Pseudomonas aeruginosa and Periodontal Pathogens in the Oral Cavity and Lungs of Cystic Fibrosis Patients: a Case-Control Study

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Cystic fibrosis (CF) is the most frequent lethal genetic disease in the Caucasian population. Lung destruction is the principal cause of death by chronic *Pseudomonas aeruginosa* colonization. There is a high prevalence of oropharyngeal anaerobic bacteria in sputum of CF patients. This study was carried out due to the lack of results comparing subgingival periodontal pathogenic bacteria between the oral cavity and lungs in patients with CF in relation with *P. aeruginosa* presence. Our first goal was to detect *P. aeruginosa* in oral and sputum samples by culture and molecular methods and to determine clonality of isolates. In addition, subgingival periodontal anaerobic bacteria were searched for in sputum. A cross-sectional pilot case-control study was conducted in the CF Reference Center in Roscoff, France. Ten CF patients with a $\Delta F508$ homozygous mutation (5 chronically colonized [CC] and 5 not colonized [NC]) were enrolled. *P. aeruginosa* was detected in saliva, sputum, and subgingival plaque samples by real-time quantitative PCR (qPCR). Subsequently, periodontal bacteria were also detected and quantified in subgingival plaque and sputum samples by qPCR. In CC patients, *P. aeruginosa* was recovered in saliva and subgingival plaque samples. Sixteen *P. aeruginosa* strains were isolated in saliva and sputum from this group and compared by pulsed-field gel electrophoresis (PFGE). Subgingival periodontal anaerobic bacteria were found in sputum samples. A lower diversity of these species was recovered in the CC patients than in the NC patients. The presence of the same *P. aeruginosa* clonal types in saliva and sputum samples underlines that the oral cavity is a possible reservoir for lung infection.

Cystic fibrosis (CF) is a severe recessive hereditary and lethal disease which is one of the most common among members of the Caucasian population (1). In Brittany (France), the incidence rate is 1:3,268 (2). The causative gene defect, cystic fibrosis transmembrane conductance regulator (CFTR), is located on the long arm of chromosome 7. Dysfunction of CFTR protein causes an imbalance of ion transport leading to hyperviscosity of mucus and a resulting mucociliary dysfunction. The main clinical manifestations involve the respiratory tract, the gastrointestinal tract, the sweat glands, and the genital tracts and are, respectively, an increased susceptibility to infections of the lower airways (LAW), malabsorption and diabetes, salt loss in sweat, and male infertility. In most cases, the severity of lung disease affects the prognosis of the patient. In the bronchopulmonary area, thick mucus and a deficient mucociliary clearance create an environment for bronchial lesions and pathogenic infections (3). Associated inflammation and bacterial infection are responsible for deterioration of the patients. The natural history includes a chronological acquisition of different bacterial species, including *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, with *P. aeruginosa* playing a major role in the morbidity and mortality of patients (4, 5). The prevalence of *P. aeruginosa* colonization increases with patient age (6, 7), and it is found in up to 80% of patients over the age of 18 years (8). The source of this bacterial acquisition in CF patients is unclear. It has been reported in the literature that patients are initially colonized by a single environmental strain that persists for several years. Then, over time, a wide variety of different genotypes are isolated, highlighting the importance of environmental reservoirs (6).

P. aeruginosa possesses several factors, such as biofilm formation (9), development of mucoid phenotype (10), and type III secretion system and quorum sensing (QS)-regulated factors (11),

that contribute to chronic pulmonary colonization. Thus, after intermittent colonization by different strains, persistent colonization by mucoid and biofilm-producing *P. aeruginosa* occurs and thus it becomes hard to eradicate. In fact, it is the biofilm formed by bacterial exopolysaccharides and mucins secreted by the host cells (11, 12) that reduces immune response and renders bacteria resistant to antibiotics (13). Therefore, early detection and treatment of *P. aeruginosa* increase the chances for efficient eradication of this pathogen. Before colonizing the lungs, respiratory pathogens may cross different anatomical sites, such as the nose, the paranasal sinuses, and the oral cavity. Recently, studies demonstrated that *P. aeruginosa* is the most common species isolated from sinuses of CF patients (14, 15). Paranasal sinuses are therefore a source of pulmonary infection (16). However, knowledge concerning the presence of *P. aeruginosa* in the oral cavity of CF patients remains very limited.

Studies of patients with mechanical ventilation in intensive

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care units showed that the oral cavity is an important reservoir of potentially pathogenic bacteria such as *S. aureus*, *P. aeruginosa*, and some *Enterobacteriaceae* (17–19). Dental plaque consists of a complex and dynamic biofilm formed on the supra- and subgingival surfaces of teeth, oral mucosa, and dorsum of the tongue. Over 500 to 700 predominant bacterial species have been detected in the oral microbiome (20). We should mention that approximately 30% to 50% are not yet cultivable and that there is a predominance of facultative and strictly anaerobic Gram-negative bacteria in the subgingival biofilm.

Fourrier and coworkers indicated that bacteria implicated in pneumonia are found in dental plaque, which thereby provides a source of pulmonary infection (21). In addition, the study of the clonal relatedness of strains isolated in plaque or saliva and bronchoalveolar lavage (BAL) fluid of these patients showed that these strains are identical (19). Passage of bacteria from the oral cavity to the lungs may occur by passive aspiration of the bacterial microbiota released in saliva or may be facilitated by the passage of medical devices such as bronchoscopes and endotracheal tubes (22).

CF pulmonary infection is polymicrobial. In fact, chronic *P. aeruginosa* lung infection generates anaerobic conditions within the endobronchial mucus (23) via intense stimulation of oxygen consumption by the accumulated polymorphonuclear leukocytes (24), resulting in zones with ongoing anaerobic metabolism (25). This status promotes the transition of anaerobic bacteria from the oral microbiota to the mucus of the airways. In 2008, Tunney and his team pointed out a higher prevalence of anaerobic bacteria in CF patients than in healthy subjects. These species are identical to those found in the oropharyngeal microbiota, such as *Prevotella* spp., *Actinomyces* spp., and *Veillonella* spp. (26).

They also suggested that anaerobic bacteria as well as *P. aeruginosa* could contribute to pulmonary infection and degradation. In contrast, in CF children who were not colonized (NC), anaerobic bacteria were also detected in the lungs. These data led to the hypothesis that anaerobic bacteria colonize lungs first, producing a favorable environment for the subsequent colonization by *P. aeruginosa* (26). Although the dynamics of colonization by this bacterium are not yet understood, it is interesting to study the relationship between these microorganisms during pulmonary infection. This knowledge will help to adapt treatment to prevent or delay the initial colonization with *P. aeruginosa*.

The main objective of this study was to detect *P. aeruginosa* by culture and quantitative PCR (qPCR) in oral and sputum samples from 10 CF patients. The genetic relatedness of these isolates was analyzed. In addition, the presence of specific periodontal bacteria in the subgingival plaque in sputum samples was explored.

MATERIALS AND METHODS

Study population. A pilot case-control study named MUCORAL was conducted between March and May 2012. The Ouest VI Ethics committee of the Brest University Hospital (France) approved this study. Patients, followed up in the Cystic Fibrosis Reference Center (CRCM) in Roscoff, France, with a $\Delta F508$ homozygous mutation who were more than 12 years old, were able to expectorate, and had not undergone lung transplantation and who (or whose parents) signed an agreement to carry out this study were included in the study. The case group consisted of patients who were chronically colonized (CC) and the control group of patients who were not colonized (NC) (Table 1) according to the criteria of Lee et al. (27). The index of decayed, missing, and filled teeth (DMFT) was measured in all patients to express the total number of teeth or surfaces that are de-

TABLE 1 Demographic data of the CF CC and NC patients

Parameter	Value(s) for indicated patient group	
	CC	NC
Total no. of patients	5	5
No. of females	2	2
No. of males	3	3
Avg age (yrs)	23.8	16.6
Age range (yrs)	16–34	12–27

cayed, missing, or filled in an individual. Scores range from 0 to 28, where 0 represents the absence of decayed, missing, or filled teeth.

Clinical samples. One saliva sample was collected from each patient in the CC and NC groups. Patients refrained from eating, drinking, or brushing their teeth for at least 2 h before samples were taken. From each patient, one sputum sample was obtained on the same day, after oral consultation, during routine chest physiotherapy. Each sputum sample was mixed with an equal volume of dithiothreitol (Digesteur Eurobio, Courtaboeuf, France), and the mixture was incubated at room temperature for 30 min. To ensure there was no contamination of sputum samples with oral bacteria, quality was verified by a preliminary microscopic examination of the Gram-stained sputum specimens according to the recommendations of the French Standard Guidelines in Medical Microbiology (REMIC) (28). Samples were classified based on the presence or absence of squamous epithelial cells (SECs) and white blood cells (leukocytes) (WBCs) under an $\times 10$ lens microscope (class 1, >25 SECs and <10 WBCs; class 2, >25 SECs and 10 to 25 WBCs; class 3, >25 SECs and >25 WBCs; class 4, 10 to 25 SECs and >25 WBCs; class 5, <10 SECs and >25 WBCs). With these criteria, class 1 and 2 samples were significantly contaminated by saliva and classified as poor quality, class 3 and 4 samples were classified as acceptable quality, and class 5 samples were classified as appropriate quality.

Microbiological assessment of periodontal pockets was performed using a commercial qPCR-based test—PerioAnalyse (Clinident Institut Biopharma, Saint-Beauzire, France). A sterile tip of paper was introduced with sterile disposable tweezers into the sulcus on the lingual side of the last mandibular molar, the periodontal site nearest to oropharynx, and the sample was sent to Clinident Institut Biopharma (France) for DNA extraction and sample analysis.

***P. aeruginosa* isolates.** For isolation of *P. aeruginosa*, liquefied sputa were immediately processed in all patients. Saliva and sputum samples (100 μ l) were incubated aerobically at 37°C for 4 days in cetrimide agar media (EMD Millipore, Darmstadt, Germany) (selective media for *P. aeruginosa* isolation) (28). Identification of *P. aeruginosa* isolates was conducted based on phenotypical and morphological criteria (colony morphology, size, and pigmentation).

Atypical *P. aeruginosa* isolates were confirmed by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry in a MALDI-TOF Biotyper (Bruker Daltonics, Bremen, Germany), conducted in the Biology Division of Brest University Hospital, France. All strains were stored at -80°C until required.

PFGE. Bacterial genomic DNA was prepared from each strain, and pulsed-field gel electrophoresis (PFGE) was performed as a modification of the method described in the Instruction Manual and Applications Guide of a CHEF-DR PFGE system (Bio-Rad). *P. aeruginosa* isolates from salivary and sputum samples of the CC patients were grown overnight at 37°C on Mueller-Hinton medium (bioMérieux, Craponne, France). Colonies were suspended in a solution (10 mM Tris [pH 7.2], 20 mM NaCl, 50 mM EDTA, pH 8) to a concentration of a McFarland standard of 5. The cell suspension was mixed 1:1 with 1% low-melting-point agarose (Bio-Rad Laboratories [Canada] Ltd., Mississauga, Ontario, Canada) to prepare the agarose plugs. The embedded cells were incubated for 2 h at 37°C in a buffer (10 mM Tris [pH 7.2], 50 mM NaCl, 0.2% sodium deoxy-

TABLE 2 Demographic and clinical data of the CF patients^a

Patient	Age (yrs)	Sex	Oral conditions	No. of cells/field				No. of isolates of <i>P. aeruginosa</i>				PFGE clonal group(s)			
				No. of DMFT	SECs	WBCs	Sputum quality	Saliva		Sputum		Saliva		Sputum	
								Wild type	Mucoid	Wild type	Mucoid	Wild type	Mucoid	Wild type	Mucoid
NC 1	12	M	Ng&Np	0	>25	<10	Poor	0	0	0	0				
NC 2	13	M	Ng&Np	0	10–25	>25	Acceptable	0	0	0	0				
NC 3	15	F	Ng&Np	1	>25	<10	Poor	0	0	0	0				
NC 4	16	M	Ng&Np	4	<10	<25	Appropriate	0	0	0	0				
NC 5	27	F	Ng&Np	12	<10	>25	Appropriate	0	0	0	0				
CC 1	16	F	Ng&Np	1	10–25	>25	Acceptable	2	0	1	0	5 and 6		5	
CC 2	17	M	Ng&Np	1	10–25	>25	Acceptable	1	0	1	0	1		1	
CC 3	24	M	Ng&Np	4	>25	10–25	Poor	0	0	1	1			7a	7b
CC 4	28	M	Ng&Np	9	10–25	>25	Acceptable	2	1	0	2	4	4		4
CC 5	34	F	Ng&Np	6	10–25	>25	Acceptable	0	0	2	2			2 and 3	2 and 3

^a NC, patients not colonized by *P. aeruginosa*; CC, patients chronically colonized by *P. aeruginosa*; M, male; F, female; Ng&Np, no gingivitis and no periodontitis; DMFT, decayed, missing, or filled teeth; SECs, squamous epithelial cells; WBCs, white blood cells.

cholate, 0.5% sodium lauryl sarcosine) with lysozyme (2 mg/ml) with constant agitation. Plugs were washed and lysed overnight at 50°C in a solution (100 mM EDTA [pH 8], 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine) with proteinase K (50 mg/ml) with constant agitation. The plugs were washed six times by gentle agitation for 15 min, twice with water and four times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and stored at 4°C. One-third to one-half of a plug was then processed with 30 U RNase XbaI (Roche, Mannheim, Germany) and prebuffer H (50 mM NaCl, 6 mM Tris-HCl [pH 7.5], 10 mM MgCl₂). Digestion was performed for 24 h at 37°C.

The gel was prepared using 1% pulsed-field-certified agarose (Bio-Rad) in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA). A 50-to-1,000-molecular-weight marker (Sigma-Aldrich) was used. PFGE was performed using a CHEF-DR III apparatus (Bio-Rad).

The gel run was performed at two different times, first at 6.0 V cm⁻¹ for 15 h at 12°C with an initial pulse time of 1 s and a final pulse time of 20 s and then for 7 h with an initial pulse time of 20 s and a final pulse time of 41 s. DNA fragments were visualized by ethidium bromide staining. Gel pictures were acquired with a Gel Doc Xr+ imaging system, and electrophoretically generated DNA profiles were analyzed using Image Lab (Bio-Rad) software.

Cluster analysis was generated using Dice's coefficient, with clustering by the unweighted-pair group method using average linkages (UPGMA), with a 1% tolerance of band positions and optimization of 1%. Isolates were defined as being of the same (clonal) PFGE type if the Dice coefficient was ≥90%.

DNA extraction of sputum samples and saliva. For molecular analysis, 2-ml aliquots were stored at -80°C. One-hundred-fifty-microliter volumes of sputum and saliva samples were pretreated by sonication in an ultrasonic bath for 5 min and centrifuged for 10 min at 5,000 × g. The pellet was recovered and resuspended in a mix containing ATL buffer (Qiagen, Courtaboeuf, France) and 20 mg/ml of proteinase K (Qiagen). Extraction was followed by use of a QIAamp DNA minikit (Qiagen) according to the instructions of the manufacturer (Tissue protocol) with a dilution volume of 100 µl. Sputum sample DNA extracts (25 µl) were sent to Clinident Institut Biopharma (France).

DNA extraction of tip paper. Each sterile tip paper was mailed to the Clinident Institute Laboratory Biopharma in France. They used a commercial kit for DNA extraction and quantification of periodontal pathogens with the real-time PCR method. The DNA extracts (50 µl) were sent back to our laboratory and frozen at -80°C.

Detection and quantification by qPCR of anaerobic periodontal pathogenic bacteria in subgingival plaque and sputum samples. Sputum sample DNA extracted in our laboratory (50 µl) was mailed to Clin-

ident Institute Laboratory Biopharma in France to be analyzed with the extracted subgingival plaque sample DNA. The amplification was done in a simplex reaction with primers and probes targeting the ubiquitous 16S rRNA gene sequences. The quantity of each organism was determined, with a detection limit of 10² CFU/ml. Another assay, performed by amplification of a universal marker of all bacteria, allowed quantification of the total bacteria in samples. Comparisons between dental flora and sputum flora were made.

***P. aeruginosa* detection and quantification in subgingival plaque, saliva, and sputum samples.** (i) *oprL* qPCR. *oprL* qPCR was performed using target sequences as described previously by Le Gall et al. (29) with minor modifications (for primer *OPRL*-F, AACAGCGGTGCCGTTGAC; for primer *OPRL*-R, GTCGGAGCTGTCGTACTCGAA; for the *oprL*-MGB probe, FAM-TGAGCGACGAAGCC-BHQ [FAM, 6-carboxyfluorescein; BHQ, block hole quencher]). The reaction mix contained 12.5 µl of Qiagen Probe master mix, 0.3 µM (each) primer (Eurogentec, Seraign, Belgium), 0.2 µM hydrolysis probe (Eurogentec, Seraign, Belgium), and 5 µl of DNA extract, with a final reaction volume of 25 µl with water. A standard curve of *P. aeruginosa* PAO1, extending from 10² to 10⁶ CFU/ml, was used (29). qPCR plates contained negative amplification controls. The results were analyzed by the use of a 7300 software SDS system (Applied Biosystems).

(ii) qPCR for *gyrB* and *ecfX*. The multiplex PCR was performed using target samples as described previously by Le Gall et al. (29) (for primer *GYRB*-F, CCTGACCATCCGTCGCCACAAC; for primer *GYRB*-R, CGC AGCAGGATGCCGACGCC; for the *gyrB*-TM probe, FAM-CCGTGGTG GTAGACCTGTTCCAGACC-BHQ; for primer *ECFX*-F, CGCATGCCT ATCAGGCGTT; for primer *ECFX*-R, GAACTGCCAGGTGCTTGC; for the *ecfX*-TM probe, yak-ATGGCGAGTTGCTGCGCTTCCT-BHQ [yak, Yakima Yellow]). The reaction mix contained 12.5 µl of Qiagen Quantitect Probe master mix, 0.4 µM (each) primer (Eurogentec, Seraign, Belgium), 0.16 µM hydrolysis probe (Eurogentec, Seraign, Belgium), and 4.5 µl of DNA extract, with a final reaction volume of 25 µl with water.

Results were analyzed by the use of a 7300 software SDS system (Applied Biosystems). The result of the qPCR for *gyrB* and *ecfX* was considered positive when at least one of the two target genes was detected.

RESULTS

Study population. Ten patients, 5 CC and 5 NC subjects, were included in this study. The average age was higher in the CC group (23.8 years, with a range of 17 to 34) than in the NC group (16.6 years, with a range of 12 to 27) (Table 1). No patients presented with gingivitis or periodontitis (Table 2). The DMFT index was

FIG 1 PFGE of salivary and pulmonary *P. aeruginosa* isolates. Analysis of the genetic relationship with the XbaI restriction enzyme was performed with the 5 CC patients. Seven clonal groups were found. The genetic profile of the reference *P. aeruginosa* PAO1 strain is also shown.

lower in the patients aged 12 to 16 years and higher in the older patients. The average DMFT index values were 3.4 and 4.2 in the NC and CC groups, respectively.

Clinical samples. Preliminary microscopic examination of the sputum was made to detect possible sputum contamination by oral microbiota. According to the number of SECs and WBCs, a quality score was assigned to each sputum sample (Table 2). The samples from patients NC 4 and 5 were of appropriate quality for analysis. The sputum samples from patients CC 1, 2, 4, and 5 were classified as acceptable, while those from patients NC 1 and 3 and CC 3 were of poor quality.

***P. aeruginosa* isolates.** No *P. aeruginosa* bacteria were found in the saliva and sputum samples from the NC patients. After a phenotypic analysis (morphology, size, and pigment production) and determination of the antibiotic resistance profiles (data not shown), 16 *P. aeruginosa* isolates (Table 2) were obtained from the CC patients. Six salivary and 10 sputum strains were isolated by selective culture. A total of 10 nonmucoid strains (wild type) and 6 mucoid strains were found. High proportions of mucoid strains were found in sputum in comparison to saliva (the quantity of data was not sufficient to demonstrate statistical significance of this result). In patients CC 3 and 5, *P. aeruginosa* was not found in saliva.

PFGE. All 16 strains restricted by XbaI were identified by PFGE. The reproducibility of the PFGE profiles was confirmed by the analysis of *P. aeruginosa* PAO1 in gel experiments, while the stability of the PFGE profiles was confirmed by multiple passages of the isolates and PFGE analysis. A dendrogram comparing the PFGE banding patterns was constructed using Image Lab software, demonstrating the rates of genomic similarity (Fig. 1). The

16 isolates were assigned to seven clonal groups. The first clonal group (group 1) was composed of one salivary strain and one pulmonary strain from patient CC 2. Patient CC 5 had two different clonal groups (groups 2 and 3), both in sputum isolates. Each clonal group had one wild-type strain and one mucoid strain. Clonal group 4 was formed by salivary and pulmonary strains of patient CC 4. Patient CC 1 had clonal groups 5 and 6. The former comprised one salivary isolate and one sputum isolate, while the latter consisted of only a sputum isolate. The last clonal group (consisting of subgroups 7a and 7b) was formed by two strains isolated from sputum from patient CC 3 which differed in only one band.

***P. aeruginosa* detection and quantification in subgingival plaque, saliva, and sputum samples.** In the CC patients, the *oprL* qPCR was positive in all samples, except in the subgingival plaque sample of patient CC 1 (Table 3). The qPCR for *gyrB* and *ecfX* confirmed the presence of *P. aeruginosa* in sputum samples of all CC patients, in the saliva of patients CC 1, 2, and 4, and in the subgingival plaque of patients CC 2 and 4. The *oprL* qPCR was taken into account for the quantification.

High proportions of the bacterial species were found in the sputum sample in comparison to the other two samples. In the NC patients, samples were analyzed by qPCR as well to exclude *P. aeruginosa* presence not detected by culture methods. *oprL* qPCR was positive in only some of the sputum samples (NC 1, 2, and 4), but the second qPCR was negative for the presence of this bacterial species. For the subgingival and saliva samples, because the first result was negative, the second qPCR was not carried out.

Detection by qPCR of anaerobic periodontal pathogenic bacteria in subgingival plaque and sputum samples. The Perio-

TABLE 3 qPCR of subgingival plaque, saliva, and sputum samples in the CF patients^a

Patient	Result of <i>oprL</i> qPCR			Result of qPCR for <i>gyrB</i> and <i>ecfX</i>			<i>P. aeruginosa</i> qPCR quantification (CFU/ml)		
	Subgingival plaque	Saliva	Sputum	Subgingival plaque	Saliva	Sputum	Subgingival plaque	Saliva	Sputum
NC 1	—	—	+	NM	NM	—	0	0	0
NC 2	—	—	+	NM	NM	—	0	0	0
NC 3	—	—	—	NM	NM	NM	0	0	0
NC 4	—	—	+	NM	NM	—	0	0	0
NC 5	—	—	+	NM	NM	—	0	0	0
CC 1	—	+	+	—	+	+	0	$4.2 \cdot 10^3$	$2.6 \cdot 10^6$
CC 2	+	+	+	+	+	+	$1.0 \cdot 10^3$	$4.8 \cdot 10^5$	$4.5 \cdot 10^9$
CC 3	+	+	+	±	—	+	$1.2 \cdot 10^1$	0	$1.1 \cdot 10^7$
CC 4	+	+	+	+	+	+	$1.12 \cdot 10^6$	$2.1 \cdot 10^7$	$1.7 \cdot 10^8$
CC 5	+	+	+	—	—	+	0	0	$1.2 \cdot 10^9$

^a NC, patients not colonized by *P. aeruginosa*; CC, patients chronically colonized by *P. aeruginosa*; —, negative detection result; +, positive detection result; ±, positive for *gyrB* and negative for *ecfX*; NM, determination not made.

Analyse test identifies nine periodontal pathogens, i.e., *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Eikenella corrodens*, *Campylobacter rectus*, and *Parvimonas micra*. These bacteria correspond to the major pathogenic bacterial species involved in periodontal disease in the Socransky complex (30). Detection and quantification of each of these bacterial species were performed by qPCR as well as total flora quantification (Table 4). Regarding both groups, we observed a decreased diversity in the CC group. Seven of the 9 targeted species were found in the NC group, while only 5 were present in the CC group. Concerning the samples of both groups, there was higher diversity in the subgingival plaque samples than in sputum.

In all patients, *F. nucleatum* was the most frequently detected bacterium, mainly in the subgingival plaque samples with the highest quantification.

E. corrodens was the second most frequently detected bacterium, predominately in the NC patients and in both samples.

P. micra and *C. rectus* were more often detected in the NC group, with no difference between samples. *P. intermedia* was found in both groups, with a clear prevalence in subgingival plaque samples.

Some species were present sparsely: *T. forsythia*, *T. denticola*, and *P. gingivalis*. The first two were detected only in the NC patients, and *P. gingivalis* was detected in the CC group. No *A. actinomycetemcomitans* organisms were found. None of the target species were found in the subgingival plaque and sputum samples of patient CC 5. These results have a descriptive interest; the quantity of data was not sufficient to apply the usual tests to demonstrate statistical significance of our observations.

DISCUSSION

Over the last few years, several studies have focused on the relationship between sinus colonization and lung colonization of *P. aeruginosa* in CF patients. Moreover, the oral cavity may represent a potential source for airway colonization. The present investigation questioned whether there is a link between the oral cavity (saliva and subgingival plaque) and *P. aeruginosa* lung colonization.

This case-control study isolated salivary and lung *P. aeruginosa*

bacteria in CF patients, detected and quantified the bacteria by qPCR, and determined clonal relatedness. In addition, an analysis of specific periodontal bacteria in the subgingival plaque and sputum samples was made.

As the sputum samples from patients CC 3 and NC 1 and 3 were of poor quality, contamination by saliva was a possibility. However, for these patients, *P. aeruginosa* was isolated in sputum samples but not in saliva, thus excluding sputum contamination by oral *P. aeruginosa*. Looking at Table 4, for patient NC 1, periodontal pathogenic bacteria were found in subgingival plaque and sputum, indicating a possible contamination of sputum. From the NC 3 sputum, only 1 of the 6 periodontal bacterial species found was located in both sites, and none of the targeted species was found in the CC 3 sputum, excluding sputum contamination by saliva for these patients.

Sixteen isolates were recovered from samples from the CC patients by selective culture. Ten of these were isolated in the sputum samples. Six isolates presented a mucoid phenotype. It is well known that, in the progression of chronic lung colonization, wild-type strains are succeeded by mucoid *P. aeruginosa* alginate-producing strains. Indeed, infection or conversion of the nonmucoid forms to mucoid strains increases in prevalence with age (31).

There was good accordance between detection by selective culture and specific *P. aeruginosa* qPCR. In patients CC 3 and 5, this bacterium was not detected and quantified by qPCR or by cetrinide agar medium.

Performance of *oprL* qPCR in CF patients was corroborated in a preliminary study. *oprL* qPCR has an estimated sensitivity of 10 CFU/ml (detection threshold) and a specificity of 70% (29). After the first quantification and detection of *P. aeruginosa* by *oprL* qPCR, the presence of this bacterium was confirmed by qPCR for *gyrB* and *ecfX* in the sputum samples of all the CC patients and in the saliva and subgingival plaque samples of three CC patients.

In patient CC 5, the positive results of the *oprL* qPCR and negative results of the qPCR for *gyrB* and *ecfX* can be explained by the differences in the detection thresholds of the two qPCRs. The threshold of qPCR for *gyrB* and *ecfX* was evaluated at 10^3 CFU/ml, so there remains an unclear zone between 10 CFU/ml and 10^3 CFU/ml. The low DNA quantity in samples and the negative results of the qPCR for *gyrB* and *ecfX* can be explained by a recent early colonization.

TABLE 4 Anaerobic species detection and quantification by qPCR in subgingival plaque and sputum samples^a

Species	Sample	qPCR quantification (CFU/ml) in sample from indicated patient									
		NC 1	NC 2	NC 3	NC 4	NC 5	CC 1	CC 2	CC 3	CC 4	CC 5
<i>Treponema denticola</i>	Subgingival plaque	0	0	0	0	3.83 · 10 ⁴	0	0	0	0	0
	Sputum	0	0	0	0	0	0	0	0	0	0
<i>Tannerella forsythia</i>	Subgingival plaque	0	0	5.78 · 10 ³	0	0	0	0	0	0	0
	Sputum	0	0	0	0	0	0	0	0	0	0
<i>Porphyromonas gingivalis</i>	Subgingival plaque	0	0	0	0	0	0	0	0	6.75 · 10 ³	0
	Sputum	0	0	0	0	0	0	0	0	0	0
<i>Campylobacter rectus</i>	Subgingival plaque	5.73 · 10 ⁵	0	8.70 · 10 ³	0	9.38 · 10 ⁵	0	0	0	0	0
	Sputum	4.07 · 10 ⁴	0	0	0	3.95 · 10 ³	0	0	0	0	0
<i>Fusobacterium nucleatum</i>	Subgingival plaque	3.70 · 10 ⁶	4.24 · 10 ⁴	2.38 · 10 ⁵	2.08 · 10 ⁴	5.82 · 10 ⁶	1.25 · 10 ⁷	3.79 · 10 ⁵	7.58 · 10 ⁴	2.77 · 10 ⁶	0
	Sputum	3.75 · 10 ⁵	4.37 · 10 ⁴	0	2.66 · 10 ⁴	0	4.12 · 10 ⁴	0	0	1.53 · 10 ⁴	0
<i>Parvimonas micra</i>	Subgingival plaque	1.41 · 10 ⁴	0	9.68 · 10 ³	0	5.34 · 10 ⁵	0	0	0	4.90 · 10 ⁵	0
	Sputum	3.16 · 10 ³	0	0	2.14 · 10 ²	1.20 · 10 ²	0	0	0	6.45 · 10 ²	0
<i>Prevotella intermedia</i>	Subgingival plaque	1.54 · 10 ⁵	0	1.92 · 10 ⁴	0	0	0	0	9.90 · 10 ⁴	1.64 · 10 ⁶	0
	Sputum	2.61 · 10 ⁶	0	0	0	0	0	0	0	0	0
<i>Eikenella corrodens</i>	Subgingival plaque	4.59 · 10 ⁶	5.35 · 10 ⁴	1.42 · 10 ⁴	9.22 · 10 ³	4.37 · 10 ⁵	1.77 · 10 ⁶	5.77 · 10 ⁴	0	0	0
	Sputum	2.07 · 10 ⁵	2.06 · 10 ⁴	5.10 · 10 ⁴	3.81 · 10 ⁵	0	4.62 · 10 ⁴	1.31 · 10 ⁵	0	0	0
<i>Aggregatibacter actinomycetemcomitans</i>	Subgingival plaque	0	0	0	0	0	0	0	0	0	0
	Sputum	0	0	0	0	0	0	0	0	0	0
Total flora	Subgingival plaque	7.13 · 10 ⁹	2.17 · 10 ⁸	7.80 · 10 ⁸	5.48 · 10 ⁸	8.18 · 10 ⁹	2.70 · 10 ⁹	2.80 · 10 ⁸	4.07 · 10 ⁹	4.18 · 10 ⁹	1.71 · 10 ⁸
	Sputum	5.20 · 10 ⁸	1.31 · 10 ⁸	4.26 · 10 ⁸	4.90 · 10 ⁸	5.35 · 10 ⁸	1.57 · 10 ⁸	8.45 · 10 ⁸	7.20 · 10 ⁷	9.60 · 10 ⁷	3.93 · 10 ⁹

^a NC, patients not colonized by *P. aeruginosa*; CC, patients chronically colonized by *P. aeruginosa*.

Also, those results can be explained by the presence of another bacterium responsible for cross-reaction. *Achromobacter xylosoxidans*, *Burkholderia cenocepacia*, *B. multivorans*, *Elizabethkingia meningoseptica*, *Roseomonas* spp., and *Stenotrophomonas maltophilia* have been described as responsible for cross-reaction in the *oprL* qPCR (29).

To our knowledge, no species cross-react with both qPCRs. The combination of the two qPCRs allowed 100% specificity. This last reason could explain the positive results in the *oprL* qPCR for the NC patients.

P. aeruginosa was detected in saliva and sputum samples of patient CC 1 but not confirmed in the subgingival plaque. In patients CC 2 and 4, *P. aeruginosa* was detected in subgingival plaque, saliva, and sputum. And in patient CC 3, *P. aeruginosa* was not found in saliva but was detected in sputum and subgingival plaque samples. Detection and quantification of *P. aeruginosa* in sputum of CC patients were related to their “chronically colonized” status.

Another observation was the presence of this bacterium in high concentrations in sputum samples and in lower concentrations in the saliva and subgingival plaque. This could be explained by the higher selective pressure and the heterogeneous environment of CF lungs (33) and the adaptability of *P. aeruginosa* (34). In addition, *P. aeruginosa* strains growing in biofilms and in a limited-oxygen environment could increase their tolerance of antibiotics (35).

For patients CC 2 and 4, *P. aeruginosa* was detected in the subgingival plaque samples and saliva but in the highest concentrations in saliva. In patient CC 3, *P. aeruginosa* detection was negative in saliva, whereas it was positive in the subgingival plaque.

An ascending oral passage of pulmonary bacteria could justify the presence of this bacterium in some of the saliva and subgingival samples. This fact allows us to ask the question about a possible oral reservoir of this bacterium for subsequent lung colonization or recolonization.

The genetic comparisons among the 16 *P. aeruginosa* isolates resulted in the formation of 7 clonal groups. In clonal group 4, all five of the isolates presented different phenotypic profiles (size and antibiotic resistance). The presence of two phenotypically different isolates of *P. aeruginosa* with the same PFGE profiles has been described. The finding of the same clone in lung and saliva samples, such as was observed in clonal groups 1, 4, and 5, indicates a possible passage of isolates between the oral cavity and lungs.

Studies of clonal diversity highlighted the extensive genome plasticity of *P. aeruginosa*. Genome plasticity is related to mutations due to local changes (single base substitutions, insertions, or deletions) and regional changes (deletions, duplications, and inversions). In CF lung isolates, these mutations are more often made by vertical gene transfer than by horizontal gene transfer, even though cross-infection between patients has been reported (36). This permits high adaptability of the bacterium to CF lungs. In our study, PFGE analysis showed that the isolates from each patient were more similar to each other than to the isolates from the other patients. These results are consistent with the literature (36), and they highlight the fact that chronic infections are due to *P. aeruginosa* strains derived from various environmental reservoirs (37). Cramer et al. (38) revealed in their study that there are 20 ubiquitous clones frequently found and that, for the CF popu-

lation, the five most commonly isolated clones belong to these 20 clones.

Cases describe cross-contamination occurring between CF patients during prolonged contact (siblings) or between uninfected and infected patients in clinics where patients are not separated (36). Our findings confirm that no cross-contamination occurred between our patients followed in the same department at the CRCM in Roscoff, France. The fact that we did not find the same clone in different patients confirmed that there are no currently widespread epidemics in Brittany, France.

Association between the oral cavity and the lower airways (LAW) in CF lung colonization has not been studied often. Some data reported in the literature may explain our results. Mainz et al. (16) showed in 2012 that lungs of two transplanted patients with CF became colonized by identical *P. aeruginosa* isolates. These isolates were genotypically identical to those found previously in the explanted lungs and in the sinuses of CF patients. Other studies showed the relationship between sinonasal bacterial colonization and subsequent lung colonization. This supports the hypothesis of bacterial propagation descending from the sinuses to the LAW (15) (16). The presence of identical genotypes in the upper airways (UAW) and LAW suggests that the UAW plays a role as a reservoir for *P. aeruginosa* in CF patients, allowing this bacterium to adapt to and to chronically colonize lungs (39). Further investigations are needed to evaluate whether the oral cavity may also function as a reservoir and transition zone for these bacterial pathogens.

Until now, PFGE studies did not allow the possibility of postulating that the oral cavity is a possible secondary reservoir for lung colonization. In CF disease, the high-calorie diet rich in carbohydrate foods recommended to provide the necessary source of energy combined with antibiotic treatment could explain abnormal oral health (40, 41). None of our CF patients enrolled in the study presented with gingivitis or periodontitis. These diseases are related to oral hygiene habits (42).

In addition, periodontal pathogenic bacteria (associated with development of gingivitis) were examined in the subgingival plaque and sputum samples. The bacteria analyzed were included in 3 different complexes, in relation to their pathogenicity, according to Socransky and coworkers in 1998 (30). *P. gingivalis*, *T. denticola*, and *T. forsythia* (formerly *Bacteroides forsythus*) formed the red complex. The analyzed species *F. nucleatum*, *P. intermedia*, *P. micra* (formerly *Peptostreptococcus micros*), and *C. rectus* composed the orange complex, while *E. corrodens* and *A. actinomyces-comitans* composed the green complex. These different complexes are related to pocket depth and signs of periodontal destruction and progression. According to Socransky and Haffajee, gingivitis is formed by a succession of bacterial species. It is initiated by bacteria of the green complex, and then bacteria of the orange and red complexes become more dominant (43). da Silva-Boghossian and coworkers (44) found *P. aeruginosa* at higher frequencies and levels in patients with periodontitis and high levels of periodontal pathogenic bacteria. They demonstrated associations between *P. aeruginosa* and *P. gingivalis* and between *T. denticola* and *T. forsythia*. According to those authors, the presence of non-oral bacteria in subgingival plaque is not a transitory event and the oral cavity may be a reservoir for pathogens (44).

In addition, Colombo et al. found that unusual species such as *Escherichia coli* and *P. aeruginosa* were more frequently observed in periodontitis patients, who have high levels of periodontal

pathogenic bacteria (45). To determine if subgingival plaque is a potential source of *P. aeruginosa* lung colonization and infection, it would have been interesting to determine the clonal relatedness of salivary, pulmonary, and subgingival plaque isolates of *P. aeruginosa* in these patients. But subgingival plaque samples were sent directly to the laboratory for analysis, and only DNA extracts were recovered. Bacteria in the red complex were not predominantly represented in any of the samples. In cases of periodontitis, the levels of bacteria of the red and orange complexes are significantly elevated. Our patients did not have periodontitis, which could explain the low prevalence of this complex. An association between the red and orange complexes was described in the Socransky study (43). The data obtained did not allow analysis of the relationships between these species in the different complexes. But the fact that predominant concentrations of green and orange complex bacteria existed in both groups leads to postulation of a later succession by the red complex bacteria and the development of gingivitis.

In patient CC 5, targeted anaerobic bacteria were not found. One possible explication for this result could be a bad sampling process or bad sample conservation. However, the total amount of flora found in this patient excludes this possibility. Another explication was the fact that this patient was the only one treated with the macrolide antibiotic azithromycin. Blandizzi et al. showed in 1999 that azithromycin penetrated into both normal and pathological periodontal tissues (46), suggesting elimination of the targeted periodontal pathogenic bacteria for this patient.

Excluding this patient, the average percentage of *F. nucleatum* present in both locations (sputum and subgingival plaque) was 53%, *F. nucleatum* being the most abundant and prevalent bacterium in this study. An elevated level of this bacterium was found in the NC group. Generally, the orange complex was present at a higher prevalence in the NC group than in the CC group. Bacteria of the green complex were also detected at high levels in all NC individuals and in only 2 CC individuals, in both sites. This species is one of the most important species in the initiation of the dental biofilm formation (47). A proportion of *P. intermedia*, classified in the complex as one of the most aggressive bacterial species in periodontal disease, was found in the 2 groups of patients. Nevertheless, it was more abundant in subgingival plaque than in sputum. In the orange complex, *P. micros* and *C. rectus* were found in decreased prevalence. *E. corrodens* was the second-most-prevalent and -abundant bacterial species. It is one of the least pathogenic periodontal bacterial species, according to Socransky and coworkers (30). *A. actinomycetemcomitans* was not found, unlike the results of the study by Colombo and coworkers, who found a high prevalence of this bacterium not only in chronic periodontitis subjects but also in healthy subjects (45). Concerning species diversity, there were 8 different species in subgingival plaque and only 5 species in sputum. These facts suggest that there was a possible descending passage of periodontal anaerobic bacterial species to the lungs with reduced proportions and diversity.

According to Colombo et al., these periodontal pathogens were observed in healthy patients but at much lower prevalences than in periodontitis patients. The data showed that healthy sites could be colonized by less-virulent clones of these species (45). Our data were in accordance with the results of a study by Kinirons, who found that CF patients presented significantly lower levels of gingival and periodontal tissue inflammation than healthy people (41).

The fact that anaerobic bacteria were found in sputum samples in the NC patients, in the absence of *P. aeruginosa*, underlines the idea presented in Tunney's work of a first and early infection by anaerobic bacteria that modifies the pulmonary environment for the subsequent *P. aeruginosa* infection (26). According to the concept of bacterial succession presented by Socransky and Haffajee, several species first colonize an ecosystem and make it more suitable for other bacteria that then replace the pioneer species (43). Comparing levels of species diversity between the two groups of patients, there was a trend toward a decrease of diversity in the CC group. These data could be explained by the presence of *P. aeruginosa* in the CC group. Taking into consideration the previous idea, we hypothesize that, after pulmonary modification by anaerobic bacteria, *P. aeruginosa* colonizes the lungs and that antagonistic relationships take place between this bacterium and the others. Faust and coworkers said that microbial coexclusion might be explained by functional competition for limited resources, housing modification, and production of toxins by phylogenetically related microbes (48). This hypothesis is confirmed by the fact that in the CC patients, those periodontal anaerobic bacteria were infrequently detected and were found in low concentrations. Socransky et al. showed that periodontal disease was associated with individual pathogens in periodontal site associations (30). Regarding the work by Charlson et al. (49), we found the same trend of modifications in microbial community composition between colonized and noncolonized patients. They also found a bacterial species community modification in saliva and sputum in relation to *P. aeruginosa* presence.

In all cases, the limited number of patients included in this pilot study precludes our expressing a level of statistical significance for our conclusions. Antibiotic treatments could influence diversity variations. Moreover, age differences between the members of the CC group and the members of the NC group, with an average higher age in the CC group, could represent a bias in this study.

Conclusions. To our knowledge, this is the first study on subgingival plaque pathogens that has compared oral and pulmonary samples and presented analyses of the genetic relatedness of saliva and sputum isolates of *P. aeruginosa* in CF patients.

The presence of the same bacterial clone in saliva and lung samples and detection of this bacterium in subgingival plaque samples suggest an ascending or descending passage of bacteria into the oral cavity and lungs. Data highlight the possible role of a *P. aeruginosa* reservoir in the oral cavity, constituting a potential source of subsequent lung colonization or recolonization in CF patients. This possibility is supported by the detection of oral bacteria in lung samples. A prospective longitudinal study would establish the dynamics and/or the chronology of acquisition of this bacterium and the role of the oral cavity in lung colonization and recolonization.

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REFERENCES

1. Scotet V, Gillet D, Duguépéroux I, Audrézet MP, Bellis G, Garnier B,

- Roussey M, Rault G, Parent P, De Braekeleer M, Férec C, Réseau Mucoviscidose Bretagne et Pays de Loire. 2002. Spatial and temporal distribution of cystic fibrosis and of its mutations in Brittany, France: a retrospective study from 1960. *Hum Genet* 111:247–254. <http://dx.doi.org/10.1007/s00439-002-0788-1>.
2. Scotet V, Dugueperoux I, Saliou P, Rault G, Roussey M, Audrezet MP, Férec C. 2012. Evidence for decline in the incidence of cystic fibrosis: a 35-year observational study in Brittany, France. *Orphanet J Rare Dis* 7:14. <http://dx.doi.org/10.1186/1750-1172-7-14>.
3. Lee TW, Matthews DA, Blair GE. 2005. Novel molecular approaches to cystic fibrosis gene therapy. *Biochem J* 387:1–15. <http://dx.doi.org/10.1042/BJ20041923>.
4. Taylor CJ, McGaw J, Howden R, Duerden BI, Baxter PS. 1990. Bacterial reservoirs in cystic fibrosis. *Arch Dis Child* 65:175–177. <http://dx.doi.org/10.1136/adc.65.2.175>.
5. Muhlebach MS, MacDonald SL, Button B, Hubbard JJ, Turner ML, Boucher RC, Kilpatrick DC. 2006. Association between mannan-binding lectin and impaired lung function in cystic fibrosis may be age-dependent. *Clin Exp Immunol* 145:302–307. <http://dx.doi.org/10.1111/j.1365-2249.2006.03151.x>.
6. Gibson RL, Burns JL, Ramsey BW. 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 168:918–951. <http://dx.doi.org/10.1164/rccm.200304-505SO>.
7. Rosenfeld M, Emerson J, McNamara S, Thompson V, Ramsey BW, Morgan W, Gibson RL, EPIC Study Group. 2012. Risk factors for age at initial *Pseudomonas* acquisition in the cystic fibrosis epic observational cohort. *J Cyst Fibros* 11:446–453. <http://dx.doi.org/10.1016/j.jcf.2012.04.003>.
8. Saiman L, Siegel J, Cystic Fibrosis Foundation Consensus Conference on Infection Control Participants. 2003. Infection control recommendations for patients with cystic fibrosis: microbiology, important pathogens, and infection control practices to prevent patient-to-patient transmission. *Am J Infect Control* 31(Suppl):S1–S62. <http://dx.doi.org/10.1067/mic.2003.78>.
9. Drenkard E, Ausubel FM. 2002. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 416:740–743. <http://dx.doi.org/10.1038/416740a>.
10. Mayer-Hamblett N, Rosenfeld M, Gibson RL, Ramsey BW, Kulasekara HD, Retsch-Bogart GZ, Morgan W, Wolter DJ, Pope CE, Houston LS, Kulasekara BR, Khan U, Burns JL, Miller SI, Hoffman LR. 2014. *Pseudomonas aeruginosa* in vitro phenotypes distinguish cystic fibrosis infection stages and outcomes. *Am J Respir Crit Care Med* 190:289–297. <http://dx.doi.org/10.1164/rccm.201404-0681OC>.
11. Sadikot RT, Blackwell TS, Christman JW, Prince AS. 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* 171:1209–1223. <http://dx.doi.org/10.1164/rccm.200408-1044SO>.
12. Cantón R, Fernández Olmos A, de la Pedrosa EG, del Campo R, Antonia Meseguer M. 2011. Chronic bronchial infection: the problem of *Pseudomonas aeruginosa*. *Arch Bronconeumol* 47(Suppl 6):8–13. (In Spanish.) [http://dx.doi.org/10.1016/S0300-2896\(11\)70029-1](http://dx.doi.org/10.1016/S0300-2896(11)70029-1).
13. Drenkard E. 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes Infect* 5:1213–1219. <http://dx.doi.org/10.1016/j.micinf.2003.08.009>.
14. Digoy GP, Dunn JD, Stoner JA, Christie A, Jones DT. 2012. Bacteriology of the paranasal sinuses in pediatric cystic fibrosis patients. *Int J Pediatr Otorhinolaryngol* 76:934–938. <http://dx.doi.org/10.1016/j.ijporl.2012.02.043>.
15. Muhlebach MS, Miller MB, Moore C, Wedd JP, Drake AF, Leigh MW. 2006. Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis? *Pediatr Pulmonol* 41:445–451. <http://dx.doi.org/10.1002/ppul.20396>.
16. Mainz JG, Hentschel J, Schien C, Cramer N, Pfister W, Beck JF, Tummeler B. 2012. Sinonasal persistence of *Pseudomonas aeruginosa* after lung transplantation. *J Cyst Fibros* 11:158–161. <http://dx.doi.org/10.1016/j.jcf.2011.10.009>.
17. Amaral SM, Cortés Ade Q, Pires FR. 2009. Nosocomial pneumonia: importance of the oral environment. *J Bras Pneumol* 35:1116–1124 (In Portuguese.) <http://dx.doi.org/10.1590/S1806-37132009001100010>.
18. Raghavendran K, Mylotte JM, Scannapieco FA. 2007. Nursing home-associated pneumonia, hospital-acquired pneumonia and ventilator-associated pneumonia: the contribution of dental biofilms and periodontal inflammation. *Periodontol* 2000 44:164–177. <http://dx.doi.org/10.1111/j.1600-0757.2006.00206.x>.
19. Heo SM, Haase EM, Lesse AJ, Gill SR, Scannapieco FA. 2008. Genetic relationships between respiratory pathogens isolated from dental plaque and bronchoalveolar lavage fluid from patients in the intensive care unit undergoing mechanical ventilation. *Clin Infect Dis* 47:1562–1570. <http://dx.doi.org/10.1086/593193>.
20. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, Wade WG. 2010. The human oral microbiome. *J Bacteriol* 192:5002–5017. <http://dx.doi.org/10.1128/JB.00542-10>.
21. Fourrier F, Duvivier B, Boutigny H, Roussel-Delvallez M, Chopin C. 1998. Colonization of dental plaque: a source of nosocomial infections in intensive care unit patients. *Crit Care Med* 26:301–308. <http://dx.doi.org/10.1097/00003246-199802000-00032>.
22. Scannapieco FA, Yu J, Raghavendran K, Vacanti A, Owens SI, Wood K, Mylotte JM. 2009. A randomized trial of chlorhexidine gluconate on oral bacterial pathogens in mechanically ventilated patients. *Crit Care* 13:R117. <http://dx.doi.org/10.1186/cc7967>.
23. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Döring G. 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 109:317–325. <http://dx.doi.org/10.1172/JCI13870>.
24. Kolpen M, Hansen CR, Bjarnsholt T, Moser C, Christensen LD, van Gennip M, Ciofu O, Mandsberg L, Kharazmi A, Döring G, Givskov M, Hoiby N, Jensen PO. 2010. Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. *Thorax* 65:57–62. <http://dx.doi.org/10.1136/thx.2009.114512>.
25. Kolpen M, Kuhl M, Bjarnsholt T, Moser C, Hansen CR, Liengaard L, Kharazmi A, Pressler T, Hoiby N, Jensen PO. 2014. Nitrous oxide production in sputum from cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. *PLoS One* 9:e84353. <http://dx.doi.org/10.1371/journal.pone.0084353>.
26. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, Wolfgang MC, Boucher R, Gilpin DF, McDowell A, Elborn JS. 2008. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *Am J Respir Crit Care Med* 177:995–1001. <http://dx.doi.org/10.1164/rccm.200708-1151OC>.
27. Lee TW, Brownlee KG, Conway SP, Denton M, Littlewood JM. 2003. Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *J Cyst Fibros* 2:29–34. [http://dx.doi.org/10.1016/S1569-1993\(02\)00141-8](http://dx.doi.org/10.1016/S1569-1993(02)00141-8).
28. Courcol R, Herrmann J, Laudat P, Pangon B, Peigue-Lafeuille H (ed). 2010. Référentiel en microbiologie médicale (Rémic), 2nd ed, p 99–104. Société Française de Microbiologie, Paris, France.
29. Le Gall F, Le Berre R, Rosec S, Hardy J, Gouriou S, Boisrame-Gastrin S, Vallet S, Rault G, Payan C, Hery-Arnaud G. 2013. Proposal of a quantitative PCR-based protocol for an optimal *Pseudomonas aeruginosa* detection in patients with cystic fibrosis. *BMC Microbiol* 13:143. <http://dx.doi.org/10.1186/1471-2180-13-143>.
30. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. 1998. Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134–144. <http://dx.doi.org/10.1111/j.1600-051X.1998.tb02419.x>.
31. Govan JR, Deretic V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 60:539–574.
32. Reference deleted.
33. Hogardt M, Heesemann J. 2013. Microevolution of *Pseudomonas aeruginosa* to a chronic pathogen of the cystic fibrosis lung. *Curr Top Microbiol Immunol* 358:91–118. http://dx.doi.org/10.1007/82_2011_199.
34. Eichner A, Gunther N, Arnold M, Schobert M, Heesemann J, Hogardt M. 2014. Marker genes for the metabolic adaptation of *Pseudomonas aeruginosa* to the hypoxic cystic fibrosis lung environment. *Int J Med Microbiol* 304:1050–1061. <http://dx.doi.org/10.1016/j.ijmm.2014.07.014>.
35. Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS. 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* 48:2659–2664. <http://dx.doi.org/10.1128/AAC.48.7.2659-2664.2004>.
36. Warren AE, Boulianne-Larsen CM, Chandler CB, Chiotti K, Kroll E, Miller SR, Taddei F, Sermet-Gaudelus I, Ferroni A, McInnerney K, Franklin MJ, Rosenzweig F. 2011. Genotypic and phenotypic variation in *Pseudomonas aeruginosa* reveals signatures of secondary infection and mutator activity in

- certain cystic fibrosis patients with chronic lung infections. *Infect Immun* 79:4802–4818. <http://dx.doi.org/10.1128/IAI.05282-11>.
37. Logan C, Habington A, Lennon G, Grogan J, Byrne M, O'Leary J, O'Sullivan N. 2012. Genetic relatedness of *Pseudomonas aeruginosa* isolates among a paediatric cystic fibrosis patient cohort in Ireland. *J Med Microbiol* 61:64–70. <http://dx.doi.org/10.1099/jmm.0.035642-0>.
38. Cramer N, Wiehlmann L, Ciofu O, Tamm S, Hoiby N, Tummeler B. 2012. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. *PLoS One* 7:e50731. <http://dx.doi.org/10.1371/journal.pone.0050731>.
39. Mainz JG, Naehrlich L, Schien M, Kading M, Schiller I, Mayr S, Schneider G, Wiedemann B, Wiehlmann L, Cramer N, Pfister W, Kahl BC, Beck JF, Tummeler B. 2009. Concordant genotype of upper and lower airways *P. aeruginosa* and *S. aureus* isolates in cystic fibrosis. *Thorax* 64: 535–540. <http://dx.doi.org/10.1136/thx.2008.104711>.
40. Narang A, Maguire A, Nunn JH, Bush A. 2003. Oral health and related factors in cystic fibrosis and other chronic respiratory disorders. *Arch Dis Child* 88:702–707. <http://dx.doi.org/10.1136/adc.88.8.702>.
41. Kinirons MJ. 1989. Dental health of patients suffering from cystic fibrosis in Northern Ireland. *Community Dent Health* 6:113–120.
42. Löe H. 2000. Oral hygiene in the prevention of caries and periodontal disease. *Int Dent J* 50:129–139. <http://dx.doi.org/10.1111/j.1875-595X.2000.tb00553.x>.
43. Socransky SS, Haffajee AD. 2005. Periodontal microbial ecology. *Periodontology* 2000 38:135–187. <http://dx.doi.org/10.1111/j.1600-0757.2005.00107.x>.
44. da Silva-Boghossian CM, do Souto RM, Luiz RR, Colombo AP. 2011. Association of red complex, *A. actinomycetemcomitans* and non-oral bacteria with periodontal diseases. *Arch Oral Biol* 56:899–906. <http://dx.doi.org/10.1016/j.archoralbio.2011.02.009>.
45. Colombo AP, Teles RP, Torres MC, Souto R, Rosalem WJ, Mendes MC, Uzeda M. 2002. Subgingival microbiota of Brazilian subjects with untreated chronic periodontitis. *J Periodontol* 73:360–369. <http://dx.doi.org/10.1902/jop.2002.73.4.360>.
46. Blandizzi C, Malizia T, Lupetti A, Pesce D, Gabriele M, Giuca MR, Campa M, Del Tacca M, Senesi S. 1999. Periodontal tissue disposition of azithromycin in patients affected by chronic inflammatory periodontal diseases. *J Periodontol* 70:960–966. <http://dx.doi.org/10.1902/jop.1999.70.9.960>.
47. Kolenbrander PE, Palmer RJ, Jr, Periasamy S, Jakubovics NS. 2010. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 8:471–480. <http://dx.doi.org/10.1038/nrmicro2381>.
48. Faust K, Sathirapongsasuti JF, Izard J, Segata N, Gevers D, Raes J, Huttenhower C. 2012. Microbial co-occurrence relationships in the human microbiome. *PLoS Comput Biol* 8:e1002606. <http://dx.doi.org/10.1371/journal.pcbi.1002606>.
49. Charlson ES, Bittinger K, Chen J, Diamond JM, Li H, Collman RG, Bushman FD. 2012. Assessing bacterial populations in the lung by replicate analysis of samples from the upper and lower respiratory tracts. *PLoS One* 7:e42786. <http://dx.doi.org/10.1371/journal.pone.0042786>.