Analysis of airway microbiota in adults from a Brazilian cystic fibrosis center

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Abstract

The application of next-generation sequencing tools revealed that the cystic fibrosis respiratory tract is a polymicrobial environment. We have characterized the airway bacterial microbiota of five adult patients with cystic fibrosis during a 14-month period by 16S rRNA tag sequencing using the Illumina technology. Microbial diversity, estimated by the Shannon index, varied among patient samples collected throughout the follow-up period. The beta diversity analysis revealed that the composition of the airway microbiota was highly specific for each patient, showing little variation among the samples of each patient analyzed over time. The composition of the bacterial microbiota did not reveal any emerging pathogen predictor of pulmonary disease in cystic fibrosis or of its unfavorable clinical progress, except for unveiling the presence of anaerobic microorganisms, even without any established clinical association. Our results could potentially help us to translate and develop strategies in response to the pathobiology of this disease, particularly because it represents an innovative approach for CF centers in Brazil.

Keywords Cystic fibrosis · Airway microbiota · Next-generation sequencing

Introduction

Chronic infection of the lower airways is the major cause of morbidity and reduced survival in cystic fibrosis (CF) patients. The progression of pulmonary disease is associated with a complex respiratory microbiota that experiences an increased worsening and succession of the infecting microorganisms [1]. Bacterial infections in the airways of these patients should be monitored not only to follow disease progression but also

for therapeutic interventions that may improve the quality of life and life expectancy of the patient [2, 3].

Traditional culture-based microbiological methods demonstrate the clinical value of infections caused by “typical CF pathogens”, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex, *Achromobacter* spp., and *Stenotrophomonas maltophilia*, as well as the referred antibiotic therapy that is chosen to target these microorganisms [4]. Cross-sectional and longitudinal studies using culture-independent methods have shown that the CF microbiome is highly diverse and represents an entangled microbial ecosystem that exceeds the complexity that was previously known based on culture-based methods [5–7]. The diversity and richness of the CF microbial community may be influenced by several factors including the age and gender of the patient, CFTR mutation type, antibiotic exposure, environmental factors, and severity of the lung disease [5–9].

In addition to the traditional isolated bacterial species documented as CF pathogens, studies employing next-generation sequencing showed complex microbial ecosystems in CF respiratory samples, including undescribed and uncultivable bacteria [10]. The diversity of the microbiota and the relative amount of each taxon have been associated with the severity

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of the disease in CF, with lower diversity being associated with the worst prognosis and increased airway inflammation [11]. Changes in the lung microbiome may be important in the pathophysiology of the pulmonary infection [12].

The structure and composition of the pulmonary microbiota in adults with CF exhibit decreased bacterial diversity than that of young patients, which possibly correlates with the progressive loss of the lung function and the emergence of dominant species such as *P. aeruginosa*, *B. cepacia* complex, and *Achromobacter* spp. [6, 13]. Besides that, several studies point out that the airways of individuals with CF present only a small number of taxa in common, demonstrating a high degree of interindividual variability in the structure and composition of the microbial community [14–16]. Understanding the changes in the microbiome over time is extremely relevant for this disease, especially in the search for a biomarker associated with an unfavorable prognosis of the clinical evolution, which could contribute to a more targeted and individualized treatment [17–21].

In this work, we examine longitudinally the airway bacterial microbiota from adult CF patients by 16S rRNA tag sequencing from DNA extracted from respiratory specimens, using Illumina technology. We verify changes in the diversity and abundance and describe the clinical evolution aspects of the CF patients. This study represents an innovative approach to CF in Brazil, with the purpose of describing the bacterial microbiota in respiratory samples using metagenomic techniques.

Methods

Patients, clinical samples, and medical record review

A total of five patients regularly monitored at the Policlínica Piquet Carneiro/Pedro Ernesto University Hospital/State University of Rio de Janeiro (PPC-HUPE-UERJ), a reference center for adult patients in Rio de Janeiro, Brazil, were selected for the airway analysis using the criteria of having at least four consecutive sampling points between June 2014 and July 2015. During this period, sputum samples and clinical demographic data were collected according to the scheduled visits, following the clinical follow-up protocol. This study was approved by the Ethics Committee of the Pedro Ernesto University Hospital: CAAE 78893515.1.0000.5259.

Demographic information was obtained from routine medical appointments as well as the genetic profile with respect to the F508del mutation, records of the presence of exocrine pancreatic insufficiency (PI), diabetes-related cystic fibrosis (DRCF), and liver disease. Regarding pulmonary function data, the predicted values for age, sex, and height of forced expiratory volume (FEV₁), obtained by spirometry, were recorded. The best FEV₁% values of each available exam were

selected. The results were based on predicted values of the lung function for the Brazilian population [22, 23]. According to the FEV₁ 1% value, the degree of pulmonary obstruction was classified as mild or normal (FEV₁ > 70%), moderate (FEV₁ > 40–70%), or severe obstruction (FEV₁ ≤ 40%), according to the classification used in the CF records [24, 25]. Another disease severity indicator used was the number of hospitalizations during the study period.

DNA extraction from sputum samples, 16S rRNA tag amplification and sequencing, and microbiota analysis

Samples were fluidized in 2% *N*-acetyl-L-cysteine solution, treated with 2% mercaptoethanol, concentrated by centrifugation for 15 min at 10 °C at 4000 rpm and the pellet was frozen at – 80 °C. DNA was extracted with the Nucleospin Tissue kit (Macherey-Nagel, Germany). For the pre-lysis, 180 µL of lysozyme-tris-EDTA-triton solution was added to the pellets. This material was then heated at 56 °C for 1 h and 30 min after which 25 µL of proteinase K were added. Subsequently, the material was incubated at 56 °C for 3 h until complete lysis. The samples were then submitted to DNA extraction according to the manufacturer's instructions. The DNA was eluted with 50 µL of sterile deionized water and the concentrations were determined with a fluorescent dye on a Qubit fluorometric quantification instrument (Thermo Fisher Scientific, Brazil).

The extracted DNA was used to amplify by PCR the V4 variable region of the 16S rRNA gene, according to Caporaso et al. (2011) [26]. The amplification reaction was done in 20 µL with the enzyme Taq AccuPrime DNA Polymerase High Fidelity (Invitrogen—Thermo Fisher, Brazil). PCR consisted of an initial cycle of 95 °C for 5 min followed by 25 cycles of 95 °C for 20 s, 50 °C for 30 s, and 68 °C for 1 min. A final cycle of 68 °C for 10 min completed the program. The PCR products were purified with the AMPure XP, PCR Purification system (Beckman Coulter) and quantified by fluorometry (Qubit, Thermo Fisher Scientific). Three single PCR reactions for each sputum sample were performed which, after purification, were combined. Samples were then pooled and sequenced on a MiSeq instrument in a 500-cycle MiSeq reagent V2 kit (Illumina, San Diego, California, USA).

The fastq files were processed with MOTHUR, version 1.35.1 following the MiSeq SOP recommendations [27]. Briefly, paired-end reads were joined using the make contigs command and filtered to exclude ambiguities (max = 0), homopolymers (maxhomop = 8), sequences over 252 bp, and to ensure correct overlapping. Then, they were aligned against the Silva database—release 128 [28], pre-clustered (diffs = 2), and screened for chimeras, using the vsearch algorithm [29]. Screened sequences were classified, and those belonging to

undesired taxa such as chloroplast, mitochondria, and Eukarya were removed. To build operational taxonomic units (OTUs), clustering was set at 97% similarity. For taxonomic description, OTUs with relative abundance lower than 1% of the total number of sequences were grouped as others. Alpha and beta diversity analysis were calculated after subsampling groups to the smallest library size. Results were plotted using GraphPad Prism v7 software (GraphPad, USA).

Results

Patient, samples collected, and medical record review

In total, 25 sputum samples collected over a 14-month period (June 2014 to July 2015) from five adult patients with CF were analyzed. The number of sputum samples per patient ranged from 4 to 6, and the time frame for sample collection per patient ranged from 287 to 378 days (average 347 days). Samples were collected according to the Laboratory Standards for Processing Microbiological Samples from People with CF from The UK Cystic Fibrosis Trust [30].

Among the five patients, three were male and two were female. The age of participants at the onset of the study varied from 18 to 32 years. One patient was homozygous and two were heterozygous for the F508del mutation, while the others presented different mutations. All patients showed pancreatic deficiency and two of them had diabetes. The number of hospitalizations varied from none to five. The FEV₁ ranged from 22.7 to 44.4% (Table 1).

Analysis of the airway microbiota and medical record

A summary of the taxonomic assignments was used to visualize changes in the microbiota over the course of the study. These community-wide profiles displayed unique features for each patient, as seen in Figs. 1 and 2 at the phylum and genus level, respectively.

The Shannon diversity index was used to illustrate the variation in the microbiota composition among the different patients and their respective samples. This index combines the measure of abundance and evenness, therefore being adequate for inter-patient comparison. Figure 3 shows that the lowest diversity is exhibited by patient C, the one for which the microbiota is constantly dominated by *Staphylococcus* (Fig. 2). For patients A and C, the microbiota diversity is quite stable, while for patients B, D, and E there was at least one sample in which a low diversity was observed.

Using the Bray-Curtis dissimilarity metric, we analyzed the community composition among the different sputum samples collected from the 5 patients by means of a

principal coordinate analysis (PCoA). Figure 4 shows that the bacterial communities observed in the samples from patients A, B, and C are clearly distinct from each other and from patients D and E. Furthermore, the sputum samples collected at different time-points for patients A, B, and C present a similar community composition. Samples from patient D also form a distinct cluster, with the exception of sample D2, which is located in the PCoA plot between two samples from patient E. Sample D2 differs from the others collected from this patient since it presents a high abundance of *Staphylococcus* (Fig. 2). Samples from patient E had more variability since they are spread through a larger area in the plot. Samples E5, E1, and E2 are close together and show a high abundance of *Staphylococcus*, while samples E4, E3, and E6 show significant compositional differences and are, therefore, more widely spread in the plot (Fig. 4). Notably, these samples show a high abundance of members of the *Alcaligenaceae* (Fig. 2).

Regarding the clinical status of patient A, the BMI was maintained in the normal range and the FEV₁ did not worsen, with a slight improvement over the study period. An episode of exacerbation of the mild pulmonary disease occurred in the final collection, with no need for hospitalization (Table 1). As previously shown, the microbiota was quite stable regarding the diversity between samples (Fig. 3), presenting *Haemophilus* and *Fusobacterium* as the predominant genera during the study (Fig. 2).

Patient B maintained his BMI classified as underweight and presented FEV₁ characterized by severe obstruction maintained throughout the study. At collection dates, there were no episodes of exacerbation, but there were three admissions in this period in his home city (Table 1). In the same period, DRFC was diagnosed and recurrent hemoptysis was observed. This patient presented *Burkholderia* as the dominant genus, but in the third collection, performed after the period of hospitalization and treatment with venous antimicrobials, a higher community diversity and a reduction in the abundance of OTUs assigned to the genus *Burkholderia* was observed (Fig. 2).

Patient C maintained the BMI in the normal range and presented three episodes of pulmonary exacerbation (second, third, and fourth collections), without hospitalization (Table 1). Pulmonary function was characterized by moderate obstruction. The microbiota was dominated by the genus *Staphylococcus* (Fig. 2) throughout the study and the diversity indexes of this patient were the lowest among the other ones (Fig. 3).

Patient D maintained the BMI (characterized as underweight) and presented three episodes of exacerbation (second, third, and fourth collections) and was hospitalized once throughout the study. The patient presented general clinical worsening in the second collection and started to receive oral antimicrobial treatment. In the following consultations (third

Table 1 Patient information and medical review

Patients (gender)	Birth date (month/year)	CFTR mutation 1	CFTR mutation 2	Sample and collection dates	BMI	Exacerbated	Antimicrobials	FEV ₁ (%)	Number of hospitalizations 2014/2015	DRCF	Pancreatic insufficiency
Patient A (Female)	May/1991	G542X	NA	06/06/2014 (A1)	21.77	No	No	37.8	0/0	No	Yes
				05/09/2014 (A2)	20.03	No	No	39.1			
				12/12/2014 (A3)	21.50	No	No	42			
				13/03/2015 (A4)	21.50	No	No	44.4			
				19/06/2015 (A5)	21.01	Yes	Yes (CIP OD)	NA			
Patient B (Male)	Jun/1982	G85E	G85E	27/06/2014 (B1)	17.66	No	No	NA	1/1	Yes	Yes
				26/09/2014 (B2)	17.17	No	No	19.1			
				09/01/2015 (B3)	17.41	No	No	23.6			
				10/07/2015 (B4)	16.8	No	No	NA			
				18/07/2014 (C1)	23.11	No	No	NA	0/0	No	Yes
Patient C (Male)	Dec/1988	delF508	delF508	05/09/2014 (C2)	ND	Yes ^a	Yes	NA			
				17/10/2014 (C3)	22.79	Yes	No	43			
				23/01/2015 (C4)	23.8	Yes	Yes (LVX OD)	NA			
				29/05/2015 (C5)	24.4	No	No	51.1			
				13/06/2014 (D1)	17.86	No	No	NA	0/1	No	Yes
Patient D (Female)	Jun/1981	delF508	R334W	12/09/2014 (D2)	17.56	Yes	Yes (CIP OD)	66.1			
				01/12/2014 (D3)	16.96	Yes	Yes (CIP OD + SXT OD)	66.7			
				20/02/2015 (D4)	17.21	Yes	Yes (CAZ IV, AMK IV, OXA IV)	65.4			
				27/03/2015 (D5)	NA	No ^b	No	64.2			
				27/06/2014 (E1)	NA	Yes	NA	NA	2/3	Yes	Yes
Patient E (Male)	Sep/1995	delF508	3120 + G-A	18/07/2014 (E2)	NA	Yes	Yes (MEM IV, AMK IV, SXT IV)	NA			
				26/09/2014 (E3)	14.95	Yes	No	29			
				09/01/2015 (E4)	14.80	No	No	NA			
				10/04/2015 (E5)	14.03	Yes	NA	NA			
				10/07/2015 (E6)	16.0	No	NA	22.7			

BMI body mass index/DRCF diabetes-related cystic fibrosis/FEV₁ forced expiratory volume in 1 s

ND not determined/NA not available

CIP ciprofloxacin/LVX levofloxacin/AMK amikacin/OXA oxacillin/CAZ ceftazidime/SXT trimethoprim-sulfamethoxazole

OD oral dose/IV intravenous

^a Patient C—hospitalized in august in a private clinic with BMI not available

^b Patient D—exams collected after hospital discharge. Exacerbation: clinical deterioration (Fuchs criteria), with oral or intravenous antimicrobial prescription

Fig. 1 Relative abundance of bacterial phyla detected in sputum samples collected from five different patients assessed by 16S rRNA V4 tag sequencing using Illumina technology. The colored segments of each bar represent the proportion of OTUs assigned to different bacterial phyla

and fourth visits), the patient presented little improvement, still with symptoms of exacerbation, and was admitted for intravenous medication (ceftazidime, amikacin, and oxacillin). The presence of the genus *Pseudomonas* in high relative abundance was constant during the study, except for the second sample, in which *Staphylococcus* was more abundant (Fig. 2).

Patient E was characterized as in a severe condition and in the year 2014 he had been transferred from the pediatric center at the Fernandes Figueira Institute (IFF-FioCruz) to the Policlínica Piquet Carneiro/Pedro Ernesto University Hospital (PPC-HUPE), a reference center for adult CF patients. The patient was characterized as presenting severe malnutrition, having maintained the same BMI throughout the study. There was a drop in FEV₁, with episodes of exacerbation in five of the six collection dates (Table 1) and five hospital admissions. The patient died on Feb 5th, 2016. Patient E showed genus *Staphylococcus* as the most abundant in samples 1, 2, and 5 (Fig. 2).

Discussion

We describe the fluctuations in the lower airway microbiota of five adult CF patients using a 14-month follow-up period. The total bacterial microbiota was identified, and multiple taxa were detected in different abundances in the 25 samples of the five patients investigated. In general, several common pathogens (*Pseudomonas*, *Staphylococcus*, *Burkholderia*, *Haemophilus*) and rare (*Rothia*, *Streptococcus*, *Neisseria*, *Gemella*) pathogens, as well as anaerobes, were identified. Analysis of changes in the microbial community showed that the community-wide profiles display unique features for each patient as observed by studies conducted in the UK [31] and the USA [32, 33].

We highlight the presence of anaerobes in the microbiota of four of the five patients (occurring in most of the samples from patients A, B, D and E), being represented by the genera *Veillonella*, *Prevotella*, and *Fusobacterium*. Although the role of anaerobes in pulmonary disease in CF remains unclear,

Fig. 2 Relative abundance of bacterial genera detected in sputum samples collected from five different patients assessed by 16S rRNA V4 tag sequencing using Illumina technology. The colored segments of each bar represent the proportion of OTUs assigned to different 20 most abundant bacterial genera present in the samples in a higher than 1% occurrence

Fig. 3 Shannon index showing diversity, driven by community richness (number of OTUs detected) and community evenness (relative abundance of OTUs). Each bar represents a patient sample collected over the study period (patients A, B, C, D, and E)

some studies showed that this group contributes to the transference of antibiotic resistance genes between microorganisms—while producing quorum sensing molecules that regulate the interspecies mediation pathways [34], and also attributed anaerobes to influence virulence characteristics in pathogens such as *P. aeruginosa* [35] or as potential predictive biomarkers in CF pulmonary infections such as for *Porphyrromonas catoniae* [1].

Here, the airway microbiota of patients with CF revealed a high difference between patients and not many variations among the samples collected from each patient over time. In addition, we did not find coincidence between the longitudinally examined samples, their microbial compositions, and the clinical data (antibiotic use, spirometry, clinical symptoms, stability periods, and episodes of exacerbation of the lung function episodes). There were no specific changes in the bacterial pattern that could predict the severity or the improvement of an exacerbation. Whelan et al. 2017 [10] found no correlation whatsoever in similar studies when samples were

examined longitudinally upon exacerbation onset, by using the Illumina MiSeq platform. They concluded that the exacerbation was not consistently associated with community-wide changes in the microbiome, nor with sample diversity. The association between chronic lung colonization and clinical status has been studied without a clear conclusion [36–38].

The follow-up time of this study—approximately 1 year—may have contributed to the lack of greater baseline data for the longitudinal analysis. Another possible explanation for the low correlation between the microbiome of CF sputum samples and the clinical course of the disease was the fact that the disease is driven by mucosal events that may be reflected slightly on the sputum, or that the activity of the microbiome changes independently from the bacterial load, being affected by other events, such as the expression of virulence factors, for example [39].

It is expected that certain differences between periods of clinical stability, exacerbation, and advanced disease might be found; however, we know little about the causal relationship

Fig. 4 PcoA plot of Bray-Curtis dissimilarity for bacterial communities from samples from the five patients collected at different time-points. Red circles = patient A; brown circles = patient B; orange circles = patient C; green circles = patient D; blue circles = patient E

between these modifications and the exacerbations. This lack of knowledge impairs the clinical applicability of such findings. It is important to highlight that, although we have some clinical data that show the evolution of patients, the study had a descriptive approach. Nevertheless, the airway microbiome information obtained from our group of patients was critical in the clinical decision-making about one of the individuals (patient D) whose clinical picture presented significant worsening without an explainable cause by the conventional microbiology laboratory methods. After discarding the possibility of an infection by an emergent microorganism, the NGS results that were concomitantly available at the clinical follow up indicated an increase in the relative abundance of *Pseudomonas* from 32% (during stability) to 85% (at exacerbation). This information was essential for decision-making, prompting clinicians to changes in the antimicrobial regimen, with an important clinical improvement in the following 8-month period [40].

Our goal is to provide data for our local reference center to cooperate with the clinical monitoring of CF airway infections. The data of microbiome itself should be interpreted with care. During the period of data analysis, we could provide some insights for clinical decision. Patient B, for example, have been followed clinically and microbiologically for 26 years. His previous history showed a chronic infection with *B. vietnamiensis*, but he has been clinically stable for the last 16 years with recent episodes of clinical deterioration. The genus *Burkholderia* was predominantly present in the microbiota of all samples analyzed by NGS in the present study, which was in concordance with the presence of *B. vietnamiensis* found by culture-dependent methods in the clinical laboratory. As described in a case report [41], the worsening of the prognosis was attributed to DRCF with the outcome of this patient resulting in death.

In the comparison of the pulmonary microbiome between individuals, we observed, as well as the studies by Kramer et al. (2015) [42], Coburn et al. (2016) [43], and Whelan et al. (2017) [10], the formation of highly specific patient groups, indicating that the pulmonary microbiota is unique from patient to patient. Likewise, these authors demonstrated that the CF pulmonary microbiota is prone to be more influenced by factors intrinsic to the individual than by other causes, making it necessary to be investigated by this using approach. Our results indicated that airway microbiome in CF is patient-specific.

In our study, the NGS did not reveal the presence of possible emerging pathogens that are predictors of pulmonary disease in CF or of its unfavorable clinical progress, except for revealing the presence of anaerobic microorganisms, even without any established clinical association. No coincidence was found between the findings of the microbiome and the clinical demographics of patients obtained during the follow-up period of this study. The number and length of sampling, together with clinical data granularity, may have been limiting

factors for obtaining more sound data about the correspondence between intra and interpatient variation data in microbiota composition and the clinical data correspondence. However, this work emphasizes the potential of this technique for the clinical approach as it was observed for patient D.

This is the first study to use the NGS sequencing method to describe the diversity and abundance of the airway microbiota from an adult CF center in Brazil. Besides its value on incorporating knowledge and concepts to current local reference clinical microbiology laboratories, the major relevance of this study is to be a precursor in the use of microbiological analysis of airway CF microbiome in our country.

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Compliance with ethical standards

This study was approved by the Ethics Committee of the Pedro Ernesto University Hospital: CAAE 78893515.1.0000.5259.

Conflict of interest The authors declare that they have no conflict of interest.

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