C–P+ and C+P+. Group M+MP, bronchiectasis patients with mucoid or mucopurulent sputum (n=16); group C–P+, bronchiectasis patients with culture-negative purulent sputum (n=16); group C+P+, bronchiectasis patients with culture-positive purulent sputum (n=73). Data from a total of three visits were analyzed: clinically stable (V), exacerbation (AE), and convalescence (R). (D) focused on clinically stable visits only, whereas (E,F) dynamically compare the changes in sputum microbial compositions at the three visits. For all bronchiectasis patients during clinically stability, the potentially pathogenic microorganisms detected included *Pseudomonas aeruginosa* (n=45, 42.9%), *Haemophilus influenzae* (n=13, 12.4%), *Haemophilus parainfluenzae* (n=7, 6.7%), Escherichia coli (n=5, 4.8%), *Klebsiella pneumoniae* (n=4, 3.8%), *Haemophilus haemolyticus* (n=1, 1.0%), *Haemophilus parahaemolyticus* (n=1, 1.0%), *Moraxella catarrhalis* (n=1, 1.0%), *Streptococcus pneumoniae* (n=1, 1.0%), *Stenotrophomonas maltophilia* (n=1, 1.0%), *Bordetella bronchiseptica* (n=1, 1.0%), *Serratia marcescens* (n=1, 1.0%), *Proteus mirabilis* (n=1, 1.0), *Actinetobacter haemolyticus* (n=1, 1.0%), *Rothia mucilaginosa* (n=1, 1.0%) and *Achromobacter xylosoxidans* (n=1, 1.0%). AE-V, the difference between exacerbation visit and clinical stability, measured by subtracting the values at exacerbation visits from those during clinical stability; R-AE, the difference between convalescence visit and exacerbation visit, measured by subtracting the values at convalescence visits from those at exacerbation visit.

further revealed a considerable heterogeneity of microbial compositions even within the subgroups classified according to sputum culture findings and purulence. Notably, sputum microbial compositions do not solely depend on sputum culture positivity. A considerable heterogeneity in microbial compositions could be found among individual bronchiectasis patients within the same subgroup (i.e., culture-positive or culture-negative group) (data not shown).

Focusing solely on purulent sputum in clinically stable bronchiectasis, microbial compositions varied substantially among individuals (e.g., the abundance of *Proteobacteria* and *Pseudomonas spp.* was 8.6~84.6% and 1.5~92.2%). Only those with purulent culture-positive sputum, which demonstrated minimal changes in bacterial diversity during exacerbations, displayed the most prominent degree of airway dysbiosis. These findings reaffirmed that sputum purulence alone was not the sole dependent factor that influences on microbial compositions. Importantly, our study suggested that stratification of patients based on the combination of sputum purulence and culture findings may help better elucidate the characteristics of airway dysbiosis and explore the possible source of variation in sputum microbial compositions. Therefore, sputum purulence and culture findings might have complementary value for guidance of antibiotics prescription in the clinical settings. In some patients, the minor degree of airway dysbiosis indicated the alternative sources of sputum purulence (13), such as exaggerated elastase release. According to another study, outgrowth of *Proteobacteria* and/or *Pseudomonas spp.* contributes to more prominent dysbiosis (14), whereas *Haemophilus spp.* and *Serratia spp.* counter the expansion of *Pseudomonas spp*. Our study has partially corroborated with these findings, and has further indicated the potential association between sputum purulence and outgrowth of *Proteobacteria* and/or *Pseudomonas spp.* in some, but not all, of the purulent sputum samples.

Interestingly, the changes in the relative abundance of *Proteobacteria* were greater in group M+MP compared with groups C–P+ and C+P+ during bronchiectasis exacerbations as compared with clinically stable visits. It is likely that the minor magnitude of airway dysbiosis (lower relative abundance of *Proteobacteria* that resulted in minor suppression of other PPMs) may be responsible for the greater changes in microbial composition during exacerbations. The minor changes in microbial compositions during exacerbations as compared with clinically stable visits have been reported in a previous study (8). However, because the sputum purulence assessment was not performed, we were unable to directly compare the findings further. According to the study by Purcell *et al.* (5), there existed a considerable difference in the patterns of changes in sputum microbial compositions during bronchiectasis exacerbations compared with clinically stable visits. Notably, the presence of *Burkholderiales*, *Pasteurellaceae*, *Streptococcaceae* and some other PPMs correlated with bronchiectasis exacerbations. In our study, we did not specifically focus on the comparison of changes in all bacterial phyla and genera, therefore we were unable to address whether the presence of the bacteria mentioned above might help interpret the greater change in microbial compositions during exacerbations in the group M+MP. More mechanistic investigations of the factors that drive the major changes in sputum microbial compositions during exacerbations are needed.

There are some limitations that should be considered. Some of the subgroup analysis might be not sufficiently powered because of the limited sample sizes. The sample size for the subgroups was unbalanced. Patients aged on average lower compared with the populations in other geographic regions. Our findings might not be completely extrapolated to patients in other countries because of the high relative abundance of *Proteobacteria* and *Serratia* and the low relative abundance of *Haemophilus spp.* We did not measure clinically relevant markers (i.e., neutrophil elastase activity) that have been correlated with sputum purulence. Finally, bronchiectasis exacerbations are highly heterogeneous events which indicated significant variations in sputum microbial compositions that cannot be adequately addressed based on the current study design.

This study documented the significant heterogeneity of purulent sputum in bronchiectasis. Culture findings coupled with sputum purulence assessment may better guide clinicians for antibiotics prescription.

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Footnote

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Ethical Statement: Our local ethics committee gave approval Medical Ethics Year 2012 (The 33rd), and patients signed informed consent.

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