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Claudia Rintelen, Marianne Borneff-Lipp, Dieter Worlitzsch*

Institute for Hygiene, University Hospital of the Martin-Luther-University of Halle-Wittenberg

Strictly anaerobic bacteria in sputum of cystic fibrosis children

Summary

Background: The facultative anaerobes *Pseudomonas aeruginosa* and *Staphylococcus aureus* are held responsible for chronic lung infections in patients with cystic fibrosis (CF). Recently also strictly anaerobic bacteria were found in adult CF patients. However, it remains unclear whether these bacteria already are present in CF children.

Method: We identified and quantified facultative and strict anaerobes in 92 sputum samples of 8 children and 31 CF adults. In addition, bronchoalveolar lavage (BAL) samples of 6 children with lung diseases other than CF and throat swabs of 7 CF patients were tested.

Result: In most of the CF children (75.0 % of the patients, 78.6 % of the sputum samples), strictly anaerobic bacteria were detected. This came close to the numbers in adults (93.5 % of the patients, 94.6 % of the samples). Bacterial counts for strict anaerobes were similar for children and adults ($5.6 \times 10^6 \pm 4.5 \times 10^6$ vs. $1.5 \times 10^7 \pm 6.2 \times 10^7$ KBE/ml, p=0,629). 15 genera with 35 species were identified in both CF children and adults. In merely one non-CF BAL and in 42.9 % of the throat swabs strict anaerobes were found.

Conclusion: The detection of strict anaerobes in most CF children implies that anaerobic growth conditions are common already early in the life of CF patients. This fact most likely will influence bacterial phenotypes and susceptibilities. However, the involvement of the strict anaerobes in CF lung infection has to be proven before specific therapies directed against strict anaerobes are performed.
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Introduction

Cystic

Cystic fibrosis (CF), which is also known as mucoviscidosis, is an autosomal-recessive hereditary disease. With an incidence of 1:2,500 newborn babies, it is the most common severe metabolic disease seen in western industrialised countries [1,2]. In 1989 the gene responsible for CF was identified on the long arm of chromosome 7 [3]. It codes for a protein known as cystic fibrosis transmembrane conductance regulator (CFTR) and in normal mucus-secreting epithelia it serves as a chloride channel [3]. To date, more than 1,500 different mutations of the CFTR gene have been identified. Whereas the majority of extrapulmonary clinical manifestations of the disease can be successfully treated at present, the main cause of premature death in CF patients is chronic lung infection [1,2,4]. While, today, patients have a considerably better prognosis compared with a few years ago, [5], the average life expectancy continues to be less than 35 years [6]. Despite the use of various antibiotics, CF continues to be a severe and potentially fatal disease [1, 7].

Chronic bacterial lung infection in CF patients

The lungs of CF patients are prone to recurrent respiratory infections [7,8]. The mot typical feature of such a disease is colonisation of the lungs with the facultative anaerobes *Pseudomonas aeruginosa, Staphylococcus aureus, Burkholderia cepacia* complex bacteria and *Haemophilus influenzae*. With advancing age, these bacteria cause increasingly more severe chronic respiratory infections accompanied by inflammation and irreversible loss of lung function [8]. Again, with advancing age of CF patients [8], the predominant cause of infection is *P. aeruginosa* (Figure 1). *P. aeruginosa* can grow as a biofilm on the surface of the respiratory epithelia [9]. The oxygen concentration in the biofilm decreases [10,11,12], giving rise to anaerobiosis, due to the fact that the oxygen supply is used up by bacteria, by the cells of the patient's immune system and of the respiratory epithelia [9]. This expanding anaerobiosis forces the local bacteria to undergo profound changes. To ensure optimal growth under changing environ-

***Corresponding author**

Dr. med. Dieter Worlitzsch
Institute for Hygiene
University Hospital of the
Martin-Luther-University
of Halle-Wittenberg
Email:
dieter.worlitzsch@medizin.uni-halle.de

mental conditions, microorganisms have to undergo myriad adaptations, giving rise to a new, more environmentally resistant phenotype [13]. Bacteria can survive under unfavourable environmental conditions only by altering their virulence characteristics and phenotype via regulatory genes [14]. *P. aeruginosa* responds to this changing environment by forming stable, alginate-producing genotypes [15]. By doing so, *P. aeruginosa* and other facultative anaerobic bacteria can persist in the hypoxic CF lungs despite antibiotic treatment [16]. The increasing anaerobiosis detracts from the efficacy of antibiotics [16–21]; for example, in the biofilms of CF lungs a reduced sensitivity of *P. aeruginosa* to tobramycin has been observed [10,20,22].

Strict anaerobic bacteria in CF patients

For a long time now it has been known that aerob-anaerobic mixed infections can be seen in a number of diseases [23]. When bacteria are exposed to oxygen, oxygen radicals are formed by a cascade of enzyme-catalysed reactions. These are toxic to bacteria; to render them harmless, they are converted to water and oxygen by the enzymes catalase and peroxide dismutase. Neither of these enzymes is present, or is present only in minute quantities, in strict anaerobes [23,24]. But by consuming oxygen, facultative anaerobes can reduce the oxygen concentration of tissues to such an extent as to permit multiplication of strict anaerobes [23].

Various research groups have collected data on the existence of strict anaerobic bacteria in CF sputum samples [22,25,26]. Rogers et al. identified rRNA of *Bacteroides gracilis, Eubacterium brachy, Mycoplasma salivarium, Porphyromonas spp., Prevotella salivae, Pr. melanogenica, other Prevotella spp.* and *Veillonella atypica* in CF sputum [25]. In 57% of the CF sputum samples examined, Harris found rRNA coding for *Prevotella spp*. [26]. In 75% of the CF sputum samples investigated, Field et al. found strict anaerobes of the genera *Bifidobacterium, Prevotella, Veillonella, Peptostreptococcus* and *Fusobacterium* [22]. The anaerobes identified were bacteria which had also been implicated in other studies as the causative organisms of anaerobic lung infections such as aspiration pneumonia [23], nosocomial pneumonia [27,28] and lung abscesses [29]. While the rRNA techniques mentioned above lend themselves very well to bacterial identification, they provide no insights into the number of strict anaerobes implicated. To date, it therefore remains unclear whether these strict anaerobes are derived from oral contamination or are due to multiplication of bacteria in the CF lung since strict anaerobes are often found, too, in the oral cavity [30,31,32]. Nor has it been clarified to date whether identical strict anaerobes can be found in patients only sporadically or over long periods of time. In particular, no studies have been conducted so far on microbiological colonisation of the lungs of CF children. Against that background, the present study investigated to what extent strict anaerobes co-exist together with facultative anaerobes such as *P. aeruginosa* and *S. aureus* in the sputum of CF children and adults. Furthermore, all facultative and strict anaerobes were precisely identified and quantified. Bronchoalveolar lavages (BALs) from children with lung diseases other than CF were examined for their microbial content. Moreover, tests were conducted to elucidate whether the sputum was being contaminated with strict anaerobes from the oral cavity or whether the bacteria were growing in the sputum of CF patients.

Materials and Methods

Patients

Thirty-nine patients from Martin-Luther University Hospital of Halle-Wittenberg participated in the study: 31 adults from the University Hospital's CF outpatient department and from the Polyclinic for Internal Medicine II (Prof. Dr. med. Bernd Osten, senior physician Dr. Bettina Wollschläger) and eight children from the children's CF outpatient department of the University Hospital and Polyclinic for Paediatric and Adolescent Medicine (Prof. Dieter Körholz, senior physician Dr. Nick Merkel). These were five girls and three boys as well as 20 women and 11 men. The average age of patients was 25.3 ± 9.0 for adults and 12.9 ± 4.3 for children. At regular intervals of between three and four months the patients were requested to present for follow-up examination. Only in exceptional cases did they attend more often for an outpatient consultation (e.g. in the event of any exacerbations).

To ascertain whether strict anaerobes were also present in the lungs of non-CF children, aliquots from BALs of six children (four boys and two girls aged 7.0 ± 5.2 years) were investigated for bacteria. All children suffered from different pulmonary diseases, other than CF. Five of these children had acute symptoms. The other patient suffered from chronic-recurrent, right side, atelectasis with retention pneumonia and status post peripartal aspiration and cerebral damage and scoliosis. Only the sixth patient produced copious amounts of purulent secretions. None of the children had received antibiotic treatment.

All patients gave informed written consent to participate in the study. Consent was given by the parents /guardian(s) of underage patients. The study was presented to the ethics committee of the Department of Medicine at the Martin-Luther University Hospital of Halle-Wittenberg.

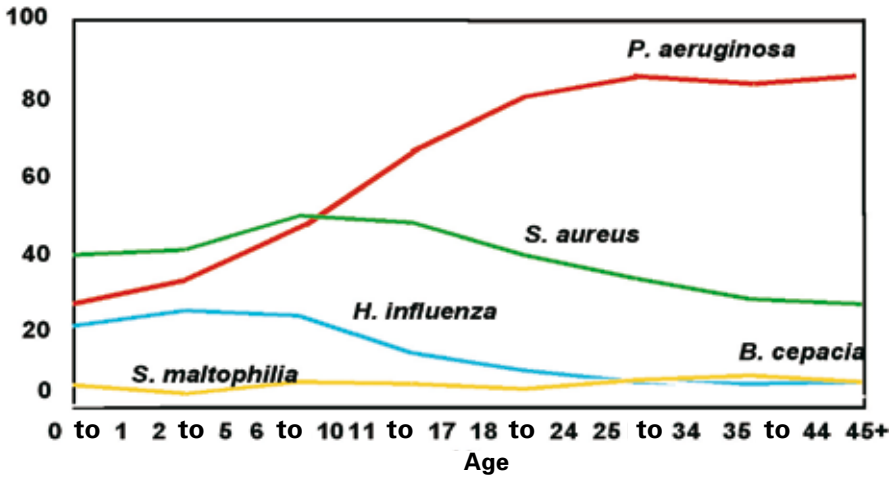

Figure 1: Age distribution of CF patients with bacterial lung colonization (as per [8]).

Sputum samples

A total of 92 sputum samples were collected and processed using a standardised method. At the time of routine follow-up examinations the CF patients were given sterile Falcon tubes and requested to produce a spontaneous (non-induced) sputum sample into these tubes.

Throat swabs

To demonstrate that sputum was not contaminated by bacteria from the oral cavity / throat during expectoration, throat swabs were taken from seven patients. The throat swabs were taken at as deep a level as possible using cotton tips. Bacteria were identified and quantified using the same procedure as for the sputum samples.

Culture media

Non-selective Columbia agar with sheep blood was used to culture facultative anaerobes for identification and quantification purposes, and a selective medium comprising cetrimide agar (Oxoid) was used for *P. aeruginosa* and *B. cepacia* complex bacteria. Various non-selective agar media were used to identify and quantify strict anaerobes: rich Schaedler agar with 5 % wether's blood (SCS, bioMérieux® sa) and non-selective CDC agar (Becton Dickinson, Heidelberg) without dextrose. In addition, Brain Heart Infusion agar (BHI, heipha Dr. Müller GmbH, Eppelheim) was used for culture of strict anaerobes.

Quantitative detection of facultative and strict anaerobes

Duplicate dilution series were produced from the sputum samples on the different agar media. The Columbia agar plates were incubated for a maximum of 24 hours at 37 °C. The following day up to three phenotypically different facultative species of anaerobic bacteria were counted. The strict anaerobic bacteria were incubated for up to 7 days at 37 °C using an anaerobic bank (Meintrup dws, Lähden-Holte). The oxygen-free mixture of gases contained 80 % nitrogen, 10 % hydrogen and 10 % carbon dioxide. Up to four phenotypically different types of strict anaerobic bacteria were counted. All bacteria were subjected to three series of growth control tests in room air to rule out facultative or aerotolerant anaerobes. Only when no growth was seen were they classified as strict anaerobes and further analysed. Sputum samples with anaerobic bacterial counts of $< 1 \times 10^5$ cfu/ml were not included in the evaluation. But that was the case for only one sample.

Identification of facultative anaerobic bacteria

To identify facultative anaerobic bacteria overnight cultures were examined using other tests. Depending on the type of bacteria, an oxidase test or coagulase test was performed. In addition, a catalase test (bioMérieux® sa) was conducted for staphylococci. Gram specimens were needed for identification of facultative anaerobes with the Vitek® system (bioMérieux®). These fixed and stained specimens were examined under the microscope (100×, Axiolab, Carl Zeiss, Jena) and placed in the Vitek® system in accordance with the manufacturer's instructions. After incubating for between 2 and 18 hours samples were evaluated while citing the probable accuracy of results. In the event of identification probabilities < 85 %, an additional confirmatory test was conducted with the BBL-Crystal™ identification system (Becton Dickinson) (probability limit: 80 %).

Identification of strict anaerobic bacteria

Strict anaerobic isolates were identified using the biochemical identification system RapIDTM ANA II and associated software (ERIC® Software, remel). A freshly cultured isolate was placed in the RapIDTM panel and incubated for four hours at 37 °C. The data collected for bacteria were processed using the Eric® software or information on them was obtained by consulting the RapID™ ANA II compendium. In the event of identification probabilities < 80 %, the entire process was repeated. Bacteria with an identification probability of < 80 % on retesting were excluded from further investigation. This was true only for four isolates. The instructions of manufacturers of the identification systems were used for taxonomic and nomenclature purposes.

Statistics

The results were given as mean values ± standard deviation (Excel, Microsoft, Redmont, Wash, USA). Significant findings (results of Student´s t-test) were likewise processed with Excel. Any p-values < 0.05 were deemed to be significant. Pearson´s correlation coefficient R2 was calculated with winstat (Microsoft) for correlation purposes.

Results

Quantitative detection of facultative and strict anaerobic bacteria in 92 sputum samples from 39 CF patients (78 samples from 31 adults and 14 samples from eight children) were investigated for the presence of facultative and strict anaerobic bacteria. Facultative anaerobes such as *P. aeruginosa, S. aureus* and *B. cepacia* complex bacteria were detected in 83 of the samples collected (90.2 %). Only in nine samples (9.8 %) were no facultative anaerobes found. The mean count of facultative anaerobes was $4.4 \times 10^7 \pm 9.0 \times 10^7$ cfu/ml (range 1×10^5 to 4×10^8 cfu/ml) (Figure 2). In 75 sputum samples (81.5 %) and in 35 out of 39 patients (89.7 %) strict anaerobic bacteria were detected. The mean bacterial counts were $1.3 \times 10^7 \pm 5.6 \times 10^7$ cfu/ml and ranged from 1×10^5 to 5.5×10^8 cfu/ml (Figure 2). The number of facultative anaerobic bacteria was similarly high (Figure 2). While the number of facultative anaerobes was slightly higher than that of the strict anaerobic bacteria, there was no correlation between these two (R2=0.0009). To elucidate whether the presence of strict anaerobes in the sputum had an influence on the facultative anaerobes *P. aeruginosa* and *S. aureus*, the numbers of facultative and strict anaerobes were correlated with each other. Comparison of the bacterial counts in sputum, containing *P. aeruginosa* together with strict anaerobes (24 samples, $5.7 \times 10^7 \pm 1.1 \times 10^8$ cfu/ml), and sputum containing P. Aeruginosa but no strict anaerobes (11 samples, $4.9 \times 10^7 \pm 8.8 \times 10^7$cfu/ml) did not show any difference (p=0.825). The same held true for the sputum samples with S. aureus with (28 samples, $1.7 \times 10^7 \pm 3.2 \times 10^7$ cfu/ml) and without strict anaerobes (5 sputum samples, $9.3 \times 10^6 \pm 1.1 \times 10^7$ cfu/ml; p=0.628). The difference between the number of strict anaerobes in sputum samples with only *P. aeruginosa* and *S. aureus* was not significant statistically (p=0.584). Of the eight children investigated, 14 sputum samples were collected. Six children (75.0 %) harboured strict anaerobes in 9 sputum samples

(78.6 %). For the adults, strict anaerobic bacteria were found in 94.9% of samples. No significant differences were discerned between the bacterial counts found for CF adults and children (facultative anaerobes p=0.238; strict anaerobes p=0.629). The mean number of bacteria found in adults was $4.8 \times 10^7 \pm 9.4 \times 10^7$ cfu/ml for the facultative anaerobes and $1.5 \times 10^7 \pm 6.2 \times 10^7$ cfu/ml for the strict anaerobes. In the children the facultative anaerobe counts found were $8.6 \times 10^6 \pm 1.9 \times 10^7$ cfu/ml and the strict anaerobe counts were $5.6 \times 10^6 \pm 4.5 \times 10^6$ cfu/ml. Five successive sputum samples from one child were examined. Four strict anaerobes (*Peptostreptococcus prevotii, S. saccharolyticus, Actinomyces odontolyticus and Actinomyces turicensis*) were repeatedly found for up to 63 days. No significant differences were noted in the bacterial counts of the sputum samples from CF patients of different age groups (Figure 3). Only in the > 40 years' age group was the number of strict anaerobes less than that of younger age groups. For the former age group it was only possible to examine one sample from a 64-year-old CF patient. That patient's age was well over that of the average age of CF patient and was possibly attributable to a "mild" mutation [5].

Identification of facultative and strict bacteria in sputum samples

Of the total of 39 CF patients, 14 patients suffered from chronic *P. aeruginosa* infections and 14 from *S. aureus* infections. Three patients were concomitantly colonised with *P. aeruginosa* and S. aureus, three other patients harboured *B. cepacia* complex bacteria, and one patient *Stenotrophomonas maltophilia*. In 42 of the sputum samples microaerophilic streptococci were found. These bacteria are generally thought to derive from contamination from the upper respiratory tract [33,34] and were excluded from further evaluation. One or several strict anaerobes were found in 35 out of 39 patients (89.7 %). In the 75 positive sputum samples a total of 136 strict anaerobes were identified (belonging to 15 different genera and 35 species) (Table 1). Up to five different species were found per sputum sample. Only those bacterial counts of 1×10^5 cfu/ml or more were included in the study. For that reason one sputum sample was excluded. For 26 out of 39 CF patients (66.7 %) two or more (maximum six) sputum samples were evaluated. Whereas in nine of these patients (34.6 %) the same species was repeatedly found, up to three strict anaerobic species were concomitantly found in two patients. An identical species was found repeatedly for up to 11 months. The most commonly identified genera were *Peptostreptococcus spp.* (32×), *Staphylococcus spp.* (23×), *Actinomyces spp.* (19×), *Veillonella spp.* (14×), *Clostridium spp.* (9×), *Streptococcus spp.* (8×) and *Bacteroides spp.* (7×). Eight other genera were seen in a total of 24 times (Table 1).

Throat swabs and comparison of organisms with those found in the sputum samples

Throat swabs were taken for seven CF patients. The patients' sputum samples harboured both facultative anaerobes as well as one or several types of strict anaerobes. The bacterial counts in sputum were $1.4 \pm 1.5 \times 10^7$ cfu/ml for facultative and $3.8 \pm 5.8 \times 10^7$ cfu/ml for strict anaerobes. *P. aeruginosa* was found in all throat swabs ($7.3 \times 10^3 \pm 9.9 \times 10^3$ cfu/ml, Figure 2). In three out of seven throat swabs strict anaerobic bacteria were found ($7.5 \times 10^3 \pm 1.1 \times 10^4$ cfu/ml). Compared with sputum, the bacterial counts in throat swabs were lower by three orders of magnitude. That rules out contamination of the sputum with bacteria from the oral cavity (Figure 2).

Bronchoalveolar lavage

To investigate whether the presence of strict anaerobes in respiratory samples were specific to CF patients, diagnostic BAL was conducted for six children with lung diseases other than CF. The lungs were sterile for three patients. Streptococci, thought to be due to contamination from the upper respiratory tract, were identified in the BALs of the three other patients [33,34]. In addition, lower counts of facultative anaerobic bacteria were found in the samples (maximum 4×10^4 cfu/ml). Only in the BAL of one patient with chronic retention pneumo-

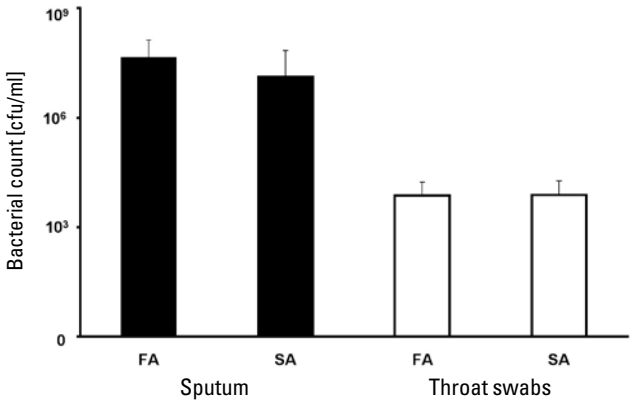

Figure 2: Number of facultative anaerobes (FA) and strict anaerobes (SA) in 92 sputum samples (black) and seven throat swabs (white).

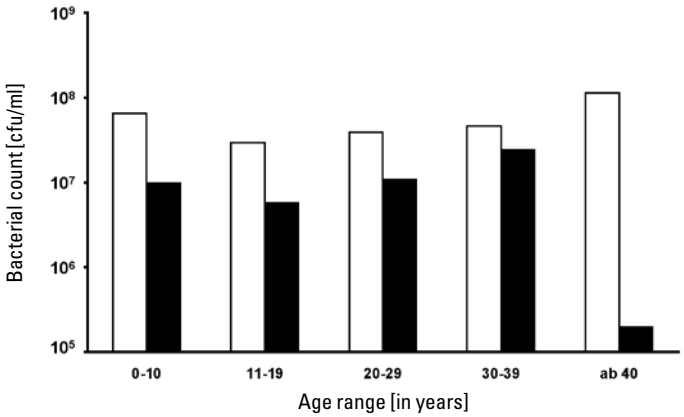

Figure 3:Mean number of facultative anaerobes (white) and strict anaerobes (black) in 39 CF patients belonging to five different age ranges

nia and purulent secretions was *S. aureus* (2×10^2 cfu/ml) found, together with the strict anaerobe *S. saccharolyticus* (1×10^2 cfu/ml). That finding suggests that large amounts of hypoxic sputum must be present in order for high counts of strict anaerobic bacteria to be found.

Discussion

Detection of strict anaerobes in the sputum of CF patients

Already in CF children, strict anaerobes can be found almost as often as in CF adults. Colonisation of the lungs with these bacteria is thus the norm for CF patients, and that is seen already at an early age. Those findings are largely in concordance with the results of studies carried out by other research groups [22, 25, 26]. While in this present study *Prevotella spp.* were not found as often as in the case of Harris et al. or Rogers et al., where *Prevotella spp.* were detected in up to 57 % [26] and 71 % [25] of sputum samples, respectively. These differences are possibly attributable to regional differences or different detection methods. Furthermore, it is possible that the rRNA method is endowed with higher sensitivity for detection of lower bacterial concentrations. Another explanation for these divergent results for detection of anaerobic bacteria may be due to the different taxonomy and nomenclature used for strict anaerobes.

Quantification of strict anaerobes

Very high bacterial counts were found for bacteria that grow under strict anaerobic conditions in CF sputum, with counts of $2.2 \times 10^7 \pm 6.9 \times 10^7$ cfu/ml (Figure 2) being seen. The use of the modern rRNA technique commonly used [25,26] was not able to rule out the possibility of oral contamination [30,31,32]. Since the sensitivity of that method is extremely high, it is possible that if the expectorated sputum comes into contact only briefly with the oral mucosa this is enough to produce a positive rRNA result for the presence of strict anaerobes.

Exclusion of oral contamination of the sputum

The fact that the number of strict anaerobes in the throat swab was less by more than three orders of magnitude than the sputum bacterial counts confirms that the

Table 1: Strict anaerobes in 92 sputum samples from CF (n=39).

Genus	Number	Species	Number
Peptostreptococcus spp.	32	*anaerobius*	6
		micros	1
		prevotii	19
		tetradius	6
Staphyloccocus spp.	23	*sacharolyticus*	23
Actinomyces spp.	19	*israelii*	2
		meyeri	3
		naeslundii	1
		odontolyticus	7
		turicensis	6
Veillonella spp.	14		14
Clostridium spp.	9	*bifermentans*	1
		butyricum	1
		clostridioforme	2
		difficile	2
		hastiforme	2
		innocuum	1
Streptococcus spp.	8	*constellatus*	7
		intermedius	1
Bacteroides spp.	7	*tectum*	6
		stercoris	1
Gemella spp.	4	*morbillorum*	4
Capnocytophaga spp.	3		3
Eubacterium spp.	3	*aerofaciens*	2
		limosum	1
Lactobacillus spp.	3	*acidophilus*	2
		jensenii	1
Mobiluncus spp.	3	*curtisii*	2
		mulieris	1
Prevotella spp.	3	*corporis*	1
		melaninogenica	2
Propionibacterium spp.	3	*acnes*	1
		granulosum	1
		propionicum	1
Wolinella spp.	2		2
Gesamtzahl	**136**		**136**

strict anaerobes, with virtually borderline certainty, were not due to contamination from the oral cavity, but rather that they had originated from the sputum, where they must have also multiplied to produce the bacterial counts measured. Nonetheless, strict anaerobes from the oral cavity can also serve as a source of lung infection, with this inevitably leading to multiplication in the sputum.

Investigation of broncholavages from patients with other pulmonary diseases

The majority of children for whom BAL samples were examined suffered from acute forms of pneumonia. Only in one of the children was the strict anaerobe *S. saccharolyticus* (1×10^2 cfu /ml) identified together with the facultative anaerobe *S. aureus* (2×10^7 cfu/ml). That 16-year-old patient had from birth suffered from chronic retention pneumonia and produced chronic purulent secretions, showing similar characteristics to those of the sputum collected from CF patients. That suggests that strict anaerobes are also possibly found in the lungs of patients with other chronic lung diseases, where sputum is produced, for example in chronic obstructive pulmonary disease (COPD) or bronchial carcinoma.

Persistence of the strict anaerobes detected

In 11 of 26 CF patients (42.3 %) from whom sputum samples were collected, the same anaerobic species was found repeatedly. Identical species were detected for a period of up to 11 months. Hence strict anaerobes, too, are able to persist for a long time in CF lungs, as has already been demonstrated for the facultative anaerobes *P. aeruginosa* and *S. aureus* [35,36]. Furthermore, our data show that strict anaerobes are present in the majority of CF patients, both adults and children. If mixed infections are present, the occurrence of strict anaerobes is promoted by the existence of facultative anaerobes such as *P. aeruginosa* or *S. aureus* . The latter bacteria use aerobic metabolism preferentially, thus protecting the strict anaerobes against oxygen, which is toxic to them [9,11,37,38]. But how the facultative anaerobes benefit from the presence of the strict anaerobes is something that to date has not been properly explained. The findings of this present study provide in principle new insights into the pathogenesis of chronic CF lung infection. The presence of a high number of strict anaerobes in the sputum of virtually all CF patients draws attention to the fact that the conventional models employed for the pathogenicity of CF lung infection are not adequate in terms of explaining the microorganisms involved. The fact that strict anaerobes can also be found in the majority of CF children would suggest that already at a young age the lungs of CF patients have anaerobic regions. All future studies focusing on the relationship between microorganisms and the immune responses seen in CF lungs should also take account of the strict anaerobes in addition to the facultative anaerobes. However, the findings available to date are not, in principle, enough to warrant radical changes in the treatment regimens of CF patients. While treatment with specific antibiotics, also endowed with efficacy against strict anaerobes, could improve treatment of chronic pulmonary infection, the role of strict anaerobes in CF pulmonary infection must first be identified before prescribing such a treatment regimen.

Conflict of Interest

The authors declare that there is no conflict of interest as understood by the International Committee of Medical Journal Editors.

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