Molecular characterization of macrolide resistance determinants [erm(B) and mef(A)] in *Streptococcus pneumoniae* and viridans group streptococci (VGS) isolated from adult patients with cystic fibrosis (CF)

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Received 17 March 2009; returned 20 April 2009; revised 15 May 2009; accepted 27 May 2009

**Objectives:** Although long-term use of azithromycin has shown a significant clinical improvement for patients with cystic fibrosis (CF), its long-term effect on the susceptibility of commensal flora within CF airways has not yet been examined. We therefore suggest that long-term use of azithromycin increases macrolide resistance in commensal streptococci.

**Methods:** Erythromycin susceptibility in naturally colonizing viridans group streptococci (VGS) was characterized, as well as macrolide resistance gene determinants through sequence analysis, in pneumococci (n=15) and VGS (n=84); i.e. *Streptococcus salivarius* (n=30), *Streptococcus mitis* (n=17), *Streptococcus sanguinis* (n=11), *Streptococcus oralis* (n=10), *Streptococcus parasanguinis* (n=6), *Streptococcus gordonii* (n=3), *Streptococcus infantis* (n=3), *Streptococcus cristatus* (n=2), *Streptococcus anginosus* (n=1) and *Streptococcus australis* (n=1) isolated from sputum from 24 adult CF patients, who were on oral azithromycin therapy for at least the previous 7 months.

**Results:** Almost three-quarters of isolates (74; 74.7%) were resistant to erythromycin, whilst a further 15 (15.2%) had reduced susceptibility, leaving only 10 (10.1%) isolates susceptible to erythromycin. The majority (89.8%) were not susceptible to erythromycin, as demonstrated by possession of the *erm(B)* gene in 25/99 (25.3%), the *mef(A)* gene in 1/99 (1.0%), the *mef(E)* gene in 75/99 (75.8%) and both *erm(B)* and *mef(E)* genes simultaneously in 11/99 (11.1%). These results indicate that genotypic resistance for macrolides is common in VGS in adult CF patients, with efflux being over three times more frequent.

**Conclusions:** Long-term treatment with azithromycin in CF patients may reduce antibiotic susceptibility in commensal VGS, where these organisms may potentially act as a reservoir of macrolide resistance determinants for newly acquired and antibiotic-susceptible pathogens.

Keywords: antibiotic resistance, azithromycin, commensal, erythromycin

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†A. T. and Y. M. played an equal role in this study.
Introduction

Cystic fibrosis (CF) is the most commonly inherited disease, leading to reduced life expectancy in persons originating from a white and European background, where the most common complication is the recurrence of chronic chest infections usually caused by bacterial pathogens.1,2 Employment of the macrolide azithromycin (Zithromax) has now become a common treatment modality in the management of patients with CF,1–3 mainly due to several reasons, including (i) its ability to inhibit mobility and quorum sensing of *Pseudomonas aeruginosa*; (ii) decreased production of mucus by epithelial cells; and (iii) decreased biosynthesis of pro-inflammatory cytokines from monocytes and epithelial cells by inhibiting nuclear factor-kB.6 Long-term employment of this macrolide may lead to the development of antibiotic resistance in the commensal flora of the CF patient, which could act as a potential reservoir of antibiotic resistance determinants for newly acquired and susceptible respiratory pathogens.

To date there have been limited reports on the level of macrolide resistance or the molecular characterization of such resistance mechanisms in the commensal flora of the upper respiratory tract in patients with CF, particularly in viridans group streptococci (VGS). Previously, Phaff et al.7 demonstrated in their study over a 4 year period that macrolide resistance levels increased in two CF respiratory pathogens, namely *Staphylococcus aureus* and *Haemophilus* spp., which was associated with azithromycin maintenance therapy. More recently, the *Streptococcus milleri* group has been shown to play an important role in the pathology of lung disease, by establishing chronic pulmonary infection, where this taxon was shown to be the dominant pathogen in 39% of acute pulmonary exacerbations.8 Hence, the objectives of this study were (i) to examine the level of resistance in VGS isolated from CF sputum; and (ii) to characterize the molecular basis for macrolide resistance in VGS, as well as in pneumococci, isolated from adult patients with CF and to assess if chronic use of azithromycin in CF may potentially have a microbiological adverse effect elsewhere in the management of the disease.

Materials and methods

Bacterial isolates

Ninety-nine isolates of streptococci, comprising *Streptococcus pneumoniae* (n=15) and VGS [n=84; i.e. *Streptococcus salivarius* (n=30), *Streptococcus mitis* (n=17), *Streptococcus sanguinis* (n=11), *Streptococcus oralis* (n=10), *Streptococcus parasanguinis* (n=6), *Streptococcus gordonii* (n=3), *Streptococcus infantis* (n=3), *Streptococcus crista* (n=2), *Streptococcus anginosus* (n=1) and *Streptococcus australis* (n=1)], were isolated from 26 sputum specimens originating from 25 adult CF patients, of whom 24 were on long-term oral azithromycin therapy (500 mg three times a week) for at least the previous 7 months. Streptococcal organisms were previously isolated from spueta on Mitis–Salivarius agar (Difco 229810, Becton Dickinson Ltd, Oxford, UK), incubated for 48 h at 37°C microaerophilic conditions, for service methods development, and hence no research approvals were required for the current study. Following incubation, visually distinct morphological variants were subcultured onto Columbia blood agar (Oxoid CM331), supplemented with 5% (v/v) fibrinated horse blood (E&O Laboratories, Bonnybridge, Scotland) for 24 h at 37°C under microaerophilic conditions. Genomic DNA was extracted from each pure cultured isolate as below. The *rnpB* gene8 and the 16S–23S rDNA internal transcribed spacer (ITS)10 were amplified, respectively, by PCR and then sequenced in order to identify each VGS and pneumococci to the species level.

Antimicrobial susceptibility test

Erythromycin discs (15 μg) were purchased from Oxoid Ltd (Basingstoke, UK). Antimicrobial susceptibility to erythromycin was detected by disc diffusion test according to the CLSI standard method.11

DNA extraction

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 (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 guidelines of Millar et al.12 in order to minimize contamination and hence the possibility of false-positive results. Bacterial genomic DNA was extracted from a few colonies of each 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.

PCR amplification of macrolide resistance

[erm(B)+mef(A/E)] determinant genes

All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and from the amplification and post-PCR room in order to minimize contamination.12 PCR mixes (25 μL) comprised: 1 μL of DNA template, containing ~25 ng of DNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP, 0.6 U of Taq DNA polymerase (New England Biolabs Ltd, Hertfordshire, UK) and 10 μM of each primer encoding regions of the *erm(B) gene and mef(A/E) gene.* Primer sequences for the *erm(B) gene and mef(A/E) are described elsewhere.13 Following a ‘hot start’, the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, annealing temperature [erm(B) 57°C; mef(A/E) 50°C] for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Following amplification, PCR products were visualized on 1–1.5% (w/v) agarose gels in 0.5× TBE buffer followed by staining with ethidium bromide (10 mg/100 mL) and UV illumination with a gel image analysis system (UVP Products Ltd, Cambridge, UK).

DNA sequencing and analysis

Amplicons for sequencing were purified with a QIAquick PCR purification kit (QIAGEN Ltd, West Sussex, UK), according to the manufacturer’s instruction. Partial sequences of the *erm(B) gene and the mef(A/E) gene were determined using the BigDye Terminator Cycle Sequencing kit and ABI 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK). Sequencing analysis was carried out with GENETYX software (Genetyx Corporation, Tokyo, Japan).
Macrolide resistance in VGS from CF patients

Table 1. Molecular and phenotypic disc susceptibility (erythromycin) properties amongst VGS and pneumococci, including GenBank accession numbers submitted

<table>
<thead>
<tr>
<th>Species</th>
<th>erm(B)</th>
<th>mef(A)</th>
<th>mef(E)</th>
<th>R</th>
<th>I</th>
<th>S</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. salivarius (n=30)</td>
<td>8</td>
<td>0</td>
<td>22</td>
<td>29</td>
<td>1</td>
<td>0</td>
<td>erm(B) FJ667781 mef(E) FJ667786</td>
</tr>
<tr>
<td>S. mitis (n=17)</td>
<td>1</td>
<td>0</td>
<td>16</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>erm(B) FJ667784 mef(E) FJ667788</td>
</tr>
<tr>
<td>S. pneumoniae (n=15)</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>erm(B) FJ667782 mef(E) FJ667787</td>
</tr>
<tr>
<td>S. sanguinis (n=11)</td>
<td>6</td>
<td>1</td>
<td>9</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>erm(B) FJ667783 mef(A) FJ667795 mef(E) FJ667791</td>
</tr>
<tr>
<td>S. oralis (n=10)</td>
<td>7</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>erm(B) FJ667780 mef(E) FJ667785</td>
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<td>S. parasanguinis (n=6)</td>
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<td>0</td>
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<td>5</td>
<td>1</td>
<td>0</td>
<td>mef(E) FJ667790</td>
</tr>
<tr>
<td>S. infantis (n=3)</td>
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<td>0</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td>mef(E) FJ667792</td>
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<tr>
<td>S. gordonii (n=3)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>mef(E) FJ667789</td>
</tr>
<tr>
<td>S. cristaus (n=2)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>mef(E) FJ667794</td>
</tr>
<tr>
<td>S. anginosus (n=1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>mef(E) FJ667793</td>
</tr>
<tr>
<td>S. australis (n=1)</td>
<td>0</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

R, resistant; I, intermediate; S, susceptible.

Results

Antimicrobial susceptibility

Among the isolates tested (n=99), almost three-quarters of isolates (74; 74.7%) were resistant to erythromycin by standard disc diffusion assay, whilst a further 15 (15.2%) had reduced susceptibility, leaving only 10 (10.1%) isolates susceptible to erythromycin (Table 1).

Prevalence of erythromycin resistance determinants

The erm(B) gene and mef(A/E) gene were successfully amplified by PCR in separate reactions. The carriage rate of each gene was: erm(B), 25/99 (25.3%); mef(A), 1/99 (1.0%); mef(E), 75/99 (75.8%); and erm(B) + mef(E), 11/99 (11.1%). Partial erm(B) gene sequences were obtained from five species, i.e. S. salivarius, S. mitis, S. pneumoniae, S. sanguinis and S. oralis (Table 1). Those sequence similarities were >99%, containing a 3 bp mutation at the most. As a result of these substitutions, two amino acid substitutions were found, which were T75I and R118H. mef(E) gene sequences were obtained from all species. The sequence similarities were almost 100%, and one mutation existed at the most in each isolate. Although there were nine mutations overall at different nucleotide positions, none of them would have led to any amino acid substitution. Only one mef(A) sequence was obtained, which originated from an S. sanguinis isolate. Where erm(B), mef(A) and mef(E) gene sequence data were absent for the VGS group, appropriate and novel sequence data were deposited in GenBank, with accession numbers FJ667780–FJ667795 (Table 1).

Discussion

VGS have three known mechanisms of resistance to macrolides:14 the first is an active drug efflux mechanism, which is mediated by a membrane-bound efflux protein, commonly referred to as the M phenotype. This mechanism of resistance is encoded by the mef(A) gene. Two subclasses of the mef(A) gene have been described, i.e. mef(A) (from S. pyogenes) and mef(E) (from S. pneumoniae), but these are now considered as a single class of the mef(A) gene. The second mechanism of resistance is a target site modification, which is mediated by the erythromycin resistance methylases, encoded by the erm(A) or erm(B) genes, and this is known as the MLS B (macrolide, lincosamide and streptogramin B) phenotype, where expression of MLS B resistance can be either constitutive (cMLS B) or inducible (iMLS B). Finally, the third mechanism of resistance is a mutation in the streptococcal 23S rRNA or ribosomal protein genes, leading to macrolide or streptogramin B resistance (MS phenotype).14 Several studies have focused on macrolide resistance in VGS, relating to the M and MLS B phenotypes, namely erm(B) and mef(E), respectively.15,16 Our study demonstrated a high level of
erythromycin resistance, with approximately three-quarters of isolates being resistant and a further 15.2% of isolates having reduced susceptibility. Only 10.1% of isolates remained susceptible to erythromycin. All isolates within seven VGS species, namely *S. anginosus*, *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. parasanguinis* and *S. salivarius*, exhibited either full or intermediate resistance, followed by isolates of *S. oralis* and *S. sanguinis* (90% resistance) and then isolates of *S. mitis* (88.2% resistance). Pneumococci were the most susceptible, with 60% of isolates being resistant. This may be related to the relevant transient or short-term colonization of pneumococci in the CF lung or oropharynx, compared with a colonization of greater longevity associated with VGS persistence. Indeed, those CF patients with pneumococcal carriage were not being actively treated for pneumococcal lung disease.

We subsequently wished to compare our CF VGS population, which originated mainly from adult CF patients taking oral azithromycin long term, with a VGS population from a non-CF patient group, which had not been exposed to long-term use of macrolides, in order to access downstream consequences relating to macrolide resistance in commensal VGS flora within the CF patient population. As a comparator, clinically significant and invasive VGS-related infection was identified in 129 consecutive patients with bacteraemia over a 9 year period (2000–2008) at Belfast City Hospital. We did not wish to determine the macrolide resistance mechanism(s) in these non-CF isolates, as we believed this to be outside the scope of our present study because we were solely interested in examining molecular resistance mechanisms in CF VGS. Such resistance mechanisms have been described previously for macrolide resistance in non-CF isolates.14,17

Table 2 highlights the marked differences, in terms of both VGS population structure and, more importantly, levels of erythromycin resistance, between these two patient populations, where VGS originating in CF patients was significantly more resistant than VGS originating from non-CF-related sources. These data suggest that long-term use of azithromycin in the CF

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**Table 2.** Comparison of levels of individual species resistance to erythromycin in VGS from a CF patient population who were on long-term azithromycin therapy versus VGS from a comparator non-CF patient population who were not receiving long-term azithromycin therapy

<table>
<thead>
<tr>
<th>VGS isolated from patients with CF on long-term azithromycin therapy (% of isolates resistant to erythromycin), n=84 isolates</th>
<th>VGS isolated from bacteraemic patients without CF not on long-term azithromycin therapy (% of isolates resistant to erythromycin), n=129 isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. anginosus (100%)</td>
<td>S. anginosus (7.4%)</td>
</tr>
<tr>
<td>S. australis (100%)</td>
<td>S. gordonii (16.7%)</td>
</tr>
<tr>
<td>S. cristatus (50%)</td>
<td>S. mitis (27.2%)</td>
</tr>
<tr>
<td>S. gordonii (100%)</td>
<td>S. oralis (39.5%)</td>
</tr>
<tr>
<td>S. infantis (100%)</td>
<td>S. parasanguinis (33.3%)</td>
</tr>
<tr>
<td>S. mitis (88%)</td>
<td>S. salivarius (54.5%)</td>
</tr>
<tr>
<td>S. oralis (90%)</td>
<td>S. sanguinis (47.3%)</td>
</tr>
<tr>
<td>S. parasanguinis (100%)</td>
<td></td>
</tr>
<tr>
<td>S. salivarius (100%)</td>
<td></td>
</tr>
<tr>
<td>S. sanguinis (100%)</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 1.** Comparison of erythromycin susceptibility zone sizes and possession of *erm*(B) and *mef*(A) resistance determinants in a population of VGS and pneumococci isolated from the sputum of adult patients with CF. Open squares, *erm*(B); open diamonds, *mef*(A) or *mef*(E); filled triangles, *erm*(B) + *mef*(E); crosses, none. R, resistant; I, intermediate; S, susceptible.
Macrolide resistance in VGS from CF patients

patient population may lead to high levels of macrolide resistance in the VGS commensal flora.

Subsequently, we examined the molecular basis for macrolide resistance in the VGS CF isolates by PCR detection of the \textit{erm}(B) and \textit{mef}(A/E) genes, followed by sequence analysis of these loci. The MLS\textsubscript{B} type was approximately three times more frequent than the M type (76:25), demonstrating that efflux-mediated resistance is more frequent than resistance due to methylation events. To date, there has been little work performed examining \textit{erm}(B) and \textit{mef}(E) genes in VGS at the sequence level, and only \textit{mef}(A/E) has been described in \textit{S. mitis}, \textit{S. oralis}, \textit{S. salivarius} and \textit{S. sanguinis}. Likewise, \textit{erm}(B) has been identified previously in \textit{S. cristatus}, \textit{S. mitis} and \textit{S. oralis}, but sequence data were not shown. Elsewhere in GenBank, there has been a previous sequence report of \textit{erm}(B) originating in \textit{S. cristatus}. We therefore now wish to present novel sequence data for \textit{mef}(E), in relation to \textit{S. anginosus}, \textit{S. australis}, \textit{S. cristatus}, \textit{S. gordonii}, \textit{S. infantis}, \textit{S. mitis}, \textit{S. oralis} and \textit{S. parasanguinis}, as well as novel sequence data for \textit{erm}(B) relating to \textit{S. mitis}, \textit{S. oralis}, \textit{S. salivarius} and \textit{S. sanguinis}. All these novel sequences have now been deposited in GenBank, with corresponding accession numbers, as described in Table 1. Therefore, sequences from this study may facilitate the further understanding of macrolide resistance among VGS species.

The homology of \textit{erm}(B) and \textit{mef}(E) amongst the VGS species and pneumococci is interesting. From the present study we are not able to indicate the origins of this resistance in VGS or pneumococci as being either \textit{de novo} or as a result of horizontal gene transfer events; however, this homology causes concern in that commensal VGS in the upper and lower respiratory tracts of the CF patient may potentially act as an important reservoir of \textit{erm}(B) and \textit{mef}(E) gene determinants and may donate these to pathogens, such as pneumococci and/or members of the \textit{S. milleri} group.

When we compared zone sizes against the presence of \textit{erm}(B)\textsubscript{+}+\textit{mef}(E), we observed a relationship between relative zone size and presence/absence of the resistance determinants (see Figure 1). Overall, mean zone diameters were as follows: \textit{erm}(B)\textsuperscript{+}, 6.3 mm; \textit{mef}(E)\textsuperscript{+}, 13.3 mm; \textit{erm}(B)\textsubscript{+}/\textit{mef}(E)\textsuperscript{+}, 9.5 mm; and \textit{erm}(B)\textsuperscript{+}/\textit{mef}(E)\textsuperscript{−}, 24.2 mm. These indicate that \textit{erm}(B)\textsuperscript{+} isolates confer a higher level of macrolide resistance than resistance due to the presence of \textit{mef}(E)\textsuperscript{−}.

Perhaps most important of all is the carriage of antibiotic resistance determinants in CF commensal VGS flora. This is important as these determinants have the ability to be promiscuous and to exchange such determinants with true respiratory pathogens, such as \textit{S. pneumoniae} or the \textit{S. milleri} group. Indeed, in our study, we identified a strain of \textit{S. anginosus} which was \textit{mef}(E)\textsuperscript{−}. What remains unclear is the frequency of such genetic exchange events and the factors which up-regulate/down-regulate such events between commensals and pathogens.

Furthermore, these determinants are highly conserved, not only inter-VGS species, but also inter-genera [see Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. For instance, the \textit{erm}(B) determinant from \textit{Staphylococcus intermedius} (738 bp; position 263–1000; AF299292) demonstrates 99% homology with \textit{erm}(B) from \textit{S. pneumoniae} AJ972605, as well as multiple other genera and species. Likewise, the \textit{mef}(E) determinant, as demonstrated in VGS in this study, has complete homology with the same gene found in \textit{S. pneumoniae}, as well as in other streptococcal species (e.g. \textit{S. agalactiae} and \textit{S. intermedius}).

Overall, the implications of this study for clinicians are that prolonged use of azithromycin for an immunomodulatory effect may have negative impacts elsewhere, namely in the development of macrolide resistance in the commensal streptococcal flora.

In conclusion, this study demonstrated high level macrolide resistance in VGS commensal organisms in adult CF patients who were on long-term oral azithromycin therapy. More studies are urgently required to help define antibiotic resistance profiles of CF commensal flora and the potential role that these organisms play as a reservoir of antibiotic resistance determinants in this patient population.

Funding

This work was financially supported through an HSC Research & Development Office commissioned grant: Antimicrobial Resistance Action Plan (AMRAP) (COM/2730/04).

Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


