Journal of Cystic Fibrosis 13 (2014) 645 – 652

www.elsevier.com/locate/jcf

Original Article

An exploratory study of microbial diversity in sinus infections of cystic fibrosis patients by molecular methods

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Received 10 October 2013; received in revised form 16 January 2014; accepted 14 February 2014
Available online 11 March 2014

Abstract

Background: For the first time microorganisms in CF sinuses are investigated by molecular methods in response to an absence of anaerobes in CF sinus samples during a two-year period at the Copenhagen CF center.
Methods: Endoscopic sinus surgery was performed in 19 CF patients. DNA from intact bacterial cells was investigated by 16S rRNA gene analysis and quantitative PCR. Results were compared to culture-dependent routine diagnosis.
Results: Molecular methods showed a large microbial diversity, which included undetected anaerobes that may play a pathogenic role. Importantly, the culture methods did not always detect known CF pathogens. Quantitative PCR showed generally a higher abundance of classic CF pathogens e.g. *Pseudomonas aeruginosa* and *Staphylococcus aureus* compared with the anaerobe *Propionibacterium acnes*.
Conclusions: The results indicate that the culture methods in some cases may not be suitable as stand-alone method for this patient group, as diversity may be underestimated and important species undetected.

Keywords: Anaerobes; Sinuses; Culture; Molecular methods

1. Introduction

Sinus infections are a commonly occurring condition afflicting up to 15% of the otherwise healthy European and American population [1]. Cystic fibrosis (CF) patients are more susceptible to infections and prone to chronic sinus infections [1], due to their increased mucus viscosity and decreased mucociliary clearance [2,3]. Consequently, most CF patients are thought to have bacteria in their sinuses [4]. The microorganisms in CF sinuses may be important treatment targets in order to improve quality of life and life expectancy of patients [5], since recent studies have suggested that sinuses serve as a reservoir for colonization and infection of the lower airways [2,6–8]. However transport from the lower airways to the sinuses may also occur. Despite the possible importance of CF sinus infections, CF research has mainly focused on chronic lung infections.

The initial motivation for this study was an absence of anaerobes as detected by culture methods in sinus samples obtained from patients undergoing sinus surgery during a two year period, which lead to a termination of anaerobic testing for this patient group. However, the knowledge from non-CF patients suggests that chronicity of sinus infections is linked to increased rates of anaerobes (as well as *Staphylococcus aureus* and Gram negative rods) [1,9]. Since CF patients have significantly lower

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http://dx.doi.org/10.1016/j.jcf.2014.02.008

oxygen tension in their sinuses compared to non-CF patients [10], it seems likely that anaerobes should be present in CF sinuses. The lack of detection of anaerobes therefore raised the question whether potentially important microorganisms might be missed by culture-dependent routine diagnosis resulting in inappropriate therapy against these infections.

In the study samples obtained by image-guided functional endoscopic sinus surgery (FESS) [4] were investigated by culture-independent molecular methods including construction of broad-range 16S rRNA gene clone libraries and species-specific quantitative real-time polymerase chain reaction (qPCR). 16S rRNA gene analysis has previously been used to investigate the bacterial flora in the sinuses of non-CF patients [9,13,14], but this study is the first to apply this method to CF sinus samples.

2. Methods

2.1. Patients and treatment

19 CF patients undergoing sinus surgery at the Department of Otorhinolaryngology, Head and Neck Surgery and Audiology (Rigshospitalet, Copenhagen, Denmark) were included in the study, 10 females and 9 males (age = 6–45 years, median = 22 years) (Table 1). All patients were followed at the Copenhagen CF Centre in Denmark by monthly visits to the outpatient clinic and were selected for surgery based on the criteria described in [4]. All patients were prescribed oral azithromycin, and none received IV antibiotics within 4 weeks prior to sinus surgery.

The study was approved by the local ethics committee (H-A-2008-141). All patients gave informed consent; for patients <18 years of age a consent was also obtained from their parents.

2.2. Surgical procedure and routine diagnosis

Surgery was performed as an extended FESS, as described previously [4]. Briefly, visible intramucosal abscess-looking structures were resected along with swollen and inflamed tissue when accessible. No local disinfectants were used in the nose. Samples for molecular investigations were obtained collectively with samples for culture-dependent routine diagnosis from the same sample site (typically the right maxillary sinus) and divided after mixing the sample material, which always consisted of tissue and secretions. Routine diagnosis was performed under aerobe conditions as described previously [4]. The samples for molecular investigations were stored in glycerol and kept at −80 °C for subsequent DNA extraction.

2.3. DNA extraction

DNA from intact bacterial cells was extracted from all 19 samples using MolYsis Basic (Molzym, Bremen, Germany) whereby human and naked microbial DNA was degraded prior to DNA extraction using the FastDNA® SPIN Kit for Soil (MP Biomedicals, France). The DNA was eluted in 75 μL DEPC-treated water.

2.4. Construction and analysis of clone libraries

Clone libraries were constructed for the individual samples and analyzed as described previously [15]. Briefly, cloned fragments of 16S rRNA genes were sequenced by Macrogen Inc. (Korea) using the M13F primer. The sequences were subjected to manual refinement, screening for abnormalities, alignment and taxonomic lineage assignment. Sequences were identified as originating from the same type of microorganism and grouped together if they had more than 97% sequence similarity [16]. One clone from each of these groups was additionally sequenced with the M13R primer to obtain near full-length sequences for phylogenetic analysis using neighbor joining, maximum parsimony and maximum likelihood methods. The phylogenetic analysis was considered robust since the three resulting phylogenetic trees showed congruent phylogenetic relationships (data not shown). The coverage ratio for each clone library was calculated, as described elsewhere [15,16]. The non-redundant, near full-length 16S rRNA gene sequences, representing the types of microorganisms identified in this study, were deposited in GenBank under the accession numbers JQ794610–JQ794658.

2.5. qPCR

The numbers of *Pseudomonas aeruginosa*, *Propionibacterium acnes* and *S. aureus* cells in the samples were measured by applying a variety of hydrolysis probe qPCR assays (Table 2) to the extracted DNA. For each sample triplicate 25 μL reactions were run, as described in [15], except that 2 μL of template DNA was used and the concentration of primers and probes were adjusted (Table 2). All experiments included no-template controls. QPCR cycling conditions were 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, using the Stratagene Mx3000p machine. Quantifications were based on standard curves of 10-fold serial dilutions (10^0 to 10^7 copies μL^{-1}) of synthesized plasmids containing the respective target gene sequences (GenScript, USA), from which the reportable range was determined [17].

2.6. Analysis of quantitative data

The number of gene copies measured by qPCR was converted to number of CFU per gram sample using $CFU/g = C_{measured}/C_{genome} * (V_{total}/V_{used}) / m_{sample}$. Here $C_{measured}$ is the number of copies measured and C_{genome} is the number of gene copies in the genome of one CFU, while V_{total} is the total volume of DNA extract (75 μL) and V_{used} is the volume added to the qPCR reactions (2 μL). M_{sample} is the mass of the initial sample material (in grams).

For samples where more than one bacterium could be quantified, a two tailed T-test was used to provide a hypothesis test of the difference between population means. A statistical value of ≤ 0.05 was considered significant.