Microbial ecology of the cystic fibrosis lung: does microflora type influence microbial loading?

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ABSTRACT

This study aims to examine the association between the numbers of culturable microbial species forming the microflora of the lung in patients with cystic fibrosis (CF) and microbial loading (i.e., type[s] versus numbers). Additionally, it examines qualitative combinations of the microflora present in a large adult CF centre (n=138) in order to ascertain ecological relationships between the taxa present. The culturable microflora of sputum from 34 adults patients with CF are enumerated using a spread plate technique on non-selective agar, and the microflora identified phenotypically employing the API 20NE scheme. Microbiological examination of the 34 adult patients demonstrated that their sputum contained between one and three taxa, with a mean cell density of 8.25±0.85 log colony-forming units (cfu)/g sputum and a range of 5.91–9.74 log cfu/g sputum. Most colonising patterns demonstrated only Gram-negative infection (22/34), followed by a mixed Gram-positive/Gram-negative infection pattern (10/34). Only 2/34 patients had a single Gram-positive infection. Most patients (53%) were colonised by only one organism, with 38% of patients colonised by two organisms, and the remainder (4%) colonised with three organisms. There was no statistical difference (P>0.05) between microbial cell density and the number of taxa present (i.e., the greater number of taxa present in sputum did not produce a higher cell density). However, there was a significantly higher cell density (log 0.59 cfu/g sputum) noted for those patients who had only Gram-negative infection, compared to those who had a mixed Gram-negative/Gram-positive infection pattern (P=0.02). Relatively little is known about the ecological interactions that exist between the microflora in the CF lung. Further work is required to explore these interactions in order to aid understanding of the succession and dominance of Gram-negatives in chronic chest infections. Ultimately, a greater understanding of such interactions may allow the opportunity to manipulate the ecology of the lung to control otherwise problematic pathogens

KEY WORDS: Burkholderia cepacia. Cystic fibrosis. Pseudomonas aeruginosa. Stenotrophomonas maltophilia

Introduction

Cystic fibrosis (CF) is the most commonly inherited lethal disease in persons originating from a Caucasian and European background and has a genetic carriage rate of 1 in 20 persons and an incidence of 1 in 2500 live births.[1] It is an autosomal recessive condition whereby two alleles carrying a polymorphism in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene phenotypically manifest the disease state through a variety of multiorgan problems, associated with a pharmacological dysfunction to regulate chloride-ion secretion across cell membranes.

The most common complication of CF is the recurrence of chronic chest infections, usually caused by bacterial pathogens.[2] Patients continue to suffer from recurrent and chronic respiratory tract infections and most of their morbidity and mortality is due to such infections throughout their life.[3] These infections are usually dominated by Gram-negative organisms, especially by the pseudomonads, including *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia*.

To date, the majority of CF clinics routinely monitor the microbiological status of patients through qualitative (presence/absence) examination of sputum specimens, employing a combination of selective and non-selective culture media. In contrast, there have been few reports in the literature on the microbial loading of sputum from CF patients, as there are relatively few centres that routinely examine the microbial loading of patients' sputum through quantitative conventional analysis.

Therefore, the main aim of this study is to examine the relationship between the resident microflora of the lung and the loading of these flora in the same lung. Additionally, the study will examine qualitative combinations of the microflora present in a large adult CF centre.

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Materials and methods

Quantitative enumeration of the culturable microflora in sputum
Duplicate sputa specimens (1 mL, minimum) were collected from 34 adult patients in sterile (100 mL) plastic disposable containers. Sputum was collected immediately after a standardised session of physiotherapy and was stored at

Table 1. Comparison of total quantitative microbiological counts from the sputum of 34 adult patients with cystic fibrosis with co-infecting/colonising microflora.

Single organism infection			Double organism infection			Triple organism infection		
Patient	Count*	Microflora present	Patient	Count*	Microflora present	Patient	Count*	Microflora present
2	9.08	*Pseudomonas aeruginosa*	1	8.17	*Streptococcus viridans*, *Burkholderia cepacia*	18	7.60	*Neisseria* sp. *Streptococcus viridans* Diphtheroids
3	8.00	*Haemophilus influenzae*	5	7.82	*Pseudomonas aeruginosa*, *Pseudomonas* sp.	19	8.79	*Burkholderia cepacia*, *Serratia marcescens*, *Pseudomonas aeruginosa*
4	5.91	*Staphylococcus aureus*	6	8.56	*Pseudomonas aeruginosa*, *Streptococcus viridans*	29	7.92	*Burkholderia cepacia*, *Candida* sp, *Neisseria* sp.
7	7.87	*Stenotrophomonas maltophilia*	8	9.58	*Pseudomonas aeruginosa*, *Burkholderia cepacia*			
10	8.36	*Burkholderia cepacia*†	9	8.29	*Burkholderia cepacia*, *Pseudomonas aeruginosa*			
12	9.41	*Pseudomonas aeruginosa*	11	8.33	*Streptococcus* sp., *Burkholderia cepacia*			
14	8.41	*Burkholderia cepacia*	13	6.90	Coliforms *Staphylococcus aureus*			
16	8.78	*Pseudomonas aeruginosa*	15	8.39	*Haemophilus influenzae*, *Pseudomonas aeruginosa*			
21	8.11	*Staphylococcus aureus*	17	8.75	*Pseudomonas aeruginosa*, *Streptococcus viridans*			
22	9.75	*Pseudomonas aeruginosa*	23	7.18	*Streptococcus viridans*, *Burkholderia cepacia*			
24	7.70	*Stenotrophomonas maltophilia*	28	7.41	*Staphylococcus aureus*, *Pseudomonas* sp.			
25	7.94	*Stenotrophomonas maltophilia*	20	8.04	*Streptococcus* sp. *Moraxella catarrhalis*			
27	7.00	*Staphylococcus aureus*	26	8.84	*Streptococcus* sp. *Burkholderia cepacia*			
30	9.15	*Pseudomonas aeruginosa*	20	8.04	*Streptococcus* sp. *Moraxella catarrhalis*			
31	8.86	*Pseudomonas aeruginosa*						
32	9.74	*Pseudomonas aeruginosa*						
33	7.30	*Pseudomonas aeruginosa*						
34	8.48	*Pseudomonas aeruginosa*						
Mean count (±1SD)		8.83±0.99 log cfu/g sputum			8.52±0.73 log cfu/g sputum			8.10±0.62 log cfu/g sputum
Range		5.91–9.74 log cfu/g sputum			6.90–9.58 log cfu/g sputum			7.60–8.79 log cfu/g sputum

*$\log_{10}$ colony-forming units/g sputum. †*B. cepacia* complex.

ambient temperature and processed within 4 h of collection. Fresh sputum (1 mL, minimum) was mixed with an equal amount of Sputasol (Oxoid SR089A, Oxoid, Poole, UK) and was incubated in a water bath at 37°C for 15 min, before further processing and enumeration. Serial dilutions of sputum were prepared in quarter strength Ringer's solution diluent (Oxoid BR52). From the 10^{-4}, 10^{-5} and 10^{-6} dilutions in triplicate, 100 µL inoculum was spread on the surface of Columbia agar base (Oxoid CM331) supplemented with 5% (v/v) defibrinated horse blood (E&O Laboratories, Bonnybridge, Scotland) and incubated at 37°C for 48 h in a 5% (v/v) CO_2 atmosphere prior to counting. All cultured flora, regardless of colonial morphology and appearance, were enumerated and the total viable count (TVC) was expressed as $\log_{10}$ colony-forming units (cfu) per gram (cfu/g) of original sputum.

Qualitative isolation of culturable microflora in sputum

In order to examine the qualitative presence of mono-, bi- or tri-microbial infections, freshly expectorated sputum from 139 adult patients was examined to determine its microbiological composition. Processed sputa (as above; 20 µL) were inoculated and incubated on several media, including Columbia blood agar (Oxoid CM0331) supplemented with 5% (v/v) defribinated horse blood, chocolate agar (incubated microaerophilically in 5% [v/v] CO_2), MacConkey agar (Oxoid CM0007), Pseudomonas isolation agar (PIA; Oxoid CM0559 + SR0102) and

Burkholderia cepacia-selective agar (BCSA; Mast DM253E + SV22, Mast Diagnostics, Merseyside, UK). All media were incubated aerobically at 37°C for 48 h unless otherwise stated. After an initial 48-h incubation, the PIA and BCSA plates were incubated at room temperature for a further three days. In addition, all different phenotypes from the sputum of each patient were identified phenotypically by a combination of conventional identification methods (e.g., oxidase) and API identification schemes (API 20NE, API 20E; bioMérieux, Les Halles, France).

Statistical analysis

Statistical analyses were performed by using an unpaired Student's *t*-test, where $P<0.05$ (5%) was considered significant.

Results

Microbiological examination of the 34 adult patients demonstrated that their sputum contained between one and three taxa, with a mean cell density of 8.25±0.85 log cfu/g sputum and a range of 5.91–9.74 log cfu/g sputum (Table 1). The mean count for *P. aeruginosa* was 8.95±0.75 log cfu/g sputum, with a range of log 7.30–9.74 cfu/g sputum. Most of the colonising patterns demonstrated sole Gram-negative infection (22/34), followed by a mixed Gram-positive/Gram-negative infection pattern (10/34), with only two patients showing a single Gram-positive infection.

Most patients (53%) were colonised by just a single organism, with 38% patients colonised by two organisms, and the remainder (4%) colonised with three organisms. There was no statistical difference ($P>0.05$) between microbial cell density and number of taxa present (i.e., the greater number of taxa present in sputum did not produce a higher cell density in sputum). However, there was a significantly higher cell density (log 0.59 cfu/g sputum) noted for those patients who had a single Gram-negative infection, compared to those who had a mixed Gram-negative/Gram-positive infection pattern ($P=0.02$).

Qualitative examination of a larger population of adult patients ($n=139$) demonstrated 25 infection/co-colonising patterns in 122 adult patients (Table 2), with 16 (11.3%) patients exhibiting no significant microbiological findings. Of these, *P. aeruginosa* and *B. cepacia* complex (BCC) organisms were the most commonly isolated pathogens, accounting for 47.1% and 22.5%, respectively.

Yeasts and filamentous fungi were isolated from 11/138 (8.0%) patients. In order to understand the possible ecological interactions between CF microbial pathogens and non-pathogenic co-colonisers, forming part of the microflora of the CF lung, Table 3 describes the potential microbe-microbe interactions that are occurring in the lung.

Table 2. Microbiological co-colonising/infection patterns isolated from the sputum of 139 adult patients with cystic fibrosis.

Microflora present	Number of patients	Percentage of adult CF population
Pseudomonas aeruginosa	50	36.2
Burkholderia cenocepacia	17	12.3
Staphylococcus aureus	14	10.1
Pseudomonas aeruginosa and *Staphylococcus aureus*	7	5.1
Stenotrophomonas maltophilia	5	3.6
Pseudomonas aeruginosa and *Burkholderia cenocepacia*	4	2.9
Burkholderia multivorans	3	2.2
Candida spp.	3	2.2
Burkholderia cenocepacia and *Staphylococcus aureus*	2	1.5
Candida sp. and *Burkholderia cenocepacia*	2	1.5
Aspergillus sp. and *Pseudomonas aeruginosa*	2	1.5
Pseudomonas fluorescens	1	0.7
Aspergillus sp.	1	0.7
Commensals	1	0.7
Escherichia colii	1	0.7
Haemophilus influenzae	1	0.7
Haemophilus parainfluenzae	1	0.7
Moraxella catarrhalis	1	0.7
Morganella morganii	1	0.7
Neisseria sp.	1	0.7
Aspergillus sp. and *Staphylococcus aureus*	1	0.7
Candida sp. and *Pseudomonas aeruginosa*	1	0.7
Haemophilus influenzae and *Burkholderia cenocepacia*	1	0.7
Staphylococcus aureus, *Burkholderia cenocepacia* and *Pseudomonas aeruginosa*	1	0.7
Candida sp., *Burkholderia cenocepacia* and *Pseudomonas aeruginosa*	1	0.7
No significant growth	16	11.3

Discussion

Chronic chest infections with bacterial respiratory pathogens, mainly *P. aeruginosa* and BCC organisms, are significant causes of morbidity and mortality in patients with CF.[2,3] Thus, it is important that stringent measures be taken in an attempt, by both the patient and the healthcare professional (following infection control guidelines), to prevent colonisation of the lung with these and other organisms.[4]

Although combination antibiotic therapy is the cornerstone of the treatment of such chronic infections, high levels of resistance have been described for Gram-negative organisms once they have been acquired by the CF patient.[5] Therefore, other approaches are being sought, such as promoting the disruption of biofilm formation in *B. cepacia* and *P. aeruginosa* through alteration of quorum sensing mechanisms, in order to help control bacterial infection.[6]

To date, there has been relatively little examination of the microbe-microbe ecological interactions of organisms in the CF lung; hence, it was the aim of this study to examine

Table 3. Possible ecological interactions between CF microbial pathogens and non-pathogenic co-colonisers, forming part of the microflora of the CF lung.

Ecological (microbe-microbe) interaction	Category	Effect on CF pathogen	Effect of non-pathogenic co-coloniser
Antagonism	I	Reduction in cell density	Increase in cell density
	II	Increase in cell density	Reduction in cell density
Neutral	III	No effect	No effect
Symbiosis	IV	Presence of non-pathogenic co-coloniser essential for growth and proliferation of pathogen	Presence of pathogen essential for growth and proliferation of non-pathogenic co-coloniser
Syngery	V	Presence of non-pathogenic co-coloniser, although not essential, promotes growth of pathogen, more so than effect of pathogen growing alone	Presence of pathogen, although not essential, promotes growth of non-pathogenic co-coloniser, more so than effect of non-pathogenic co-coloniser growing alone

the effect of simultaneous co-colonisation of the CF lung with one, two and three taxa on microbial loading, as well as to examine the infection patterns associated with a large adult CF unit. The data presented show that the microbial loading of the lung is not dependent on the numbers of different taxa present, whereby the presence of a single taxon gave an equal loading (approximately log 8 cfu/g sputum) of organisms as when two or three different taxa were present simultaneously. This is important because it suggests that chronic chest infections in CF patients are maintained at a steady state and it may be that additions and/or subtractions of taxa present or acquisition of new virulence determinants for the existing microflora are responsible for pulmonary exacerbations and not significant changes in microbial loading.

Overall, this study demonstrates that a large number (n=25) of infection patterns may exist in the CF lung, with the presence of one to three organisms, thus complicating the antimicrobial management of such infections. However, the presence of two or three co-colonising taxa in the CF lung did not result in higher bacterial loading.

The effect of Gram-positive flora in combination with only Gram-negatives lowered the microbial loading by approximately half a log unit. Furthermore, as only 25/138 (18.1%) of patients were infected with a Gram-positive organism, the dominance of the Gram-negative flora suggests that Gram-positives may find co-colonisation hostile.

Ecological interactions between organisms in the CF lung may operate at various taxonomic levels (i.e., between viruses, bacteria and fungi), as well as at the inter-genus, inter-species and intra-species level. Such interactions potentially are antagonist, neutral, symbiotic or synergistic, as detailed in Table 3. At present, these interactions in the microflora of the CF lung are largely unknown.

However, artificial manipulation of the ecology of the CF lung could be attempted in order to achieve category I (i.e., reduction of CF pathogen cell density; Table 3). Alternatively, attempts could be made to eliminate the co-coloniser to avoid category IV (Table 3), whereby it might be easier to eliminate and displace the essential co-coloniser from the microflora of the CF lung, rather than eliminate the CF pathogen (e.g., the co-coloniser might be more susceptible to antibiotic therapy than the pathogen).

In addition, artificial manipulations might alter the physiology so that established CF pathogens, including *P. aeruginosa* and BCC organisms might be displaced or alternatively be prevented from initial airways colonisation, as has been demonstrated in the prevention of adherence of *P. aeruginosa* to epithelial cells by *Lactobacillus crispatus* in the urinary tract.[7]

In conclusion, relatively little is known about the ecological interactions that exist between organisms in the CF lung. Therefore, further work is required to explore these interactions in order to aid understanding of the succession and dominance of Gram-negatives in chronic chest infections. Ultimately, a greater understanding of such interactions might permit the opportunity to manipulate the ecology of the lung to control otherwise problem pathogens and thus reduce morbidity and mortality in the CF patient. □

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