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Short Communication

Population structure and characterization of viridans group streptococci (VGS) including *Streptococcus pneumoniae* isolated from adult patients with cystic fibrosis (CF)

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Abstract

A study was undertaken to examine the population structure of viridans group streptococci (VGS) in the sputum of adult patients with cystic fibrosis (CF). Freshly expectorated sputa ($n=58$) from 45 adult CF patients were examined by selective conventional culture on Mitis–Salivarius agar and yielded 190 isolates of VGS. Sequence analyses of the *rpn*B and 16-23S rRNA ITS genes identified these isolates to belong to 12 species of VGS and included *S. anginosus*, *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. pneumoniae*, *S. salivarius* and *S. sanguinis*. The most frequently VGS organism isolated was *S. salivarius* (47/190; 24.7%), followed by *S. mitis* (36/190; 19%), *S. sanguinis* (25/190; 13.2%), *S. oralis* (20/190; 11.0%), *S. pneumoniae* (19/190; 10.0%), *S. parasanguinis* (16/190; 8.4%), *S. infantis* (11/190; 5.8%), *S. gordonii* (7/190; 3.7%), *S. anginosus* (4/190; 2.1%), *S. cristatus* (2/190; 1.1%), *S. australis* (1/190; 0.5%), *S. mutans* (1/190; 0.5%) and *S. agalactiae* (1/190; 0.5%). All, but four, patients harboured at least one VGS species, which ranged from one to five streptococcal species, with a mean of 2.85 species per patient. There was no clonality at the subspecies level employing ERIC RAPD PCR. Antibiotic susceptibility was determined by Minimum Inhibitory Concentration (MIC) testing against penicillin, erythromycin and ciprofloxacin. Overall, resistance to penicillin with all VGS was 73/190 (38.4%) and 167/190 (87.9%) for erythromycin. With regard to ciprofloxacin, 27/190 (14.2%) were fully resistant, whilst a further 21/190 (11.1%) showed intermediate resistance, which equated to approximately three quarters (74.7%) of isolates being fully sensitive to this agent. In addition, as a comparator control population, we examined antibiotic susceptibility, as above, in a non-CF population comprising 12 individuals (50 VGS isolates), who were not receiving chronic antibiotics. In comparison, 8% and 38% of VGS isolates from non-CF individuals were resistant by disk susceptibility testing to penicillin and erythromycin, respectively. None of the non-CF VGS organisms were resistant to ciprofloxacin, but 42% showed intermediate resistance.

1. Introduction

Oral streptococci are largely composed of members of the viridans group streptococci (VGS), which currently encompasses 20 species, which are commensal inhabitants of the

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oropharyngeal cavity and the gastrointestinal and genital tracts of mammals [1]. On the basis of 16S rRNA sequence homology, these bacteria are categorized in four groups: the salivarius rRNA homology group, including *Streptococcus thermophilus*, *Streptococcus vestibularis* and *Streptococcus salivarius*; the mitis group, including *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus sanguinis* and *Streptococcus parasanguinis*; the anginosus group, including *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*; and the mutans group, including *Streptococcus mutans*, *Streptococcus criceti*, *Streptococcus downei*, *Streptococcus ferus*, *Streptococcus macacae*, *Streptococcus ratti* and *Streptococcus sobrinus* [1].

Historically, VGS organisms were not considered first line pathogens of the immunocompetent host, with the exception of VGS involvement in infective endocarditis. Recently, several groups have reported on the clinical significance of bacterial genera, which until now, were not regarded as important in the CF lung pathology setting, including the anaerobes [2] and the *Streptococcus milleri* Group [3]. Current opinion suggests that these novel groups may play a crucial role in maintaining a stable microbial ecology within the CF lung, and enhance virulence associated with polymicrobial interactions, as well as directly contributing to the pathology of the lung. However, to date, there have been no reports on the ecological structure, distribution, virulence, clinical role and frequency of viridans group streptococci (VGS), which are known to act both as commensal bacteria, as well as opportunistic pathogens, in CF sputum. Therefore, the aim of this study was to investigate distribution and frequency of VGS and pneumococci in CF sputum, as well as to determine their antibiotic susceptibility, genetic relatedness and cytotoxic effects on epithelial cells.

2. Materials and methods

2.1. Bacterial isolates

In this study, 58 sputa from 45 adult CF patients attending the Northern Ireland Regional Adult Cystic Fibrosis Unit at Belfast City Hospital were examined culturally by conventional microbiological techniques for the presence of VGS organisms and pneumococci. Fresh expectorated sputa were collected from patients by physiotherapists following either in-patient or out-patient physiotherapy procedures. All sputa were plated onto selective Mitis–Salivarius agar (cat no: 229810, Becton Dickinson Ltd., Oxford, UK) containing 1% [w/v] tellurite solution and were incubated for 48 h at 37 °C under microaerophilic conditions in a CO_2 incubator regulated at 5%[v/v] CO_2. Following incubation for 48 h, presumptive VGS isolates resembling small and minute blue colonies, as well as "gum drop"-like blue colonies and visually distinct morphological variants, were subcultured onto Columbia Blood agar (CM0331 Oxoid Ltd., Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37 °C under microaerophilic conditions, as detailed above. All isolates were subsequently frozen in defibrinated horse blood (2 ml) at −80 °C and stored as part of the Northern Ireland Public Health Laboratory (NIPHL) Strain Repository.

2.2. Molecular identification

Purified isolates were subcultured on Columbia Blood Agar, as detailed above, for 24 h at 37 °C. All DNA isolation procedures were carried out in a Class II Biological Safety Cabinet (Micro-Flow, UK) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the "post-PCR" room in accordance with the Good Molecular Diagnostic Procedures (GMDP) guidelines of Millar et al. [4], in order to minimise contamination and hence the possibility of false positive results. Bacterial genomic DNA was extracted from few colonies of each purified isolate, by employment of the Roche High Purity PCR Template Preparation Kit (Roche Diagnostics Ltd., Sussex, UK), in accordance with the manufacturer's instructions. Extracted DNA was stored at −20 °C prior to PCR amplification. Two gene loci were employed to identify the VGS to the species level, namely the *rnpB* gene [5] and the 16S-23S rDNA ITS [6]. Following PCR amplification, amplicons for sequencing were purified with a QIAquick PCR purification kit (Qiagen Ltd., UK) and eluted in Tris–HCl (10 mM, pH 8.5) prior to sequencing. Following labelling of PCR amplicons using Big Dye cycle sequencing chemistry (ABI, Applied Biosystems Ltd., Warrington, UK), automated sequencing was performed on an ABI Capillary Sequencer (3740 platform). Resulting sequences were confirmed from chromatogram analysis and confirmed sequences were compared with those stored in the GenBank using the BLASTn alignment software (http://www.blast.genome.ad.jp/).

2.3. Serotyping of pneumococcal isolates

All isolates which were identified molecularly as pneumococci were serotyped conventionally by a latex aggregation assay (Pneumotest-Latex) (Statens Serum Institut, Copenhagen, Denmark).

2.4. Determination of antimicrobial susceptibility

Antibiotic susceptibility testing was performed on all VGS isolates by standard disk susceptibility, as well as by determination of the minimum inhibitory concentration (MIC) value, against three classes of antibiotics, namely a β-lactam (penicillin), a macrolide (erythromycin) and a fluoroquinolone (ciprofloxacin). The MIC against the same agents was determined by broth microdilution method according to CLSI standard method [7]. In order to estimate ciprofloxacin resistance, interpretive criteria for ofloxacin was used. Lyophilised antimicrobial tablets were purchased from MAST Ltd. (Merseyside, UK). In addition, as a comparator control population, we examined antibiotic susceptibility, as above, in a non-CF population comprising 12 individuals (50 VGS isolates), who were not receiving chronic antibiotics.

2.5. Determination of genetic-relatedness between VGS

Genomic DNA was subjected to enterobacterial repetitive intergenic consensus (ERIC)-PCR. PCR reaction mixes (25 μl) contained: 2 μl of DNA template, containing circa. 50 ng DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.8 mM MgCl2, 200 μM of each dNTP, 1.5 U of Taq DNA polymerase (New England Biolabs Ltd., Hertfordshire, UK) and 100 μM of each primer. The primers ERIC 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were previously described [8]. Reaction mixtures were subjected to the following thermal cycling parameters in a GeneAmp 9700 thermal Cycler (Applied Biosystems, Warrington, UK): 95 °C for 5 min followed by 35 cycles of 95 °C for 45 s, annealing temperature 52 °C for 1 min, 72 °C for 5 min, followed by a final extension at 72 °C for 20 min. Following amplification, PCR products were visualized on 1.5% (w/v) Certified Low Range Ultra Agarose (Bio-Rad Laboratories Ltd., Hertfordshire, UK) containing ethidium bromide (1 μg/ml). Gels were analysed by UV illumination with a gel image analysis system (UVP Products Ltd., Cambridge, UK) and banding profiles were determined manually, where a unique genotype was defined as having one or greater differences in its banding profile.

2.6. Determination of VGS-related cytotoxicity

Cytotoxicity of the 13 species of streptococci isolated from adult CF sputa (*S. agalactiae*, *S. anginosus*, *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. pneumoniae*, *S. salivarius* & *S. sanguinis*) was assessed, as well as *Pseudomonas aeruginosa* NCTC10662. A single representative isolate of each species was randomly selected and was cultured individually in 10 ml of Brain Heart Infusion (BHI) broth (CM1032, Oxoid Ltd., UK), supplemented with yeast extract (5 g/l; LP0021, Oxoid Ltd., UK) at 37 °C under microaerophilic conditions for 24 h. Following this, 25 ml of fresh sterile BHIYE broth were inoculated with 1 ml of pre-culture and incubated at 37 °C under microaerophilic conditions for 48 h. After incubation, spent culture broth was filtered using a 0.22 μm syringe filter (Millipore, Watford, UK). For cytotoxicity testing, filtered spent broth (33 μl) was added to CF bronchial epithelial (CFBE) cell suspension (66 μl ≅ 5×10^4 cells) and incubated at 37 °C with 5% [v/v] CO_2 for 24 h. Cytotoxicity was measured by release of lactate dehydrogenase (LDH) employing the LDH-Cytotoxicity Assay Kit II (Bio Vision, California, USA) and plate reader (450 nm), as per the manufacturer's instructions. % cytotoxicity was defined as:–

$$Cytotoxicity(\%) = \frac{(Test\ sample - Low\ control)}{(High\ Control - Low\ Control)} \times 100$$

3. Results

3.1. Species identification and distribution

From the 58 sputa from 45 adult CF patients examined, 190 VGS isolates were cultured. Twelve members of the viridans

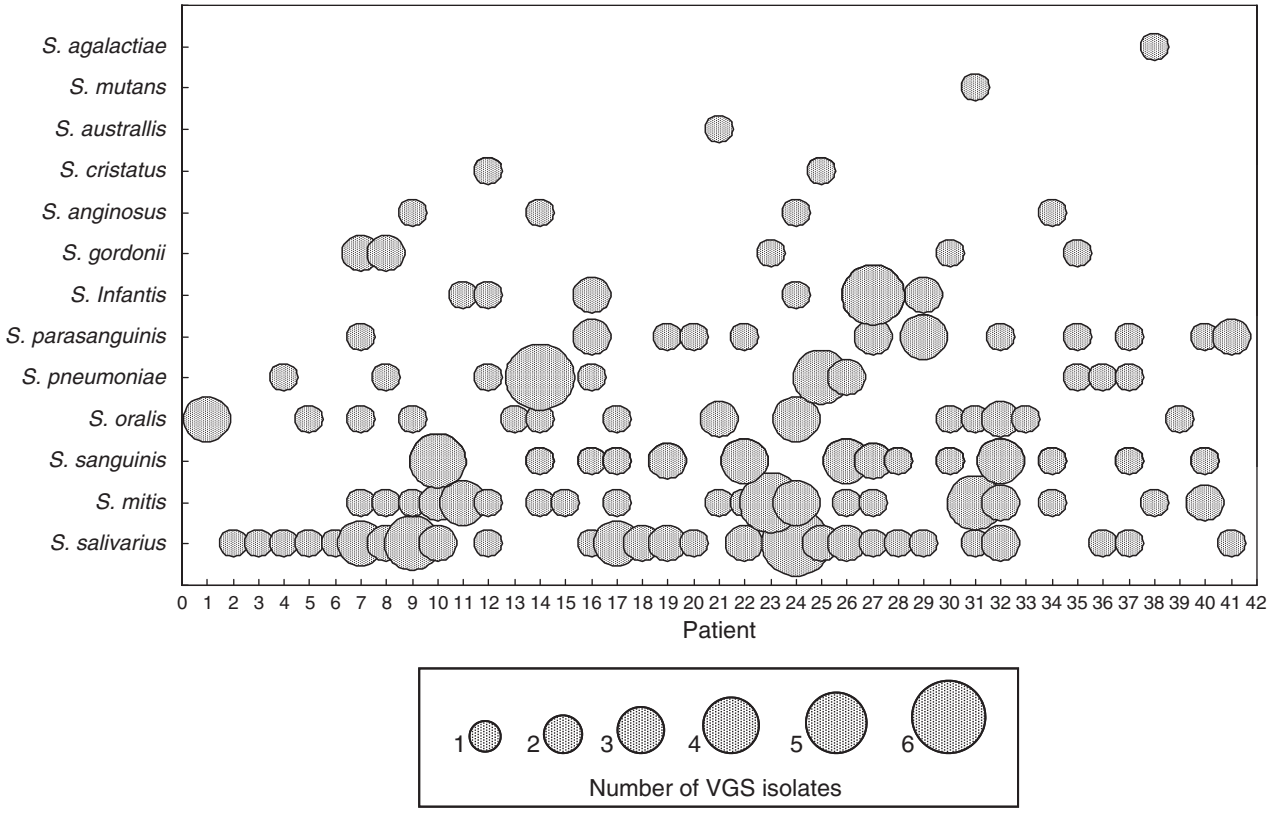

Fig. 1. Distribution and frequency of each VGS species in each CF patient.

group streptococci were culturally recovered and included *S. anginosus*, *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. pneumoniae*, *S. salivarius* and *S. sanguinis*. *S. agalactiae* was also recovered from one patient. The most frequently VGS organism isolated was *S. salivarius* (47/190; 24.7%), followed by *S. mitis* (36/190; 19%), *S. sanguinis* (25/190; 13.2%), *S. oralis* (20/190; 11.0%), *S. pneumoniae* (19/190; 10.0%), *S. parasanguinis* (16/190; 8.4%), *S. infantis* (11/190; 5.8%), *S. gordonii* (7/190; 3.7%), *S. anginosus* (4/190; 2.1%), *S. cristatus* (2/190; 1.1%), *S. australis* (1/190; 0.5%), *S. mutans* (1/190; 0.5%) and *S. agalactiae* (1/190; 0.5%). All, but four, patients harboured at least one VGS species, which ranged from one to five streptococcal species, with a mean of 2.85 species per patient (Fig. 1). A representative sequence of the *rnp*B gene and 16-23S rRNA ITS region for each species has now been deposited in GenBank with the respective accession numbers, as detailed in Table 1.

3.2. Serotyping of pneumococcal isolates

When the serotypes of the isolated pneumococci ($n=19$) were further examined (see Table 2), it can be seen that 12 of these fell immunologically outside the coverage of the 23-valent vaccine.

3.3. Antimicrobial susceptibility

The antibiotic susceptibility of all 190 isolates was determined by Minimum Inhibitory Concentration (MIC) testing against penicillin, erythromycin and ciprofloxacin. Overall, resistance to penicillin with all VGS was 73/190 (38.4%) and 167/190 (87.9%) for erythromycin. With regard to ciprofloxacin, 27/190 (14.2%) were resistant, whilst a further 21/190 (11.1%) showed intermediate resistance, which equated to approximately three quarters (74.7%) of isolates being fully sensitive to this agent. Table 3 details the MIC range, geometric MIC mean, MIC_{50} and MIC_{90} for each streptococcal species identified against these three antibiotic agents. Table 4 details the proportion of VGS streptococci resistant to single agents, as well as to combinations of double agents and for all three antibiotic agents. In comparison, 8% and 38% of VGS isolates from non-CF individuals were resistant by disk susceptibility testing to penicillin and erythromycin, respectively. None of the non-CF VGS organisms were resistant to ciprofloxacin, but 42% showed intermediate resistance.

Table 1
Deposited accession numbers in GenBank relating to partial *rpn*B and 16-23S rRNA ITS sequences for all VGS identified in this study.

Species	Strain	*rnpB*	*16S-23S rRNA ITS*
S. anginosus	C61	GU907491	GU907504
S. australis	C89	GU907492	GU907505
S. cristatus	C122	GU907493	GU907506
S. gordonii	C163	GU907494	GU907507
S. infantis	C50	GU907495	GU907508
S. mitis	C42	GU907496	GU907509
S. mutans	C169	GU907497	GU907510
S. oralis	C12	GU907498	GU907511
S. parasanguinis	C86	GU907499	GU907512
S. pneumoniae	C30	GU907500	GU907513
S. salivarius	C5	GU907501	GU907514
S. sanguinis	C62	GU907502	GU907515

Table 2
Serogroups and serotypes of *S. pneumoniae* isolated from adult CF patients in this study.

Strain no.	Serotype
C10	33(**33F**[a],33A,33B,33C,33D)
C30	24(24F,24A,24B),31,40
C52	18(18F,18A,18B,**18C**)
C57	19(**19F,19A**,19B,19C)
C58	29,34,35(35F,35A,35B,35C),42,47(47F,47A)
C59	19(**19F,19A**,19B,19C)
C60	19(**19F,19A**,19B,19C)
C63	19(**19F,19A**,19B,19)
C69	25(25F,25A),38,43,44,45,46,48
C118	27,32(32F,32A),41(41F,41A)
C119	9(9A,9L,**9N,9V**)
C120	27,32(32F,32A),41(41F,41A)
C121	27,32(32F,32A),41(41F,41A)
C124	13,28(28F,28A)
C125	13,28(28F,28A)
C138	27,32(32F,32A),41(41F,41A)
C212	29,34,35(35F,35A,35B,35C),42,47(47F,47A)
C217	27,32(32F,32A),41(41F,41A)
C220	27,32(32F,32A),41(41F,41A)

[a] Enlarged and bold serotypes are included in the current 23-valent PPSV vaccine.

3.4. Determination of genetic-relatedness between VGS

ERIC-PCR produced unique genotypes with all VGS species and also amongst isolates within each species, indicating a lack of clonality of the VGS in the CF patient population examined. Although, there were seven occasions that two ERIC-PCR products from same species showed indistinguishable banding patterns, all of these isolates were sampled from the same patient at the same time and from the same sputum specimen. Table 5 details the number of ERIC-PCR genotypes generated for each VGS species.

3.5. Determination of VGS-related cytotoxicity

None of the VGS species were considered to have elicited a cytotoxic response against the cell line tested.

4. Discussion

Recently, the employment of sophisticated molecular techniques has allowed the identification of a diverse range of bacterial families, genera and species in sputum from the airways of patients with cystic fibrosis (CF), which are generally not isolated by conventional cultural techniques in the clinical microbiology laboratory or are isolated by such laboratories but do not get reported, as they are considered clinically not relevant. One such group are the viridans group streptococci. Historically, there have been very few reports in the literature of VGS associated with CF patients. Previously,

Table 3
MIC range, geometric MIC mean, MIC_{50} and MIC_{90} for each VGS streptococcal species identified against penicillin, erythromycin and ciprofloxacin.

	Penicillin				Erythromycin				Ciprofloxacin			
Species	MIC range (μg/ml)	Mean	MIC50	MIC90	MIC range (μg/ml)	Mean	MIC50	MIC90	MIC range (μg/ml)	Mean	MIC50	MIC90
S. anginosus	0.015–0.125	0.05	0.03	0.125	0.06–>16	9.515	2	>16	0.5–1	0.875	1	1
S. australis	0.125	0.125	0.125	0.125	4	4	4	4	1	1	1	1
S. cristatus	0.125–0.25	0.188	0.25	0.125	0.03–4	2.015	0.03	4	2	2	2	2
S. gordonii	0.015–0.25	0.051	0.015	0.25	4–8	6.857	8	8	0.5–32	5.786	1	32
S. infantis	0.015–8	1.967	1	4	4–8	4.727	4	8	1–>64	24.64	2	64
S. mitis	0.015–8	0.507	0.06	1	0.06–>32	4.675	4	8	0.5–32	4.319	2	8
S. mutans	0.015	0.015	0.015	0.015	0.06	0.06	0.06	0.06	0.125	0.125	0.125	0.125
S. oralis	0.015–>8	2.879	0.06	>8	0.03–>16	12.31	4	>16	1–>64	17.65	4	>64
S. parasanguinis	0.03–8	1.119	0.25	4	0.06–>16	7.504	8	8	2–64	9.625	2	32
S. pneumoniae	0.015–2	0.225	0.125	0.5	0.03–>16	8.647	4	>16	0.5–8	1.711	1	4
S. salivarius	0.015–1	0.315	0.125	1	0.03–>16	15.62	8	>16	0.5–16	1.723	1	2
S. sanguinis	0.06–1	0.447	0.125	1	0.06–>16	16.25	4	>16	0.5–8	1.82	1	8
S. agalactiae	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	2	2	2	2

Rogers et al. [9] demonstrated through T-RFLP techniques that 6% of CF patients had a DNA fragment that was due to organisms in the Assignation group (*S. pneumoniae, S. salivarius, S. pyogenes, S. macedonicus, S. sanguinis*) and Klepac-Ceraj et al. [10] recently reported the presence of *Streptococcus* group (AF009482), *Streptococcus constellatus* (AF104676) and *Streptococcus gordonii* (AF003931) from oropharyngeal swabs from children with CF employing phylochip hybridization technology.

Recently, a Canadian group has published several reports on the clinical significance of members of the "*S. milleri*" or *S. anginosus* group (*S. constellatus, S. intermedius and S. anginosus*) [3,11]. In these reports, these workers argue that the *S. milleri* group of organisms have been overlooked as respiratory pathogens, due their difficulty in being differentiated from the VGS, and where *S. milleri* infection led to malodorous sputum in pulmonary exacerbations.

With this background and given that there have not been any reports on VGS from patients with CF, it was the aim of this study to specifically examine the population structure and characteristics of resulting VGS organisms isolated from the sputum of adult CF patients.

The majority (41/45 [91%]) of adult CF patients examined were colonized with at least one species of VGS. In all, 12 species of VGS were identified, including four patients (8.9%) with *S. anginosus* and 10 patients (22.2%) with *S. pneumoniae*. As it was not the intention of this study, at this stage, to attempt to attribute clinical significance to these two species in these CF patients, it has not been possible to estimate how significant these organisms are in these patients. Nevertheless, both of these organisms have been described as pathogens in CF patients and hence, these two species would probably represent the most virulent of the VGS species *per se* isolated in this study. Hence, approximately one quarter of all adult CF examined cultured a viable pneumococcus. Pneumococci are not regarded as common respiratory pathogens in CF patients and therefore their presence in CF airways may be akin to their close phylogenetic neighbours, namely the VGS organisms. As approximately 20–50% of healthy individuals may also be colonized asymptomatically with pneumococci, it is difficult to interpret the significance of these organisms in CF airways. Equally, the value of pneumococcal vaccination remains unclear in this patient group, although recommended as a precautionary measure in such a respiratory group.

Perhaps, the most important finding of the current study is the very high level of antibiotic resistance seen in the isolated VGS, particularly resistance towards the macrolides.

Table 4
Proportion of VGS streptococci solely resistant to each single agent, as well as their resistance to combinations of double agents and for all three antibiotic agents.

MIC break point criteria	
Resistance pattern	Number of isolates (including intermediate resistance)
Penicillin	1
Erythromycin	71
Ciprofloxacin	1
Penicillin + Erythromycin	50
Penicillin + Ciprofloxacin	1
Erythromycin + Ciprofloxacin	25
Penicillin + Erythromycin + Ciprofloxacin	21
All susceptible	20
Total	190

Table 5
Details of ERIC-PCR genotypes generated for each VGS species amongst the 190 VGS isolates obtained from 42 adult CF patients.

Species	Patient no. of origin	Genotype no.	Identical genotype no.
S. salivarius ($n=47$)	28	44	3
S. mitis ($n=36$)	19	35	1
S. sanguinis ($n=25$)	13	23	2
S. oralis ($n=20$)	14	20	0
S. pneumoniae ($n=19$)	10	18	1
S. parasanguinis ($n=16$)	10	16	0
S. infantis ($n=11$)	7	11	0
S.gordonii ($n=7$)	5	5	0
S. anginosus ($n=4$)	4	4	0
S. cristatus ($n=2$)	2	2	0
S. australis ($n=1$)	1	1	0
S. mutans ($n=1$)	1	1	0
S. agalactiae ($n=1$)	1	1	0

The long-term use of several classes of antibiotic agents for the prophylaxis, maintenance and treatment of bacterial respiratory pathogens causing chronic chest infections in CF patients has important consequences for the persistence of VGS flora of the treated patient. In order to survive, the VGS organisms colonising the patient can evolve resistance mechanisms in response to the chronic use of these antibiotic agents. What is not known at present is what resistant mechanisms do commensal organism use and are these mechanisms potentially transferable to hitherto sensitive pathogens. Therefore, antibiotic resistance within the VGS flora of CF patients potentially may be an important reservoir of genetic material for exacerbating antibiotic resistance in CF pathogens.

Equally, with the ability to survive intense and prolonged antibiotic pressure, such VGS organisms may become dangerously poised to become potential pathogens, if (i) there is a downward shift in the immune status of the patient e.g. following lung transplantation, (ii) where such organisms are genetically promiscuous in acquiring virulence determinants from co-habiting with true pathogens and (iii) where horizontal gene transfer events occur, leading to the acquisition of antibiotic resistance determinants by newly colonising pathogens. The acquisition of virulence determinants is also a significant cause for concern in antibiotic resistant VGS organisms and where such commensal flora dominate. One reason for their success is the relative plasticity of their genomes to adapt to varying host immune responses, as well as selective antibiotic pressure. With this genomic plasticity and the ability to naturally transform, VGS organisms have the ability to take up virulence determinants, which then potentially can transform their status from commensal organism to opportunistic pathogen to true pathogen.

Given that most CF patients are treated regularly with both oral, iv and nebulised antibiotics for maintenance, as well as acute pulmonary exacerbations, it is not surprising that their commensal VGS organisms have become resistant to several classes of antibiotics. Recently, we have shown that long-term use of azithromycin in adult CF patients has led to a highly macrolide resistant population of VGS isolated from patients' sputum, in comparison to similar VGS populations originating in non-CF patients, who were not treated with azithromycin for long periods of time [12]. In addition, complete gene homology in the macrolide resistance determinants, particularly *erm*(B) and *mef(A)* is also shared with other closely-related genera, including *Gemella, Enterococcus* and *Granulicatella*. Presence of multiple macrolide-resistance determinants occurring at high frequency in VGS commensal organisms is potentially important for the CF patient, other CF patients and the non-CF population. The presence of bacterial pathogens in CF sputum generally reflects those bacterial genera which are commonly associated with CF lung disease, including *Pseudomonas aeruginosa* and *Burkholderia cenocepacia.* Normally these pathogens do not constitute an infection risk to the healthy, non-CF individual. However, the presence of such macrolide-resistance determinants in VGS organisms may be problematic for the healthy non-CF individual, as these may act as a reservoir of resistance determinants for other respiratory pathogens, particularly *S. pneumoniae,* where these commensal flora are transmitted from the CF patient to non-CF individuals, for example, between CF and non-CF siblings within a household.

We subsequently wished to compare our CF VGS population, which originated mainly from adult CF patients taking oral azithromycin long-term, as well several courses of iv β-lactam and oral ciprofloxacin antibiotic agents, with a VGS population from a non-CF patient group, which had not been exposed to long term use of macrolides or other antibiotics, in order to access downstream consequences relating to macrolide, as well as β-lactam and fluoroquinolone resistance in commensal VGS flora within the CF patient population. As a comparator, clinically non-significant and non-invasive VGS organisms were examined in 12 consecutive patients in the community, who were attending their primary care doctor. These data suggests that long-term use of antibiotics in the CF patient population may lead to high levels of antibiotic resistance in the VGS commensal flora.

Finally, VGS organisms may be important in the CF lung, in that they may have the ability to coaggregate with other important CF bacterial pathogens. Coaggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules, with the main aim of leading to the formation of a multi-species biofilm (For a comprehensive review on coaggregation, see Rickard et al. [13]). Oral streptococci are widely known to be important coaggregators in the mouth resulting in some form of mutualism for the VGS and its coaggregating bacterial partner. Previously, Chalmers et al. [14] described that coaggregation required a receptor polysaccharide (RPS), which is a cell surface molecule found on many strains of *S. oralis* and *S. mitis* [15], which mediates coaggregation by its role as the recognition molecule for lectin-like adhesins found on many other bacterial genera including *Actinomyces, Veillonella*, and other streptococci. Six RPS types have been identified in oral streptococci [16]. Each type is composed of a distinct hexa- or heptasaccharide repeating unit which contains one of two host-like disaccharide recognition motifs, GalNAcβ1-3Gal (Gn type) or Galβ1-3GalNAc (G type). The lectin-like adhesins on *Actinomyces* [17] and on veillonellae [18] recognize the Gn and G types of RPS, whereas certain streptococci bear GalNAc-specific adhesins that recognize only the Gn types [16]. Intergeneric coaggregation of RPS-bearing streptococci and *Actinomyces* [16] or veillonellae [18] is prevalent and is thought to contribute to the formation of pioneer multispecies communities on enamel [19,20]. *Pseudomonas aeruginosa* have been reported to produce lectin PA-IL (galactophilic lectin, LecA) and PA-IIL (fucose>fructose/mannose-binding lectin, LecB), which mediate auto- as well as hetero cell-to-cell interactions for biofilm formation and adherence to target cells/organisms [21]. To date, intergenic coaggregation of RPS-bearing VGS and *Pseudomonas aeruginosa* has not yet been reported. Therefore, studies to help elucidate a potential coaggregation effect between VGS species and *Pseudomonas aeruginosa* are urgently required to help define the role VGS as an initiator of *Pseudomnas* attachment and colonization.

The presence of VGS in the airways of CF patients may be significant due to (i). the presence of known VGS species within the "*S. milleri*" (*S. anginosus* group), as well as from the presence of *S. pneumoniae*, which have been recently regarded as pathogenic within the CF community, (ii). their relatively high level of antibiotic resistance, especially with the macrolides, ciprofloxacin and penicillin, (iii). their ability for horizontal gene transfer, which could allow the recycling of these resistances in newly colonising CF microflora, (iv). VGS's potential coaggregation events with CF pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* may lead to initial attachment and eventual colonisation with these pathogens and the promotion and development of several different multispecies biofilms in the CF airways.

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