Original article

Changes of Bacterial Spectrum of Lower Respiratory Tract in Patients with Rhinovirus Induced AECOPD

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Abbreviation: AECOPD, acute exacerbation of chronic obstructive pulmonary disease; RhV, rhinovirus.

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Objective: To evaluate the bacteria pathogenic spectrum in the lower respiratory tract of Acute Exacerbated Chronic obstructive pulmonary diseases (AECOPD) patient with Rhinovirus infection. **Methods:** Sputum samples were collected from patients with AECOPD hospitalized. Respiratory viruses were detected by fluorescent quantitative PCR. According to the results of RhV detection, the patients were divided into two groups: AECOPD patients with rhV positive (RhV+AECOPD group) and AECOPD patients with rhV negative (RhV-AECOPD group) with 11 cases in each group. DNA was extracted from patient sputum. Meta rDNA amplification library was constructed and validated. Variable V4 region of 16S rDNA gene was sequenced. Operational taxonomic unit (OTU) and bacterial species were analyzed between the two groups. **Results:** There was no significant difference in the average OTUs between the two groups. According to the classification of bacterial pathogens, the level of actinomycetes in sputum of RhV+AECOPD group was significantly higher than that of RhV-AECOPD group ($P<0.05$); *Corynebacterium*, *Neisseria* and *Roxiella* had significant difference in genus level between the two groups; The species level of *Roxiella mucosa* had a significant difference in the two groups. The differences between the two groups, from the specific results of complements, indicated that the bacterial pathogens in the experimental group were less than those in the control group: The variable V4 region of 16S rDNA gene sequenced by two-index and two-terminal sequencing, showed that there was the difference in the diversity of lower respiratory tract bacteria between the two groups, indicating that there were significant differences in the microbial community structure between the two groups.

Key Words: Rhinovirus; chronic obstructive pulmonary disease; exacerbation; microorganism.

COPD is a chronic inflammatory disease within the respiratory tract. The incidence of COPD is increasingly common in recent years. It is expected that COPD will become the top four contributors to mortality in the world by 2020.¹

AECOPD can further aggravate the pulmonary function problem, leading to the decline of patients life quality-life and the increase in the mortality rate.² Most of the AE COPD patients relate to infection, and 40% of the AECOPD relate to RhV-based viral infection.³ In addition, co-infection of viruses and bacteria can be detected in 25% of acute exacerbated COPD cases, and studies have shown that co-infection of viruses and bacteria can further aggravate the lung function problem and prolong hospitalization time.^{4,5}

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Respiratory microorganisms themselves are believed to be beneficial for the host to promote the differentiation of mucosal structure and function, stimulate innate immunity and adaptive immune response, and resist the invasion of pathogens through the "anti-colonization" effect.⁶ Virus infection may break the immune balance between host and respiratory microorganisms, it creates favorable conditions for the growth of pathogenic bacteria. About 60% of COPD patients had secondary sputum bacterial culture positive after RhV infection.⁷

Molyneux et al.³ Longitudinally compared the changes of respiratory flora before and after RhV infection in COPD patients, in order to elaborate on the influence of RhV on respiratory flora. This study compared the bacterial spectrum of the lower respiratory tract between RhV-positive AECOPD patients and virus-negative AECOPD patients, it reveals the differences of bacterial flora between two testing groups, and provide guidance for clinical prevention and treatment of secondary bacterial infections.

Materials and methods

Ethical description

The study was approved by the Ethics Committee of Shanghai Fifth People's Hospital. All the volunteers have signed written consent.

Study subjects

Patients admitted from the Fifth People's Hospital of Shanghai in June 2015 -2016 in May. Sputum samples were collected from patients without treatment on the first day of admission. The sputum of all patients was screened and detected by fluorescent quantitative PCR in Shanghai Public Health Clinical Center for 10 respiratory viruses, including influenza virus type A (FluA), influenza virus type B (FluB), adenovirus (ADV), respiratory syncytial virus type A (RSV-A), (respiratory syncytial virus type A, RSVA), respiratory syncytial virus type B (RSVB), rhinovirus (RhV), parainfluenza virus (PIV), coronavirus (CoV), Boca virus (BoV), and metapneumonia virus (m). Etapneumovirus, MPV). Eleven RhV positive sputum samples from patients with AECOPD were selected as the experimental group and 11 virus negative sputum samples as control group.

Entry criteria and exclusion criteria

Inclusion criteria: all patients met the diagnostic criteria⁸ for COPD guidelines. Acute exacerbation of COPD is defined as the continuous deterioration of COPD patients beyond their daily conditions and the need to change the routine medication of basic COPD. In the course of the disease, cough, sputum, shortness of breath and/or wheezing are aggravated in the short term, sputum volume is increased, purulent or viscous purulent, and inflammation such as fever can be significantly aggravated.

Exclusion criteria: Patients who cannot cooperate with pulmonary function tests, those who do not meet the definition of acute exacerbation of COPD mentioned above, those with asthma, bronchiectasis, cystic fibrosis, active pulmonary tuberculosis or other respiratory diseases requiring treatment or diagnosis, those with mental disorders or any related immune inflammation reaction. Other diseases and patients who received antibiotics or hormone therapy during the past 4 weeks. In the control group, 1

sample was removed after it was detected that it did not meet the requirement of building meta rDNA amplification library.

Collection of clinical data

The general information of patients, including age, sex, smoking history and complications, laboratory and examination results, including blood routine, liver function, coagulation items, blood gas analysis, antibiotic use time, prognosis and hospitalization time were collected.

Experimental method

(1) methods for sample testing

The sputum metagenic DNA was extracted according to the standard operating procedure of Meta DNA extraction, and the sample concentration was quantified accurately by the qubit fluorimetry. The sample integrity was detected by agarose gel electrophoresis (agarose concentration: 1%; voltage: 150V; electrophoresis time: 40min).

(2) building Meta rDNA amplicon Library

Genomic DNA was standardized to 30 ng for each PCR reaction, and then the primer mixture of V4 double index fusion PCR and the precursor of PCR (NEB Phusion high fidelity PCR) were added to run the PCR. The fusion temperature is 56 C and the PCR cycle is 30 times. PCR products remove non-specific products with Ampure XP beads (AGINCOURT).

(3) verify the Meta rDNA amplicon library.

The final library was quantified in two ways: using the Agilent 2100 Bioanalyzer (Agilent). The average molecular length was determined by DNA 1000 reagent and the library was quantified by real-time quantitative PCR (Eva GreenTM).

(4) sequencing of Meta rDNA amplicon Library

Qualified libraries will be sequenced in the MiSeq system using the sequencing strategy PE250 (PE251+8+8+251) (MiSeq kit) by double exponential double-ended sequencing of the variable V4 region of 16S rDNA gene.

(5) bioinformatics analysis

During data processing and filtering, we used the internal written procedures of the Chinese big gene company through Illumina

The platform (Hiseq) carries out Paired-end sequencing to filter low-quality reads and obtain high-quality Clean Data. Sequence splicing software FLASH v1.2.11 is used to assemble pairs of reads obtained by double-terminal sequencing into a sequence and obtain Tags with the high variable region. Tags were clustered into OTUs by USEARCH (v7.0.1090). Then the OTU representative sequences were compared with the database Release 9 201203 by RDP classifier (v2.2). Species complexity and species diversity were analyzed based on OTU and species annotation results.

(6) statistical analysis

Discrete variables are represented by counting (percentage) and continuous variables by means of mean + standard deviation. The t test is used to compare the mean of two independent samples, and Fisher's exact probability method is used to compare discrete variables. Alpha diversity analysis is the analysis of species diversity in a single sample. ACE index, Chao index, Shannon index and Simpson index are used to evaluate species diversity. Beta diversity analysis is used to compare the differences in species diversity between a pair of samples, and Bray-Curtis distance is used to assess the differences. Alpha diversity and Bray-Curtis distance are calculated by Mothur (v1.27). $P < 0.05$ is considered to be

meaningful in all statistical analyses. SPSS 18 and GraphPad. cameyo 6 are used for data analysis and processing.

Result

Clinical characteristics analysis

The clinical characteristics of AECOPD patients in the rhinovirus positive group (11 cases) and the virus negative group (10 cases) were compared, the characters include age, sex, smoking history, complications, body temperature, blood routine, liver function, coagulation function, antibiotic usage time, prognosis and hospitalization time. The result shows that there are no significant statistical differences to be found. The differences was ($P > 0.05$) (see Table 1).

OTUs diversity analysis

We generated an average of 25,587 sequences from RhV-positive AECOPD patients and 27,654 sequences from virus-negative AECOPD patients, and the corresponding OTUs averaged 132 and 134 in 97% similarity between the two groups, but there was no statistical difference between the two groups (see Figure 1). The alpha diversity analysis (such as ACE index, Chao index, Shannon index and Simpson index) was presented in Table 2. The

comparison of alpha diversity index between the two groups showed no statistical difference. Beta diversity was analyzed by Bray-Curtis clustering tree graph (see Figure 2). In this graph, no RhV positive or virus negative samples were found clustering in a branch of the tree. Two groups of samples were intersected and distributed in different branches.

Microbial composition.

In this study, the total sequence measured provided a taxonomic map of sputum microorganisms showing 20 phyla, 41 classes, 65 orders, 108 families, 160 genera and 62 species.

At the door level, 20 doors were presented in sputum samples (see Fig. 3a). Higher taxa included the posterior wall bacteria (42.34% of RhV-positive microorganisms and 45.91% of virus-negative microorganisms), Bacteroides (27.35% and 22.71%, respectively), Proteus (20.23% and 21.66%, respectively), Fusobacterium (4.60% and 4.07%, respectively) and Actinomycetes (2.67% and 1.55%, respectively). The gates in the two groups constitute 97.19% and 95.90% of the total sequence respectively. Only RhV positive group was found to be verruca microphylla and GNO2 gate, accounting for about 0.002% and 0.012% of total microorganism respectively. However, only in the viral negative group were found to be Curvularia and Phytophthora, accounting for about 0.002% and 0.004% of the microorganisms, respectively.

Table 1. comparison of clinical characteristics between rhinovirus positive and virus negative AECOPD patients

Parameter	RhV positive AECOPD patients (n=11)	virus negative AECOPD patients (n=10)	Chi square value and P value
Age (year)	71±3.1	70±2.8	$P=0.889$
Sex (male)	8 (72.73)	7 (70.00)	$P=1.000$
Smoking	7 (63.64)	5 (50.00)	$P=0.670$
Complications	6 (54.55)	9 (90.00)	$P=0.149$
Body temperature ≤ 37.3 C	10 (90.91)	8 (80.00)	$P=0.587$
Body temperature > 37.3 C	1 (9.09)	2 (20.00)	
White blood cell count ($>10 \times 10^9/L$)	2 (18.18)	1 (10.00)	$P=1.000$
White blood cell count ($\leq 10 \times 10^9/L$)	9 (81.82)	9 (90.00)	
Neutrophil count ($10^9/L$)	5.60±0.86	4.91±0.55	$P=0.130$
Lymphocyte count ($10^9/L$)	1.62±0.32	1.70±0.19	$P=0.839$
Eosinophils ($10^9/L$)	0.14±0.09	0.16±0.06	$P=0.887$
Hemoglobin (g/L)	127±4.6	120±5.7	$P=0.349$
ESR(mm/h)	33±8.0	36±13.0	$P=0.710$
CRP (mg/L)	17.06±7.15	18.13±5.02	$P=0.906$
Procalcitonin (ng/mL)	0.05±0.02	0.07±0.03	$P=0.649$
D- two dimer (ug/L)	0.67±0.16	0.96±0.25	$P=0.197$
ALT (U/L)	20±4.9	23±6.9	$P=0.447$
LDH (U/L)	281±60.1	271±40.1	$P=0.889$
PH	7.42±0.01	7.39±0.01	$P=0.169$
PaO ₂ (mmHg)	89.6±6.7	92.4±8.8	$P=0.802$
PaCO ₂ (mmHg)	45.3±4.1	48.4±2.5	$P=0.552$
SAO ₂ (%)	96.0±0.7	96.2±0.8	$P=0.894$
PHT (mmHg)	37±6.5	45±7.5	$P=0.306$
HOD (d)	10±0.9	10±1.1	$P=0.844$
Days of antibiotic use (d)	12±3.2	11±3.6	$P=0.532$
Mortality (individual)	0 (0.00)	0 (0.00)	$P=1.000$

Note: $P < 0.05$ indicates that there is a significant difference between the two groups, with statistical significance; the above values are expressed by mean $\pm$ standard deviation or counting (percentage), using t-test and Fisher's exact probability method.

Table 2. Alpha Diversity Analysis of All Samples at 97% Similarity

Sample number	chao	ace	shannon	simpson
RA1	134.5	136.20961	2.431979	0.136777
RA2	236.857143	240.663379	3.513141	0.057304
RA3	124.5	125.392	3.10924	0.098695
RA4	135.866667	132.962317	2.549182	0.122363
RA5	163.857143	155.244874	3.129914	0.099724
RA6	161.25	158.678073	2.347577	0.202257
RA7	166	166.163767	3.45852	0.064706
RA8	83	83.373446	2.365371	0.164682
RA9	195.2	169.37627	1.94734	0.28224
RA10	143.071429	143.739587	1.90196	0.415508
RA11	136.153846	133.412765	2.07753	0.239247
A1	103	115.249581	1.930024	0.318995
A2	144.315789	157.026115	2.426431	0.145361
A3	121.909091	150.177887	1.22708	0.560757
A4	144.857143	145.370895	2.935694	0.115315
A5	59.111111	63.879509	0.832303	0.676633
A6	187.5	188.156065	3.493712	0.055526
A7	226.882353	221.113872	2.634352	0.257711
A8	180.071429	200.469099	3.114787	0.083656
A9	159.111111	158.687289	3.033778	0.148011
A10	201.076923	205.599802	2.570417	0.168522

Note: RA1-RA11 was rhinovirus positive AECOPD group, A1-A10 was virus negative AECOPD group.

Figure 1. Comparison of average OTUs between rhinovirus-positive AECOPD and virus-negative AECOPD

Note: $P < 0.05$ indicates that there is statistical significance.

Abbreviations: AECOPD, acute exacerbation of chronic obstructive pulmonary disease; OTU, operational taxon; RhV, rhinovirus.

At the generic level, 160 genera were found in sputum samples, 127 genera were found in RhV positive group samples, and 145 genera were found in virus-negative group samples (see Figure 3b). Of these genera, 112 genera were shared among the two groups. Seven genera (*Streptococcus*, *Puccinia*, *Neisseria*, *Chitinophaga*, *Oligotrophomonas*, *Veronococcus* and *Ciliarium*) comprised about 69.20% of RhV positive samples and 52.67% of sputum microorganisms in virus-negative samples. A total of 20 genera (including the seven genera mentioned above and *Streptococcus granulosus*, *Staphylococcus*, *Haemophilus*, *Roxiella*, *Clostridium*, *Staphylococcus*, *Carbonophilus*, *Lautropia*, *Sediminibacterium*,

Figure 2. Bray-Curtis Cluster Tree of Subjects

Note: RA1-RA11 was rhinovirus positive AECOPD group, A1-A10 was virus negative AECOPD group.

Figure 3. Abundance and prevalence of different phyla (A), genus (B) and species (C) in 21 samples

Note: RA1-RA11 was rhinovirus positive AECOPD group, A1-A10 was virus negative AECOPD group.

Oribacterium, Acinetobacter, Delftia, Campylobacter) constitute RhV positive. There were about 87.51% microorganisms in the group and 66.36% in the virus negative group. Fifteen genera were detected only in RhV positive group, accounting for a relatively low abundance of microorganisms (about 0.102%) and 33 genera were detected only in virus-negative group, accounting for a relatively low abundance of microorganisms (about 0.272%).

At the species level, a total of 62 species were present, 50 species were found in RhV positive group samples and 58 species were found in virus-negative group samples (see Figure 3c). In these species, 46 species are shared in the two groups. Four genera were detected only in RhV positive group, which were ornithine-eating bacteriophage, anaerobic bacillus, Corynebacterium and Flavobacterium, accounting for the relatively low abundance of microorganisms (about 0.130%). Twelve species were detected only in virus-negative group, which were arthrobacterium, bacteroides, Butyrivibrio, desulfurizing bacterium and purple bacillus.

Fragrant bacteria, psychrophilic bacteria, radish bacteria, rumen coccus-bromii, rumen coccus-flarefaciens, rumen coccus-gnavus and Streptococcus accounted for the relatively low abundance of microorganisms (about 0.151%).

Bacterial taxonomic analysis and comparison.

Samples of microbes from rhinovirus positive group and virus negative group were also compared. Among all taxa, analysis at different taxonomic levels showed that the microorganisms in positive rhinovirus group were significantly higher than those in negative rhinovirus group ($P < 0.05$): actinomycetes at the phylum level, actinomycetes at class level, actinomycetes and Neisseria at the order level, Corynebacteriaceae and Micrococcus at discipline level. Neisseria and Corynebacteriaceae at the generic level, Neisseria and Rochella at the species level, Rochella mucinosa at the species level; microorganisms in the rhinovirus-positive group were significantly lower than those in the virus-negative group ($P < 0.05$): Bdellovibrio at order level, Vibrio bacteriophage and Fibrobacter at family level, and at generic level. It belongs to non-myxotropic fungi, bacteria, radish fungi and bacillus, and at the species level it belongs to radish fungi (see Fig. 4).

Discussion

Timely and accurate detection of bacterial spectrum within the lower respiratory tract is for understanding the interaction between viruses and bacteria in AECOPD and to prevent and treat secondary bacterial infections after virus infection. Early research

Figure 4. Average bacterial abundance with significant differences between RhV+AECOPD and Virus-AECOPD patients

Note: A-F shows the average abundance of bacteria at different taxonomic levels (A: gate level; B class level; C: order level; D: family level; E: genus level; F: species level); $P < 0.05$ indicates statistical significance.

Abbreviation: AECOPD, acute exacerbation of chronic obstructive pulmonary disease; RhV, rhinovirus.

is mainly based on traditional microbial culture technology, but many bacteria cannot be cultured, it limits identifying bacteria. Compared with the traditional culture methods, the molecular biology methods represented by macro genomics have obvious advantages in detecting bacteria that are difficult to cultivate.⁹

This study explored the effect of rhinovirus on the microbial composition of the lower respiratory tract in COPD patients from a transverse perspective. It was found that there were significant changes in the abundance of many pathogenic and non-pathogenic bacteria after rhinovirus infection. The pathogenic bacteria were *Rochella mucosa*, belonging to the actinomycetes. Huang et al¹⁰ also found that there was a significant increase in actinomycetes in sputum specimens of AECOPD patients compared with stabilizers. Molyneux et al reported³ COPD patients were infected with rhinovirus. The results showed that the number of *Proteus* increased significantly 15 days after rhinovirus infection, and there was significant growth of *Haemophilus*. This suggests that rhinoviruses may cause significant increases in different pathogenic bacteria in the respiratory tract, but whether they are

specific to rhinovirus infection is unknown. Due to the impairment of respiratory immune function in COPD patients, including the removal of damaged mucosal cilia,¹¹ increased epithelial infiltration,¹² dysfunction,¹³ and weakened interferon production,¹⁴ these deficiencies may lead to inefficient bacterial clearance and allow excessive growth of pathogenic bacteria in the community. It also shows that COPD patients are more likely to cause secondary bacterial infection after virus infection than normal people, which increases the difficulty of acute exacerbation treatment of COPD. It was found that the pathophysiology and mechanism of the local immune response in the respiratory tract are virus-specific.¹⁵ A virus-specific bacterial classification may occur in the respiratory tract. The discovery of specific bacterial infections that depend on any kind of virus will be beneficial to the prevention and treatment of secondary bacterial infections of the respiratory tract caused by the virus in the future. However, up to now, no specific bacterial changes corresponding to different viruses have been clearly identified, which still requires us to continue to study and summarize.¹⁶

Rochella mucosa found in this study is Gram-positive coccus, belonging to *Rochella* and *Actinomycetes*. It has the characteristics of coagulase-negative, sporeless, encapsulated in capsule and facultatively anaerobic. It is usually found in the oral cavity and upper respiratory tract,¹⁷⁻¹⁹ and occasionally in the gastrointestinal tract,²⁰ intestinal epithelium,²¹ breast milk,²² and dental plaque.^{23,24} Although *Rochella mucosa* is considered to be a normal microorganism in the oral and upper respiratory tracts, it is associated with a wide range of diseases, such as bacteremia, central nervous system infection, meningitis, peritonitis, osteomyelitis, necrotizing fasciitis of the neck, endophthalmitis, pneumonia and endocarditis.²⁵⁻³⁰ More and more of them are recognized as opportunistic pathogens, most of which affect the host³¹ of immunodeficiency. Clinical studies have found that single beta-lactam antibiotics or vancomycin or combined with other antibiotics can successfully treat infection caused by *Rochella mucosa*.³² In this study, the local lung defense barrier of COPD patients is damaged, which may provide favorable conditions for the colonization of *Rochella mucosa*.³¹ Rhinovirus infection aggravates the damage of the barrier and promotes the change of local environment, which may be more conducive to the colonization of the bacteria and eventually lead to secondary bacteria following lower respiratory tract virus infected.³³⁻³⁴

Figure 5. Venn diagram of common/unique OTUs in two groups

Note: The interior of each circle symbolically represents the number of OTUs observed in a group; the overlap area will represent the set of OTUs that occur together in the corresponding group; similarly, the single-layer region represents the unique number of OTUs found in a group.

Abbreviation: OTU, Operational Classification Unit.

In this study, the variable V4 region of prokaryotic 16S ribosomal DNA (16S rDNA) gene was sequenced on Illumina MiSeq platform by double exponential two-terminal sequencing.

The advantage of this sequencing is that its fusion primers are designed, including suitable P5 and P7 Illumina assembly sequences, an 8nt index sequence and gene-specific primers, which can reduce the cost of long-term customization of primers. In addition, this method can produce a large number of high-quality sequences, which can describe the characteristics of bacterial community composition in the respiratory tract more broadly, and discover the existence of potentially difficult-to-cultivate low-abundance microorganisms. The disadvantage is that the measured microbial data may only reflect the DNA fragments of bacteria rather than live bacteria, which may lead to the data containing partially dead bacteria, and then affect the results of sequencing.¹³

One limitation of this study is that intensive microbiological analysis is performed only in a small number of COPD patient samples from 21 sources. Sequencing results do not fully reflect the changes in the composition of common respiratory microflora caused by rhinovirus infection in COPD population. Another limitation was that the upper and lower respiratory tracts were not tested to see if the microbial changes caused by rhinovirus infection were consistent. Our strengths include controlled rhinovirus research, horizontal research and the use of high-throughput sequencing techniques.

In conclusion, this study investigated the composition of bacterial microorganisms in the lower respiratory tract of patients with rhinovirus-positive AECOPD and virus-negative AECOPD from a transverse point of view by double-index double-end sequencing of the variable V4 region of 16S rDNA gene. It was found that there was no significant difference in microbial diversity between the two groups, but there was a significant difference in community structure. We observed a significant difference in actinomycetes between the two groups, and a significant increase in the abundance of *Rochella mucosa*. Through this sequencing method, we can find the bacterial microorganisms with higher specificity in patients with COPD infected by rhinovirus, and provide guidance for clinical prevention and treatment of secondary bacterial infections.

Conflict of interest statement

The authors declare they have no conflicts of interest relevant to this manuscript.

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Ethical approval

Written informed consent was obtained from the patient for publication of this paper. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

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