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Detection of Anaerobic Bacteria in High Numbers in Sputum from Patients with Cystic Fibrosis

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***Rationale*: Pulmonary infection in cystic fibrosis (CF) is polymicrobial and it is possible that anaerobic bacteria, not detected by routine aerobic culture methods, reside within infected anaerobic airway mucus.**

***Objectives*: To determine whether anaerobic bacteria are present in the sputum of patients with CF.**

***Methods*: Sputum samples were collected from clinically stable adults with CF and bronchoalveolar lavage fluid (BALF) samples from children with CF. Induced sputum samples were collected from healthy volunteers who did not have CF. All samples were processed using anaerobic bacteriologic techniques and bacteria within the samples were quantified and identified.**

***Measurements and Main Results*: Anaerobic species primarily within the genera *Prevotella, Veillonella, Propionibacterium,* and *Actinomyces* were isolated in high numbers from 42 of 66 (64%) sputum samples from adult patients with CF. Colonization with *Pseudomonas aeruginosa* significantly increased the likelihood that anaerobic bacteria would be present in the sputum. Similar anaerobic species were identified in BALF from pediatric patients with CF. Although anaerobes were detected in induced sputum samples from 16 of 20 volunteers, they were present in much lower numbers and were generally different species compared with those detected in CF sputum. Species-dependent differences in the susceptibility of the anaerobes to antibiotics with known activity against anaerobes were apparent with all isolates susceptible to meropenem.**

***Conclusions*: A range of anaerobic species are present in large numbers in the lungs of patients with CF. If these anaerobic bacteria are contributing significantly to infection and inflammation in the CF lung, informed alterations to antibiotic treatment to target anaerobes, in addition to the primary infecting pathogens, may improve management.**

Keywords: cystic fibrosis; anaerobe; infection; pathogenesis

Chronic bacterial pulmonary infection leading to an irreversible decline in lung function is the main cause of mortality and morbidity in patients with cystic fibrosis (CF), with more than 95% of deaths due to respiratory failure (1). The bacteria most frequently isolated from the sputum of patients with CF and pulmonary infection by standard aerobic microbiological methods are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia* complex (2).

Recently, it has been shown that there are steep oxygen gradients in mucus in the lungs of patients with CF and that proliferation of *P. aeruginosa* within hypoxic mucus generates fully hypoxic (anaerobic) conditions in the lungs of patients with CF and with persistent respiratory infection (3, 4). Because anaerobic conditions exist in the lungs of patients with CF, there is the real possibility that CF pulmonary infection is polymicrobial, with anaerobic bacteria that are not detected by routine aerobic culture methods residing within the anaerobic airway mucus and contributing to infection. Although a number of studies have detected potentially pathogenic anaerobic bacteria in significant numbers in the lungs of patients with CF by culture (5–7), the assumption that the easily grown aerobic bacteria (e.g., *P. aeruginosa* and *B. cepacia* complex) are the principal pathogens in progressive CF lung disease may have obscured the potential contribution of anaerobes. Significantly, recent molecular studies indicate that there is a highly diverse bacterial community within the CF lung, with many different bacterial species, including anaerobes, present (8–10). Furthermore, the anaerobes detected were similar to those found in other studies of anaerobic pulmonary infection, such as nosocomial pneumonia (11, 12), lung abscesses (13, 14), and empyema (15), in which aerobes and anaerobes were present in a polymicrobial infection and were considered to be of significance.

The presence of anaerobes in the lungs of patients with CF could be important because current antibiotic treatment targeted at aerobic bacteria may be ineffective against anaerobic bacteria, which may result in a less than optimal treatment outcome for patients. In this study, we used strict anaerobic bacteriologic culture techniques to detect anaerobic bacteria in CF sputum and bronchoalveolar lavage fluid (BALF) and determined the sus-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although it has been shown that anaerobic conditions exist in the lungs of patients with cystic fibrosis (CF), no studies have used strict anaerobic bacteriologic culture to determine, in a large number of patients with CF, if anaerobic bacteria are present in the CF lung.

What This Study Adds to the Field

This study shows that potentially opportunistic anaerobes are present in the lungs of patients with CF. If these anaerobes contribute to infection and inflammation in the CF lung, alterations to antibiotic treatment to target them may improve management.

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ceptibility of these anaerobes to a range of antibiotics. We also collected and processed, using strict anaerobic bacteriologic culture techniques, induced sputum samples from healthy volunteers who did not have CF. Some of the results of these studies have been previously reported in the form of abstracts (16, 17).

METHODS

Collection of Sputum Samples from Adult Patients with CF and Healthy Volunteers

There are currently 180 adults attending the regional Adult Cystic Fibrosis Centre at the Belfast City Hospital (Belfast, UK). At the time of this study, 90 of these patients had chronic *P. aeruginosa* infection, 20 had chronic *B. cepacia* complex infection, with the remainder infected by a number of different pathogens. All clinically stable patients attending the outpatient clinic who were capable of expectorating a sputum sample were invited to take part in the study, with written, informed consent acquired from all patients. Clinical stability was defined as no change in symptoms, FEV_1 within 10% of best value in the previous 6 months, and no new antibiotics started. Ethical approval for the study was received from the School of Medicine Research Ethics Committee at Queen's University Belfast.

Sputum samples were collected in sterile containers, under the supervision of a physiotherapist, from patients with CF. The sputum samples were examined macroscopically for the presence of salivary contamination, with no samples rejected on this basis. Induced sputum samples were collected from healthy volunteers who did not have CF, with sputum production induced by inhalation of 5% hypertonic saline for 15 minutes before expectoration. After collection, all samples were transferred within 15 minutes to an anaerobic cabinet in the adjacent research laboratory for processing.

Collection of BALF from Pediatric Patients with CF

Ethical approval was received from the Institutional Review Board of the University of North Carolina, Chapel Hill, with written, informed consent acquired from the parents/guardians of all children joining the study and written assent from children old enough to read. BAL was performed during general anesthesia at University of North Carolina Hospitals, Chapel Hill, as previously described (18, 19). Additional details on the collection of BALF are provided in the online supplement. After collection, BALF samples were frozen within 5 to 10 minutes of collection and stored at −80°C. When all samples had been collected, they were transported in dry ice at −80°C to Belfast for processing. The condition of the samples was checked on receipt in Belfast with all samples remaining frozen. Samples were subsequently thawed in the anaerobic cabinet at 37°C before processing.

Bacterial Isolation and Identification

Sputum and BALF samples were treated for 15 minutes with Sputolysin (Calbiochem, La Jolla, CA) in accordance with the manufacturer's instructions, and serial 10-fold dilutions prepared in quarter-strength Ringers lactate (Oxoid, Basingstoke, UK) supplemented with 0.05% (wt/vol) L-cysteine (Sigma-Aldrich, Dorset, UK). One hundred–microliter aliquots were spread plated onto anaerobic blood agar (Oxoid), kanamycin–vancomycin laked blood agar, phenylethyl alcohol agar (Sigma-Aldrich), and nutrient agar (Oxoid). Anaerobic blood agar, phenylethyl alcohol agar, and kanamycin–vancomycin laked blood agar plates were incubated anaerobically for 5 to 7 days at 37°C with nutrient agar plates incubated aerobically at 37°C for 2 to 4 days. After incubation, the total viable counts of each distinct colony type were determined and all isolates present with a colony count greater than 10^4 cfu/g sputum were identified. Additional details of the quantitative microbiology methods used for isolation of bacteria are provided in the online supplement.

Aerobic isolates were initially screened for *P. aeruginosa* using polymerase chain reaction (PCR) detection of the *oprL* gene as described by Xu and colleagues (20). Any aerobes that were negative for *oprL* were then identified by colony PCR and sequencing of 16S ribosomal RNA genes using universal primers as described by LiPuma and associates (21). Anaerobic isolates were also identified by colony PCR and sequencing of 16S ribosomal RNA genes (21). Additional details of the molecular methods used for identification of bacteria are provided in the online supplement. Any anaerobic isolates that could only be identified to genus level by sequencing were further examined using the RapID Ana II identification system (Remel, Lenexa, KS). If this system was unable to identify the isolate to species level, it was recorded at genus level.

Antimicrobial Susceptibility

The susceptibility of selected anaerobic isolates cultured from the sputum of adult patients with CF to ampicillin, clindamycin, meropenem, metronidazole, and piperacillin/tazobactam was determined using E-test strips (Bio-Stat, Stockport, UK) according to the manufacturer's instructions. Minimum inhibitory concentrations (MICs) were read after incubation at 37°C for 48 hours in an anaerobic cabinet and the MIC_{50} and MIC_{90}, the antibiotic concentrations required to inhibit growth of 50 and 90% of isolates, respectively, determined for all of the isolates and for each genera. The percentage of isolates susceptible to each antibiotic was also calculated using the Clinical and Laboratory Standards Institute breakpoints for antimicrobial susceptibility of anaerobic bacteria (22). *Bacteroides fragilis* (American Type Culture Collection 25285) was used as a reference strain for susceptibility testing.

Statistical Analysis

Statistical analysis (independent *t* test, Mann-Whitney test, and chi-square test) was performed with SPSS version 14 (SPSS, Inc., Chicago, IL) software package, with *P* values of less than 0.05 considered to be significant.

RESULTS

Isolation and Identification of Bacteria in Sputum from Patients with CF

A total of 66 sputum samples were collected from 50 patients with CF who provided between one and four sputum samples each. Anaerobic bacteria were isolated in high numbers (defined as $10^4 \leq n \leq 9 \times 10^7$ cfu/g of sputum) from 42 of 66 (64%) samples, representing 33 of 50 (66%) patients (*see* Table E1). Identification of the anaerobes isolated revealed 14 different genera with those in the genera *Prevotella*, *Veillonella*, *Propionibacterium*, and *Actinomyces* most frequently isolated (Table 1). Of these 14 genera, 3 genera (*Streptococcus*, *Rothia*, and *Staphylococcus)* are

TABLE 1. BACTERIA ISOLATED BY CULTURE FROM THE SPUTUM OF ADULT PATIENTS WITH CYSTIC FIBROSIS

Anaerobic Isolates			Aerobic Isolates		
Genera*	No. Isolates	No. Patients	Genera	No. Isolates	No. Patients
Prevotella	20	11	*Pseudomonas*	51	28
Actinomyces	9	8	*B. cepacia* complex	4	4
Veillonella	8	7	*Rothia*	30	17
Propionibacterium	7	7	*Streptococcus*	27	19
Peptostreptococcus	2	2	*Staphylococcus*	23	20
Bulleidia	2	2	*Micrococcus*	4	3
Bifidobacterium	2	1	*Nessieria*	4	4
Gemella	2	2	*Bacillus*	3	3
Lactobacillus	2	2	*Escherichia*	3	2
Fusobacterium	1	1	*Stenotrophomonas*	2	1
Clostridium	1	1			
Staphylococcus	2	2			
Streptococcus	19	13			
Rothia	1	1			
Total	78			151	

* Examples of species detected: *Staphylococcus* (aerobic: *aureus, hominis, epidermidis*; anaerobic: *saccharolyticus*); *Streptococcus* (aerobic: *salivarius, mitis, oralis*; anaerobic: *thermophilus, sanguinis, parasanguinis*); *Rothia* (*dentocariosa*); *Prevotella* (*salivae, corporis, melaninogenica, disiens*); *Actinomyces* (*odontolyticus*); *Veillonella* (*atypica, dispar*).

known to contain aerobic and microaerotolerant members, with the remaining genera containing obligate or facultative anaerobes. The *Staphylococcus* species identified, *S. saccharolyticus*, is anaerobic (23, 24) and therefore only those isolates in the genera *Streptococcus* and *Rothia* were considered microaerotolerant. Accordingly, obligate or facultative anaerobic bacteria were isolated from 37 of 66 (56%) sputum samples, representing 28 of 50 (56%) of the patients examined (*see* Table E1). Aerobic bacteria were isolated from all sputum samples; identification of these aerobes revealed 10 different genera, with *P. aeruginosa* and bacteria in the genera *Staphylococcus*, *Streptococcus*, and *Rothia* most frequently isolated (Table 1).

Anaerobes were cultured from 27 of 39 (69%) samples from which *P. aeruginosa* were cultured. In contrast, anaerobes were only cultured from 11 of 27 (41%) samples from which *P. aeruginosa* were not cultured ($P < 0.01$, chi-square analysis). For sputum samples in which both *P. aeruginosa* and an anaerobe were isolated, comparison of the total viable counts revealed that, in 21 of 27 (78%) samples, the anaerobe or anaerobes were present in equal or greater numbers than *P. aeruginosa* (Figure 1).

Characteristics of Adult Patients with CF

The mean (range) age and lung function (FEV_1) of the patients was 26.5 (18–50) years and 1.96 (0.53–3.96) L, respectively. For further analysis, patients were subdivided into two groups: group 1, anaerobes cultured from sputum, and group 2, anaerobes not cultured from sputum. There was no significant difference (independent *t* test) in lung function in patients from whom anaerobes were (mean FEV_1, 2.05 L) and were not cultured (mean FEV_1, 1.81 L). Patients in both groups received similar intravenous antibiotics for treatment of an acute exacerbation, which was usually a combination of tobramycin and ceftazidime, with some patients receiving meropenem, aztreonam, or piperacillin/tazobactam as an alternative to ceftazidime. There was also no significant difference (independent *t* test) in the time from last treatment of an acute exacerbation with intravenous antibiotics between patients in whom anaerobes were cultured (mean time, 31 wk) and not cultured (27 wk). Details of chronic maintenance antibiotic treatment were available for 63 of 66 samples collected with patients primarily receiving azithromycin and colomycin, either alone or in combination. There was no relationship between use of maintenance antibiotics and culture of anaerobes, with anaerobes detected in 20 of 34 (59%) samples in which chronic antibiotics were used and in 19 of 29 (66%) samples in which chronic antibiotics were not used.

Isolation and Identification of Bacteria in BALF from Pediatric Patients with CF

BALF samples were collected from 10 pediatric patients with CF with a mean (range) age of 9.2 (2–14) years. The mean (range) lung function of these patients, as determined by FEV_1 and percentage predicted for age and height, was 1.78 (1–2.8) L and 83% (42–117%), respectively. No bacteria were detected in 2 of 10 samples, with aerobic and anaerobic bacteria detected in the remaining samples in lower numbers than in sputum from adult patients with CF. The most frequently isolated aerobic bacteria was *S. aureus*, which was present either alone or concomitantly with bacteria including *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Streptococcus* spp., and *Rothia* spp. in five of eight remaining samples. Bacteria that grew anaerobically

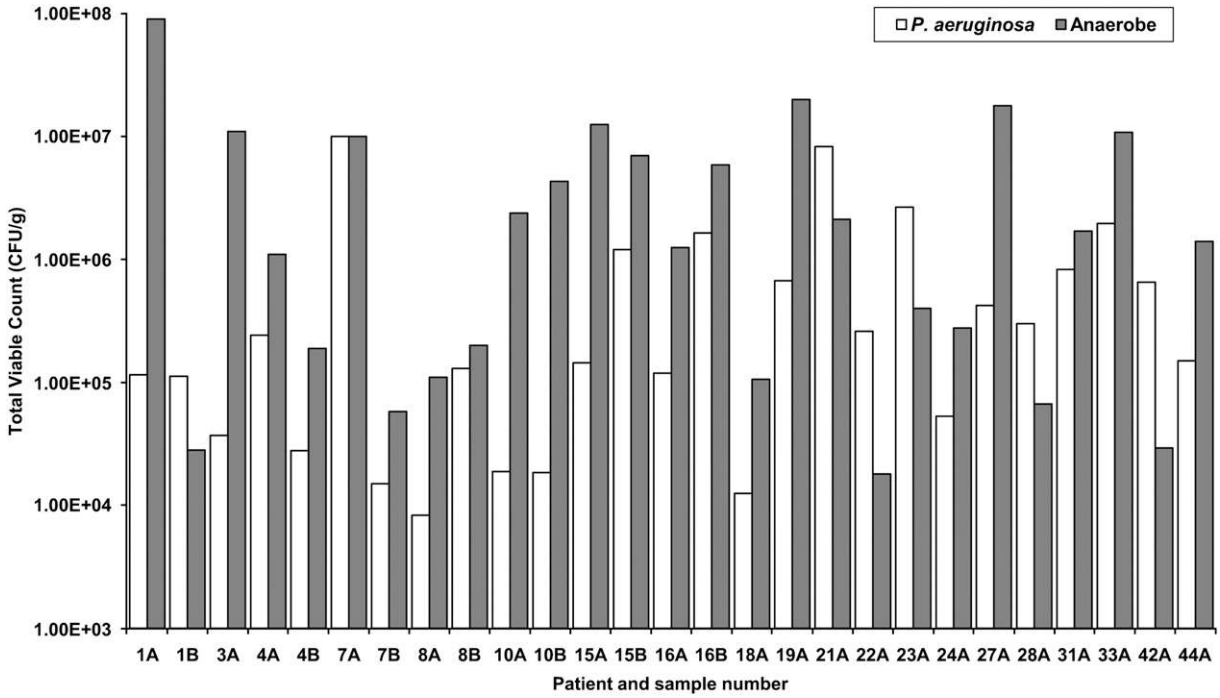

Figure 1. Comparison of total viable counts per gram of sputum of *P. aeruginosa* and anaerobic isolates cultured from the sputum of adult patients with cystic fibrosis. After treatment with Sputolysin (Calbiochem) for 15 minutes, sputum samples were processed using strict anaerobic bacteriologic techniques, and bacteria within the samples were detected by plating on selective agars, quantified by total viable count and identified by colony polymerase chain reaction and sequencing of 16S ribosomal RNA genes. Anaerobes were cultured from 27 of 39 (69%) samples from which *P. aeruginosa* was cultured, and in 21 of 27 (78%) samples, the anaerobe(s) were present in equal or greater numbers than those of *P. aeruginosa*. When multiple sputum samples were processed from the same patient, they are identified alphabetically in the order in which they were processed. If a sputum sample contained more than one *P. aeruginosa* or anaerobic isolate, only the isolate with the highest total viable count is presented.

were detected in five of eight samples from which aerobic bacteria were cultured. Anaerobes detected belonged to the genera *Prevotella*, *Veillonella*, *Propionibacterium*, and *Atopobium* (*see* Table E2).

Isolation and Identification of Bacteria from Induced Sputum from Healthy Volunteers

Induced sputum samples were collected from 20 healthy adult volunteers with a mean (range) age of 29.3 (21–54) years. Anaerobes were not detected in the induced sputum samples from four volunteers, and in the remaining samples they were present in much lower numbers, ranging from 10^2 to 10^5 cfu/g of sputum, compared with CF sputum (*see* Table E3). The total bacterial count for anaerobic bacteria in induced sputum from healthy volunteers was significantly less than that in expectorated sputum samples from patients with CF ($P = 0.037$, Mann-Whitney test). The most commonly isolated bacteria from these induced sputum samples were the *Actinomyces* and *Streptococcus*, with *Prevotella* cultured from only two samples and no *Veillonella* or *Propionibacterium* cultured. When bacteria in genera considered to be aerobic or microaerotolerant are excluded, anaerobic or potentially anaerobic bacteria were isolated from 10 of 20 (50%) induced sputum samples.

Antimicrobial Susceptibility

Although the MIC_{50} for all antibiotics tested were within the susceptible range, meropenem was the only antibiotic for which the MIC_{90} fell within the susceptible range (Table 2). Examination of the susceptibility data by genera shows species-dependent differences in susceptibility to antibiotics with known activity against anaerobes. *Propionibacterium* were the most resistant to metronidazole ($MIC_{50} > 256$ mg/L), whereas the *Veillonella* were the most resistant to piperacillin/tazobactam ($MIC_{50} > 256/4$ mg/L). Furthermore, a number of *Prevotella* spp. isolates were resistant to ampicillin, clindamycin, and metronidazole, with several isolates demonstrating high levels of resistance (MIC > 256 mg/L) to clindamycin and metronidazole.

DISCUSSION

This study is the first to use strict anaerobic bacteriologic culture techniques to systematically determine, in a large number of patients with CF, if anaerobic bacteria are present in the CF lung. Our results provide compelling evidence that the lungs of patients with CF are not only chronically colonized with pathogens, such as *P. aeruginosa*, but also by a range of other bacterial species, many of which are anaerobes, with a high frequency of the patients with stable CF examined having numbers of anaerobes in sputum equal to or greater than those of *Pseudomonas*. In a number of samples, more than one anaerobic species or genus was detected, indicating that the anaerobes exhibited far greater microbial diversity than previously associated with the CF lung. Furthermore, we detected similar anaerobic species in multiple samples collected from the same patients at different time points, which suggests persistence of these bacteria within the CF lung. These culture results support the results of recent molecular studies, which also indicated that there is a highly diverse and metabolically active bacterial community within the CF lung, with many different bacterial species, including anaerobes, present (8–10, 25).

Many potentially opportunistic pathogenic anaerobic species were detected in high numbers by culture, with *Prevotella* species most frequently isolated, followed by *Actinomyces*, *Propionibacterium*, and *Veillonella* spp. Similar species were among the most prevalent anaerobes previously detected in culture studies that examined sputum (7), transtracheal aspiration (5), and thoracotomy samples (6) from small numbers of patients with CF. Moreover, the predominant species detected by culture in the present study have also been detected in a high proportion of patients with CF by molecular analysis (8–10, 25). For example, *Prevotella* spp. identified by culture in the present study (e.g., *P. salivae*, *P. melaninogenica*) were also detected by Rogers and colleagues in sputum samples from 57 to 71% of patients using terminal restriction fragment length polymorphism (8), providing further convincing evidence that anaerobes are present in the sputum of patients with CF. The anaerobes and the facultative aerobes detected by culture in the present study and previously by molecular methods were those that would normally be found colonizing the oropharynx (26, 27). It is possible, therefore, in patients with CF who have impaired mucociliary clearance mechanisms, that these bacteria are carried from the oropharynx into the lower airways where they colonize the lung and potentially contribute to infection and lung damage. Furthermore, the anaerobes detected were similar to those found in other studies of anaerobic pulmonary infection, such as nosocomial pneumonia (11, 12), lung abscesses (13, 14), and empyema (15), where aerobes and anaerobes were present in a polymicrobial infection and were considered to be of significance.

Colonization with *P. aeruginosa* significantly increased the likelihood that anaerobic bacteria would be present in the sputum. This suggests that a preceding bacterial infection, with *Pseudomonas* spp., renders airway secretions frankly anaerobic and creates the environment required for subsequent anaerobic infection. Consistent with this hypothesis is the observation that proliferation of *P. aeruginosa* within mucus creates anaerobic conditions in the lungs of patients with CF with persistent res-

TABLE 2. ANTIMICROBIAL SUSCEPTIBILITY OF ANAEROBIC BACTERIA ISOLATED FROM THE SPUTUM OF ADULT PATIENTS WITH CYSTIC FIBROSIS

	Ampicillin			Clindamycin			Meropenem			Metronidazole			Piperacillin/Tazobactam		
Genera (no. isolates)	MIC_{50}	MIC_{90}	Suscept* (%)	MIC_{50}	MIC_{90}	Suscept (%)	MIC_{50}	MIC_{90}	Suscept (%)	MIC_{50}	MIC_{90}	Suscept (%)	MIC_{50}	MIC_{90}	Suscept (%)
All isolates (39)	0.25	16	67	0.25	> 256	79	0.064	0.5	100	8	> 256	53	0.38	> 256	87
Prevotella (14)	0.094	24	64	0.19	> 256	64	0.047	0.094	100	0.094	> 256	54	0.125	2	93
Veillonella (5)	0.25	1	60	0.125	1	100	0.094	0.19	100	0.75	1	80	> 256	> 256	20
Propionibacterium (5)	0.064	0.125	80	0.032	0.19	100	0.064	0.25	100	> 256	> 256	20	0.094	0.5	100
Actinomyces (8)	0.125	1.5	62.5	0.19	2	87.5	0.032	1	100	8	64	62.5	0.038	6	100
Other (7)	0.125	1.5	71	0.19	> 256	71	0.047	0.125	100	6	> 256	43	0.25	0.5	100

Definition of abbreviations: MIC_{50} = minimum inhibitory concentration required to inhibit growth of 50% of isolates; MIC_{90} = minimum inhibitory concentration required to inhibit growth of 90% of isolates; Suscept = susceptibility.

* Percentage of isolates susceptible calculated using the Clinical and Laboratory Standards Institute breakpoints for antimicrobial susceptibility of anaerobic bacteria.

piratory infection (3). Furthermore, *P. aeruginosa* has been previously shown to rapidly reduce oxygen levels in media during growth under either batch or continuous culture conditions (28).

In addition to aerobic bacteria, such as *P. aeruginosa* and *B. cepacia* complex, which are assumed to be the key pathogens in CF pulmonary infection, a diverse array of other potentially pathogenic facultative bacteria, including *Streptococcus* spp. and *Rothia dentocariosa*, were also detected in the sputum of patients with CF. These facultative aerobes would not be routinely considered as primary pathogens in CF pulmonary infection, with the presence of *Streptococcus* spp. in CF sputum conventionally believed to be as a result of oral contamination (29). However, in the present study, these bacteria were detected in sputum in numbers equal to *Pseudomonas*, which suggests that they may be contributing to infection and lung damage. This finding is similar to that reported by Duan and colleagues (30) who also performed quantitative microbiology on CF sputum samples and found that the concentration of oropharyngeal strains, such as *Streptococcus* and *Staphylococcus* spp., was regularly equal to or higher that that of *Pseudomonas*. Interestingly, Duan and colleagues (30) also demonstrated that the presence of *Streptococcus* spp. isolated from the sputum of patients with CF enhanced lung damage caused by *P. aeruginosa* in a rat lung infection model (30). Furthermore, *R. dentocariosa* has been described previously in human infection (31) and more recently is being recognized as a cause of bacteremia (32) and endocarditis (33). It is unlikely that these facultative aerobes would be isolated in routine diagnostic microbiology laboratories where the types of bacteria monitored are limited (34).

Because anaerobic bacteria constitute a major component of the normal oral microbiota, concerns have been expressed regarding contamination of sputum samples by the anaerobic microbiota during sampling. Indeed, the majority of the anaerobic species cultured from sputum in this study have been associated with the oropharynx (26, 27). However, comparison of the total viable count of the anaerobes and *P. aeruginosa* isolated from each sample revealed that, in a high percentage of samples, the anaerobe or anaerobes were present in equal or greater numbers than *P. aeruginosa*. Because *P. aeruginosa* is recognized as a primary pathogen frequently isolated from CF sputum, it is extremely unlikely that a significant number of anaerobic bacteria would be acquired during expectoration to equal or exceed the number of *P. aeruginosa* present in the sputum. Although too invasive for routine culturing (35), we also collected and processed a number of samples from pediatric patients using BAL. In addition to being recognized as an extremely useful method for detecting the bacterial flora present in the lower airways of patients with CF (25, 36), it has the added advantage that it greatly reduces the risk of oral or upper respiratory tract contamination associated with sputum sample collection (36). Bronchoscopy was performed using a laryngeal mask airway to further reduce risk of contamination. Significantly, although present in lower numbers in BALF, we cultured similar anaerobic species in BALF and sputum samples. Given that the total viable count of aerobes detected in BALF was also less than in sputum, it is likely that the lung secretions have been diluted by lavage fluid. Estimates of dilution of lung secretions in children with CF range from 9- to 78-fold (37). Furthermore, although only a small number of samples were processed, anaerobes were detected in the lungs of pediatric patients with CF in the absence of *P. aeruginosa*. This suggests that anaerobes may cause early infection and then, with time, produce an environment favorable for subsequent infection with *P. aeruginosa*. As a further control for potential oral contamination, induced sputum samples from healthy volunteers who did not have CF were examined for the presence of anaerobes. Significantly, anaerobes were present in lower numbers and with different species, as compared with CF, with no *Veillonella* and *Propionibacterium* species isolated, and *Prevotella* species isolated infrequently. Given that the salivary content of induced sputum samples collected from healthy control subjects is much greater than that of expectorated sputum samples collected from patients with CF (38, 39), these results taken together with the culture results from BALF samples, clearly indicate that the anaerobic bacteria cultured from CF sputum samples were derived from the lungs and were not contaminants derived from the anaerobic oral microbiota. Furthermore, they confirm the results of a recent molecular study that compared the bacterial communities in the oral cavity and the lungs of patients with CF and found that sputum samples were not contaminated to a significant effect by bacterial species found within the oral cavity (10).

Although lung function was similar in patients from whom anaerobes were and were not cultured, the presence of anaerobes in the lungs of patients with CF could still be of important clinical relevance, both as a pure anaerobic infection and in coinfection with *P. aeruginosa*. The anaerobic bacteria that we have detected in the sputum of patients with CF possess several virulence factors that may be important in the pathogenesis of CF pulmonary infection. These factors include the following: secretion of a variety of extracellular enzymes, such as proteases and β-lactamases, which may have a detrimental effect on host defense mechanisms (40–42) and antibiotics (43), respectively; capsule production (44); biofilm formation (45–47); neutrophil chemotaxis (48); and resistance to phagocytosis (49). Furthermore, anaerobes, even when present in low numbers in mixed infections, such as chronic sinusitis and lung abscesses, interact synergistically with aerobic or facultative bacteria and enhance virulence (30, 50).

It follows that if the aerobic and anaerobic bacterial species detected in our study are contributing to infection and inflammation in the CF lung, quantitative microbiology, performed under strict anaerobic conditions, would be necessary to detect them in sputum. Although quantitative microbiology performed under strict anaerobic conditions is time consuming and labor intensive, with appropriate facilities, well-developed and validated standard operating procedures, and necessary expertise, it would be feasible to perform in routine clinical microbiology laboratories.

Moreover, if anaerobic bacteria are contributing to a polymicrobial infection within the CF lung, their presence may require a change in the antibiotics used for both treatment of pulmonary exacerbations and for eradication of initial colonization to ensure optimal outcome for the patient. In the present study, the use of chronic maintenance antibiotic therapy had no effect on culture of anaerobic bacteria. This finding was not unexpected given the fact that the antibiotics used primarily for maintenance therapy—colomycin, azithromycin, and tobramycin—have poor activity against anaerobic bacteria, including those that we have cultured from CF sputum (51).

We determined the susceptibility of selected anaerobic isolates to a range of antibiotics with known activity against anaerobes. Because gram-positive anaerobic bacteria, such as *Actinomyces* and *Propionibacterium* species, are commonly resistant to nitromidazoles (52, 53), it was not surprising that a high percentage of isolates within these species were resistant to metronidazole. However, the finding that approximately half of the *Prevotella* isolates examined were resistant to metronidazole was unexpected given that metronidazole resistance among gram-negative anaerobic bacteria is reported to be extremely low (54). Interestingly, all of the anaerobic isolates tested were susceptible to meropenem, which is used in the treatment of CF pulmonary exacerbations caused by *P. aeruginosa*, where it shows some

superiority over other antipseudomonal antibiotics (55). These findings clearly demonstrate that antibiotics with known activity against anaerobic bacteria may not be effective against anaerobes isolated from CF sputum and, coupled with the need for better patient outcomes, highlights the importance of both culturing anaerobes from sputum and subsequently targeting antibiotic treatment against both anaerobes and aerobes colonizing the lungs of patients with CF.

In summary, the results of this study show that a range of potentially pathogenic anaerobic species is present in large numbers in the lungs of patients with stable CF. If these anaerobic bacteria are contributing significantly to infection and inflammation in the CF lung, informed alterations to antibiotic treatment to target anaerobes, in addition to the primary colonizing pathogens, may improve the management of infective exacerbations.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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