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Paper

Population structure and characterization of viridans group streptococci (VGS) isolated from the upper respiratory tract of patients in the community

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ABSTRACT

A study was undertaken to examine the population structure of viridans group streptococci (VGS) isolated the upper respiratory tract of adult and paediatric patients within the community. VGS are common commensal bacterial inhabitants of the upper respiratory tract and valuable sentinel reporters of underlying antibiotic resistance (AR). Laboratory examination of the colonising VGS species may provide a valuable ecological description of the species isolated from the upper respiratory tract and their antibiotic susceptibility, including an estimation of the AR reservoir in this population. Freshly obtained nasal and oropharyngeal swabs from 84 patients were examined by selective conventional culture on Mitis-Salivarius agar and yielded 363 isolates of VGS. Sequence analyses of the *rpn*B and 16-23S rRNA ITS genes identified these isolates to belong to 10 species of VGS and included *S. anginosus*, *S. australis*, *S. constellatus*, *S. infantis*, *S. mitis*, *S. oralis*, *S. parasanguinis*, *S. salivarius, S. sanguinis* and *S. vestibularis*. The most frequent VGS organisms isolated was *S. salivarius* (282/363; 78.0%), followed by *S. sanguinis* (23/363; 6.3%), *S. parasanguinis* (21/363; 5.8%), *S. mitis* (18/363; 5.0%), *S. anginosus* (5/363; 1.4%), *S. vestibularis* (5/363; 1.4%), *S. australis* (3/363; 0.8%), *S. oralis* (3/363; 0.8%), *S. infantis* (1/363; 0.3%) and *S. constellatus* (1/363; 0.3%). All patients examined carried at least one VGS organism, where there were 17 combination patterns of carriage of the 10 species of VGS species isolated, where 54.2%, 37.3%, 7.2% and 1.2% of patients harboured one, two, three and four different VGS species, respectively. Antibiotic susceptibility was determined by standard disk diffusion assay testing against four classes of antibiotics, including the β-lactams [cefotaxime, cefuroxime], the tetracyclines [doxycycline], the fluoroquinolones [levofloxacin] and the macrolides [erythromycin]. Overall, there was no resistance to levofloxacin and cefuroxime, with limited resistance to cefotaxime (3.3%) and doxycycline (9.8%). Antibiotic resistance was highest in erythromycin, where 40.9% of isolates were resistant. *S. vestibularis* was the most antibiotic resistance of all VGS species examined (*S. vestibularis* v *S. salivarius* p=0.011), followed by *S. anginosis*. *S. salivarius* was the most antibiotic susceptible VGS species examined.

Overall, given their infrequency in causing infection, relatively few studies to date have attempted to examine their ecology in their preferred body niche, namely the upper respiratory tract. However, knowing their prevalence is becoming increasingly important in relation to their ability to exclude significant respiratory pathogens, including *Streptococcus pneumoniae.*

In conclusion, these data indicate that VGS colonisation of the upper respiratory tract in individuals within the community is dominated mainly with relatively antibiotic susceptible *S. salivarius*.

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 oropharyngeal cavity and the gastrointestinal and genital tracts of mammals (Hardie & Whiley, 1997). 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*

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intermedius; and the mutans group, including *Streptococcus mutans*, *Streptococcus criceti*, *Streptococcus downei*, *Streptococcus ferus*, *Streptococcus macacae*, *Streptococcus ratti* and *Streptococcus sobrinus* (Hardie & Whiley, 1997).

Historically, VGS organisms were not considered primary pathogens of the immunocompetent host, with the exception of VGS involvement in infective endocarditis. Therefore, their role has been considered mainly as commensal in nature. More recently, the importance of commensal organisms, including the VGS species has been highlighted (Moore *et al*., 2009), where it has been suggested that such commensal flora are under selective pressure to evolve elaborate antibiotic resistance mechanisms, when the host is being treated to eradicate infecting bacterial pathogens or face eradication.

However, to date, there have been no reports on the ecological structure, distribution and antibiotic susceptibility of viridans group streptococci (VGS) in patients in the community. Therefore, the aim of this study was to investigate the distribution and frequency of VGS in the upper respiratory tract in patients within the community, as well as to determine their antibiotic susceptibility.

MATERIALS AND METHODS

Ethical approval & patient population

In this study, 84 upper respiratory tract (URT) swabs from 84 patients within the community were examined culturally by conventional microbiological techniques for the presence of VGS organisms.

This study was approved by the Office for Research Ethics Committees for Northern Ireland (ORECNI) with Study Reference Identifier 06/NIR01/16. In accordance with the ethical approval, full consent was obtained from all enrolled patients by the Research Nurse at the General Practice or Nursing Home. The individuals examined consisted of 57 females (68%) and 27 males (32%), with an age distribution of <10 years 19.3%, 11-60 years 43.4% and above 60 years 37.3%.

Bacterial isolates

Fresh URT swabs and oral rinses were collected from patients within the community attending their General Practitioner (GP), by a dedicated research nurse. URT swabs consisted of two swabs, namely a nasal swab, as well as oropharyngeal swab. All of these specimens 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 48h at 37°C under microaerophilic conditions in a CO_2 incubator regulated at 5%[v/v] CO_2. Following incubation for 48h, presumptive VGS isolates (n=5 per patient (Hedges *et al*., 1977)) 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 24h at 37°C under microaerophilic conditions, as detailed above. All isolates were subsequently frozen in defibrinated horse blood (2ml) at -80°C and stored as part of the MicroARK culture collection, within the Northern Ireland Public Health Laboratory (NIPHL) Strain Repository.

Molecular identification

Purified isolates were subcultured on Columbia Blood Agar, as detailed above, for 24h at 37°C. All DNA isolation procedures were carried out in a Class II Biological Safety Cabinet (MicroFlow, 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*. (2002), 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 (Maeda *et al*., 2011) and the 16S-23S rDNA ITS (Maeda *et al*., 2011; Chen *et al*., 2004). Following PCR amplification, amplicons for sequencing were purified with a QIAquick PCR purification kit (Qiagen Ltd., UK) and eluted in Tris-HCl (10mM, 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/).

Determination of antimicrobial susceptibility

Antibiotic susceptibility testing was performed on all VGS isolates by standard disk diffusion assay, against four classes of antibiotics, including the β-lactams [cefotaxime (disk strength =5μg), cefuroxime (30μg)], the tetracyclines [doxycycline (30μg)], the fluoroquinolones [levofloxacin (5μg)] and the macrolides [erythromycin (5μg)]. Isolates were scored as being either susceptible or non-susceptible, as based on BSAC (British Society for Antimicrobial Chemotherapy) criteria available at http://bsac.org.uk/wp-content/uploads/2012/02/Version-11.1-2012-Final-1.pdf

RESULTS

Species identification and distribution

A study was undertaken to examine the population structure of viridans group streptococci (VGS) isolated the upper respiratory tract of adult and paediatric patients within the community and yielded 363 VGS isolates. Sequence analyses of the *rpn*B and 16-23S rRNA ITS genes identified these isolates to belong to 10 species of VGS and included *S.*

Table 1:

Co-habitation of species viridans group streptococci (VGS) in the upper respiratory tract of 84 individuals.

Viridans group streptococci species combination	Number of patients with combination	Percentage of patient population with VGS combination
S. salivarius	43	51.2%
S. salivarius + *S. parasanguinis*	10	11.9%
S. salivarius + *S. mitis*	8	9.5%
S. oralis + *S. parasanguinis*	4	4.8%
S. salivarius + *S. anginosus*	3	3.6%
S. salivarius + *S. mitis* + *S. parasanguinis*	3	3.6%
S. salivarius + *S. australis*	2	2.4%
S. vestibularis	1	1.2%
S. parasanguinis	1	1.2%
S. salivarius + *S. vestibularis*	1	1.2%
S. salivarius + *S. sanguinis*	1	1.2%
S. salivarius + *S. infantis*	1	1.2%
S. salivarius + *S. mitis* + *S. vestibularis*	1	1.2%
S. salivarius + *S. parasanguinis* + *S. vestibularis*	1	1.2%
S. salivarius + *S. parasanguinis* + *S. sanguinis*	1	1.2%
S. constellatus + *S. anginosus*	1	1.2%
S. salivarius + *S. mitis* + *S. parasanguinis* + *S. vestibularis*	1	1.2%

anginosus, *S. australis*, *S. constellatus*, *S. infantis*, *S. mitis*, *S. oralis*, *S. parasanguinis*, *S. salivarius, S. sanguinis* and *S. vestibularis*. The most frequent VGS organisms isolated was *S. salivarius* (282/363; 78.0%), followed by *S. sanguinis* (23/363; 6.3%), *S. parasanguinis* (21/363; 5.8%), *S. mitis* (18/363; 5.0%), *S. anginosus* (5/363; 1.4%), *S. vestibularis* (5/363; 1.4%), *S. australis* (3/363; 0.8%), *S. oralis* (3/363; 0.8%), *S. infantis* (1/363; 0.3%) and *S. constellatus* (1/363; 0.3%). All patients examined carried at least one VGS organism, where there were 17 combination patterns of carriage of the 10 species of VGS species isolated (Table 1), where 54.2%, 37.3%, 7.2% and 1.2% of patients harboured one, two, three and four different VGS species, respectively. There were no associations between species distribution and age of population.

Antimicrobial susceptibility

Antibiotic susceptibility was determined by standard disk diffusion assay testing against four classes of antibiotics, including the β-lactams [cefotaxime, cefuroxime], the tetracyclines [doxycycline], the fluoroquinolones [levofloxacin] and the macrolides [erythromycin] against 61 VGS isolates, whereby every sixth consecutive VGS isolate was selected for susceptibility testing. Overall, there was no resistance to levofloxacin and cefuroxime, with limited resistance to cefotaxime (3.3%) and doxycycline (9.8%). Antibiotic resistance was highest in erythromycin, where 40.9% of isolates were resistant. *S. vestibularis* was the most antibiotic resistant of all VGS species examined (*S. vestibularis* v *S. salivarius* p=0.011), followed by *S. anginosis*. *S. salivarius* was the most antibiotic susceptible VGS species examined. None of the *S. vestibularis* species were isolated from patients under 10 years, but were all however, isolated from elderly patients. When the zone diameters of antibiotics were compared between children (<10 years) and the mean of the total patient population, there was no significant differences between antibiotic resistance in children versus the entire patient population (Table 2).

DISCUSSION

The viridans groups streptococci are important organisms of the upper respiratory tract. To date, their existence is mainly believed to be commensal in nature, where their presence and physiology leads to acidification of their immediate environment, thus making colonization and subsequent infection of such sites difficult by other pathogens, e.g. *Haemophilus influenzae*. Where this ecological balance within the VGS is upset due to antibiotic selective pressure, then respiratory tract pathogens may colonise and effect the host. Therefore, it is very much in the interest of the host to maintain the integrity of the ecology of such sites, namely the oropharynx and vagina. In addition, there has been a recent report demonstrating the protective effect of *S. salivarius* against *Candida albicans* mucosal invasion in the upper airways Ishijima *et al*., 2012).

To date, there have not been any reports describing the population structure of these alpha-haemolytic streptococci in the upper airways, mainly due to their perceived lack of clinical importance with their largely non-pathogenic role, as well as difficulties in the taxanomy and laboratory identification of species within this VGS group. Recently, our group reported on optimal molecular identification methods, which aided identification of VGS species, within this current study (Maeda *et al*., 2011). In order to select the representative VGS flora from the sites which we sampled, we purposely were guided by the work of Hedges *et al* (1977), which indicated that we should select at least five colony

picks from each selective plate examined. Resulting data indicated that there is much taxonomical diversity within the VGS in the upper airways, where the dominant species present was *S. salivarius*.

The use of several classes of oral antibiotic agents in General Practice, including β-lactams, tetracyclines, fluoroquinolones and the macrolides, may have important consequences for the persistence of VGS flora of the treated patient. For example, Fantin *et al* (2009) demonstrated that fluoroquinolone resistance developed in the pharyngeal flora of approximately one third of study patients receiving oral ciprofloxacin, particularly when local concentrations of ciprofloxacin were less than the MIC and that the selection of antibiotic resistance in these commensal organisms is a frequent ecological side effect of such therapy, even when doses were optimized.

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 patients treated with oral antibiotics may potentially be an important reservoir of genetic material for exacerbating antibiotic resistance in respiratory pathogens, in particular, *S. pneumoniae.*

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 innate immune status of the patient e.g. aging, immunosuppression, (ii) where such organisms are genetically promiscuous in acquiring virulence determinants from co-habiting with true pathogens and (iii) where horizontal gene transfer events may 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.

From above, we note the importance of the host in maintaining a healthy commensal flora of the upper airways to help avoid the colonisation and subsequent infection with major pathogens, including the pneumococcus. The close phylogenetic relationship between the pneumococcus and the VGS species makes this commensal-pathogen relationship further complicated, as the pneumococcus may therefore be more poised to take up genetic elements, particularly antibiotic resistance determinants, which have evolved within the host VGS population, as a result of intake of oral antibiotics. Previously, we have seen evidence of this, where Zolezzi *et al* (2004) demonstrated the successful transfer of *mef*(E) and *mel* genes, conferring resistant to the macrolide-lincosamide-streptogramin B (MLS(B)) antibiotics, from VGS organism to pneumococci. These workers concluded that VGS organisms may be important reservoirs of resistance genes for pneumococci.

In conclusion, this current study demonstrated that the upper airways of patients in the community may be colonised with a diverse combination of VGS, particularly *S. salivarius*, which are largely sensitive to several classes of antibiotic agent. There should be continued surveillance of antibiotic resistance within these populations, as a marker of potential resistance acquisition in *S. pnuemoniae*.

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The authors have no conflict of interest.

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TABLE 2:

Comparison of antibiotic zone sizes in five antibiotic agents with VGS organisms isolated from children (<10 years) and the total patient population

Antibiotic	Mean zone diameter (mm) in VGS organisms isolated from	
	Children (<10 years)	Total Patient Population
Cefotaxime	30.80	31.79
Cefuroxime	35.86	36.20
Doxycycline	29.47	30.44
Levofloxacin	25.06	25.52
Erythromycin	25.40	27.08

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