Characterization of macrolide resistance genes in 
*Haemophilus influenzae* isolated from children with cystic fibrosis

Marilyn C. Roberts*, Olusegun O. Soge and David B. No

Department of Environmental and Occupational Health Sciences 357234, School of Public Health, University of Washington, Seattle, WA 98195-7234, USA

*Corresponding author. Tel: +1-206-543-8001; Fax: +1-206-543-4873; E-mail: marilynr@u.washington.edu

Received 14 July 2010; returned 16 September 2010; revised 20 September 2010; accepted 15 October 2010

**Objectives:** To determine the mechanism(s) of macrolide resistance in *Haemophilus influenzae* isolated from cystic fibrosis (CF) patients participating in a randomized placebo-controlled trial of azithromycin.

**Methods:** Macrolide susceptibility, mutations and carriage of the macrolide resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A) were determined using PCR assays and sequencing or hybridization of the PCR products. *H. influenzae* isolates were used as donors in conjugation studies with *H. influenzae* and *Enterococcus faecalis* recipients. Transconjugant susceptibility and the macrolide resistance genes carried were determined.

**Results:** Of the 106 *H. influenzae* isolates, 27 were resistant and 78 intermediate resistant to azithromycin and/or erythromycin. All isolates carried one or more macrolide resistance gene(s), with the *mef*(A), *erm*(B) and *erm*(F) genes found in 74%, 31% and 29% of the isolates, respectively. None of the selected isolates had L4 or L22 mutations. Twenty-five donors, with various macrolide MICs, transferred macrolide resistance genes to *H. influenzae* Rd (3.5×10⁻⁷–1×10⁻¹⁰) and/or *E. faecalis* (1×10⁻⁷–1×10⁻⁸) recipients. The *H. influenzae* transconjugants were phenotypically resistant or intermediate to both macrolides while *E. faecalis* transconjugants were erythromycin resistant.

**Conclusions:** This is the first identification of *erm*(A), *erm*(C) and *erm*(F) genes in *H. influenzae* or bacteria from CF patients and the first characterization of macrolide gene transfer from *H. influenzae* donors. The high level of *H. influenzae* macrolide gene carriage suggests that the use of azithromycin in the CF population may ultimately reduce the effectiveness of continued or repeated macrolide therapy.

**Keywords:** azithromycin resistance, mobile *erm* and *mef*(A) genes, macrolides

**Introduction**

Untypeable *Haemophilus influenzae* is a common cause of community-acquired respiratory tract infections in adults and may cause acute sinusitis and/or acute otitis media.¹ *H. influenzae* is found in the lungs of cystic fibrosis (CF) patients and a 2009 study reported that between 1995 and 2005 the prevalence of *H. influenzae* increased in these patients.² In recent years, macrolides have been used for the treatment of a variety of untypeable *H. influenzae* infections.³,⁴ Antibiotics have also become important in the treatment of CF patients and randomized placebo-controlled trials of azithromycin in CF patients chronically infected with *Pseudomonas aeruginosa* have shown an improvement in lung function.⁵ The use of long-term azithromycin maintenance therapy in CF populations has been associated with increased macrolide resistance in CF patients. These isolates had no mutations in the ribosomal L4 or L22 genes or the 23S rRNA gene. The results suggested that...
acquired macrolide resistance genes found in other Gram-positive bacteria from CF patients could be another route for H. influenzae to become macrolide resistant.8

In the current study, 106 untypeable H. influenzae isolates from children with CF, between 6 and 18 years of age, enrolled in a double-blind, randomized, placebo-control treatment study of azithromycin were characterized for the presence of mutations in the ribosomal protein L4 and L22 genes and for carriage of commonly acquired macrolide resistance genes. One hundred and five of the H. influenzae isolates were either resistant or intermediately resistant to macrolides. All the H. influenzae isolates carried one or more acquired macrolide resistance gene(s) regardless of their phenotypic expression of azithromycin and/or erythromycin resistance. None of the selected isolates carried mutations in either their L4 or their L22 gene. Twenty-five H. influenzae were able to transfer these macrolide resistance genes to both H. influenzae and Gram-positive Enterococcus faecalis recipients, generating macrolide-resistant transconjugants as found in other studies with Gram-negative donors.9,10

Methods

Study design and bacterial isolates

The source of the H. influenzae isolates was a multicentre, multinational (USA and Canada), double-blind, randomized, placebo-control treatment study of children between 6 and 18 years of age with a diagnosis of CF. The study had human subject approval at each site. One hundred and thirty-one of the participants received azithromycin 250 or 500 mg three times each week, depending on the patients’ weight, and 129 participants were given placebo tablets three times each week. H. influenzae was isolated from the patients’ sputum at Seattle Children’s Hospital microbiology laboratory and forwarded to the University of Washington for further characterization. Isolates were obtained between February 2007 and June 2008. No demographic information was linked to the isolates so it is unknown how many of the isolates came from participants in the treatment and placebo groups. We also do not know if we received multiple isolates from any one participant. A total of 106 H. influenzae isolates were characterized in the current study.

Media

The H. influenzae isolates were grown on chocolate agar with brain heart infusion agar (BHI; Difco, Division of Becton Dickinson & Co., Sparks, MD) supplemented with 5% lysed sheep blood, or brain heart infusion agar supplemented with 10 mg/L haemin, 10 mg/L l-histidine and 2 mg/L nicotinamide adenine dinucleotide and incubated at 36.5°C in 5% CO2, as previously described.11 E. faecalis JH2-2 was grown on BHI agar (Difco).

Macrolide resistance

H. influenzae ATCC 49247 (MIC range 0.008–0.03 mg/L) was used as the control strain.12 The erythromycin and azithromycin MICs for H. influenzae and H. influenzae transconjugants were determined by Etest. Breakpoints of azithromycin MIC of >4 mg/L and erythromycin MIC of >8 mg/L (macrolide resistant), azithromycin 0.5–4 mg/L and erythromycin 1–8 mg/L (macrolide intermediate) and azithromycin <0.2 mg/L and erythromycin <1 mg/L (macrolide susceptible) were used in accordance with current BSAC guidelines.13 The E. faecalis JH2-2 transconjugants’ erythromycin MICs were determined by agar dilution using E. faecalis JH2-2 as a susceptible control.12

Detection of antibiotic resistance genes

Previously described PCR assays were used to detect the presence of the macrolide resistance genes erm(B), erm(F), msr(A) and mef(A) in all 106 isolates.14–16 Selected isolates were tested for the presence of erm(A) and erm(C) using PCR assays previously described.14 The PCR products were verified using hybridization with an internal 32P-labelled probe as described previously.14,15 Positive controls for the PCR assays were plasmids with cloned erm and msr(A) genes and E. faecalis JH2-2 was used as a negative control for all the PCR assays.16

L4 and L22 PCR assays

Selected isolates were used as templates for L4 and L22 PCR assays using PCR conditions of 1 cycle at 94°C for 5 min, 94°C for 30 s, 53°C for 30 s, 72°C for 45 s for 35 cycles, and 1 cycle at 72°C for 7 min and primers. The resulting PCR products were sequenced and compared with those from H. influenzae Rd as previously described.8

Conjugal gene transfer

The mobilities of the erm and mef(A) genes were examined using H. influenzae Rd (azithromycin MIC 1 mg/L, erythromycin MIC 2 mg/L) and/or E. faecalis JH2-2 (erythromycin MIC 2 mg/L) as the recipients as previously described.5,10,11 Twenty-five randomly selected donors that differed in their phenotypic resistance to azithromycin and erythromycin and the type and number of acquired macrolide resistance genes were used. Matings were performed at H. influenzae donor:recipient ratios of 1:1 and/or 1:10, while H. influenzae donor to E. faecalis recipient ratios of 25:1 and/or 50:1 were used. Transconjugants obtained from the H. influenzae–H. influenzae matings were selected on chocolate agar supplemented with erythromycin (5 mg/L) and streptomycin (250 mg/L) or rifampicin (10 mg/L). The transconjugants from the H. influenzae–E. faecalis matings were selected on BHI supplemented with erythromycin (10 mg/L) and nalidixic acid (25 mg/L) as previously described.14,15,17 The acquired macrolide gene(s) were confirmed by PCR assay and hybridization with an internal 32P-labelled probe as previously described.14,15

Results

Macrolide susceptibility, L4 and L22 mutations

Using BSAC macrolide standards, 27 (25.5%) of the H. influenzae isolates were resistant to azithromycin and erythromycin (Azi’ Erm’), 78 (73.6%) of the H. influenzae isolates were intermediate to azithromycin and erythromycin (Azi’ Erm) and one isolate (0.9%) was susceptible to both macrolides (Erm’ Azi’). (Table 1). It was previously found that 26 of 31 H. influenzae strains, with increased macrolide MICs, from community-acquired respiratory tract infections, had mutations in the ribosomal protein L4 and/or L22 genes and 3 isolates had no mutations.5 Therefore, 25 of the 27 Azi’ Erm’ and 22 of the 78 Azi’ Erm’ isolates were tested for mutations in the L4 and L22 genes using the PCR assays developed by Peric et al.13 None of these isolates had mutations in either L4 or L22 sequences when compared with H. influenzae Rd (Table 1).

Presence of acquired macrolide resistance

We had previously shown that Azi’ Erm’ H. influenzae isolated between 2002 and 2004 from CF patients carried the acquired macrolide–lincosamide–streptograminB (MLSB) resistance genes erm(B) and mef(A) and had no mutations in the
The carriage level of \textit{erm}(A) and \textit{erm}(C) is likely to be underestimated since only those isolates that were negative for \textit{erm}(B), \textit{erm}(F) and \textit{mef}(A) PCR assays were tested for \textit{erm}(A) and \textit{erm}(C).\textit{ATM, azithromycin; ERY, erythromycin.} 
\textit{a}None of the isolates sequenced had either \textit{L4} or \textit{L22} mutations. 
\textit{b}One donor transferred only the \textit{mef}(A) gene.

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>MIC (mg/L)</th>
<th>No. of isolates used as donors (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>&gt;4–64 &gt;8 to &gt;256</td>
<td>2 \textit{erm}(B), \textit{mef}(A), \textit{erm}(F) 2 \textit{erm}(B), \textit{mef}(A) 4 1 1 1 \textit{erm}(B), \textit{erm}(F) 1 0 1 8 \textit{mef}(A) 7 1 3 2 \textit{erm}(F) 2 0 1 1 \textit{erm}(C) 1 0 1 1 \textit{erm}(A) 1 0 1</td>
</tr>
<tr>
<td>78</td>
<td>0.5–4 1–8</td>
<td>5 \textit{erm}(B), \textit{erm}(F), \textit{mef}(A) 2 3 2 8 \textit{erm}(B), \textit{mef}(A) 3 5 5 6 \textit{erm}(F), \textit{mef}(A) 3 3 0 1 \textit{mef}(A), \textit{erm}(C) 0 1 1 34 \textit{mef}(A) 5 29 1 13 \textit{erm}(B) 3 10 1 6 \textit{erm}(F) 3 3 0 4 \textit{erm}(C) 2 2 4 1 \textit{erm}(A) 1</td>
</tr>
<tr>
<td>1</td>
<td>&lt;0.25 &lt;0.5</td>
<td>1 \textit{erm}(B), \textit{mef}(A) 0 1 0</td>
</tr>
</tbody>
</table>

The carriage level of \textit{erm}(A) and \textit{erm}(C) is likely to be underestimated since only those isolates that were negative for \textit{erm}(B), \textit{erm}(F) and \textit{mef}(A) PCR assays were tested for \textit{erm}(A) and \textit{erm}(C).\textit{ATM, azithromycin; ERY, erythromycin.} 
\textit{a}None of the isolates sequenced had either \textit{L4} or \textit{L22} mutations. 
\textit{b}One donor transferred only the \textit{mef}(A) gene.

The carriage level of \textit{erm}(A) and \textit{erm}(C) is likely to be underestimated since only those isolates that were negative for \textit{erm}(B), \textit{erm}(F) and \textit{mef}(A) PCR assays were tested for \textit{erm}(A) and \textit{erm}(C).\textit{ATM, azithromycin; ERY, erythromycin.} 
\textit{a}None of the isolates sequenced had either \textit{L4} or \textit{L22} mutations. 
\textit{b}One donor transferred only the \textit{mef}(A) gene.

Four domain V 23S rRNA or ribosomal protein L4 and L22 genes.\textsuperscript{8} Therefore it was of interest to determine whether the 106 CF isolates in the current study carried either of these genes. The \textit{erm}(F) gene was included because it has previously been identified in other Gram-negative bacteria and is a commonly found \textit{erm} gene.\textsuperscript{16} Ninety-nine (93%) of the 106 isolates carried one or more of the three macrolide resistance genes and included 25 of the \textit{Azir Ermr H. influenzae}, 73 of the \textit{Azii Ermi H. influenzae} and the 1 \textit{Ermi H. influenzae} (Table 1). The \textit{erm}(F) gene was found in 78 (74%), the \textit{erm}(B) gene in 33 (31%) and the \textit{erm}(F) gene in 27 (29%) of the isolates (Table 1). The remaining seven isolates, including two \textit{Azir Ermr H. influenzae} and five \textit{Azii Ermi H. influenzae}, were negative for the three genes.

The \textit{erm}(C) and \textit{erm}(A) genes are the third and fourth most commonly found macrolide resistance genes and thus it was of interest to determine whether the seven negative isolates carried either of these two genes.\textsuperscript{18} These, along with 10 randomly selected isolates that carried the \textit{erm}(F), \textit{erm}(B) and/or \textit{mef}(A) genes, were tested for carriage of the \textit{erm}(A) and \textit{erm}(C) genes. Two isolates carried \textit{erm}(A), five isolates carried \textit{erm}(C), one isolate carried both \textit{erm}(C) and \textit{mef}(A) and the remaining isolates did not carry \textit{erm}(A) and/or \textit{erm}(C) (Table 1). Thus, all 106 \textit{H. influenzae} isolates were positive for one or more of the macrolide resistance genes examined (Table 1). Fifteen (56%) of the \textit{Azir Ermr H. influenzae} isolates, 20 (26%) of \textit{Azii Ermi H. influenzae} isolates and the 1 \textit{Azii Ermr H. influenzae} isolate carried two or three macrolide resistance genes, 12 of the \textit{Azir Ermr H. influenzae} (44%) and 58 (74%) of the \textit{Azii Ermi H. influenzae} isolates carried one of the macrolide resistance genes tested (Table 1).

### Macrolide gene transfer

The \textit{erm}(A), \textit{erm}(B), \textit{erm}(C), \textit{erm}(F) and \textit{mef}(A) genes are normally associated with mobile elements.\textsuperscript{18} Therefore it was of interest to determine whether selected \textit{H. influenzae} isolates would be able to transfer their macrolide resistance genes to recipients and if the resulting transconjugants would be macrolide resistant. We chose to use \textit{H. influenzae} Rd and \textit{E. faecalis} JH2-2 as recipients. \textit{E. faecalis} JH2-2 has been used as a recipient in a number of conjugation studies previously since these genes are known to be expressed in the strain.\textsuperscript{9,16} Ten \textit{Azir Ermr} and 15 \textit{Azii Ermi H. influenzae} isolates were used as donors in the mating experiments (Table 2). Transfer frequencies between the \textit{H. influenzae} donor and the \textit{H. influenzae} Rd recipient ranged from 3.5×10\textsuperscript{-7} to 1×10\textsuperscript{-10} per recipient and for the \textit{H. influenzae} donor and \textit{E. faecalis} ranged from 1×10\textsuperscript{-7} to 1×10\textsuperscript{-8} per recipient (Table 2). Twelve of the donors carried two macrolide resistance genes and 11 (92%) of the donors transferred both macrolide resistance genes to the transconjugants (Table 2). The \textit{H. influenzae} transconjugants had 4- to 48-fold increased MICs of azithromycin and 4- to 24-fold higher MICs of erythromycin compared with the recipient (Table 2). All the \textit{H. influenzae} transconjugants were resistant to erythromycin and resistant or intermediate to azithromycin. All \textit{E. faecalis} transconjugants were erythromycin resistant.
had macrolide therapy, since this did not disqualify them from participating in the trial. In addition, macrolide resistant H. influenzae isolation has increased in recent years both in community-acquired infections and from CF patients. This increase might also explain the differences between the H. influenzae from the 1999–2004 study and the current study.3

There are a number of possible reasons why the CF H. influenzae in the current study carried macrolide resistance genes while the earlier study did not find acquired macrolide resistance genes.7 In the previous study the H. influenzae were isolated from an adult population while the current study looked at CF children, of whom half were on azithromycin therapy. These two populations have different microbiota and it is likely that all the CF children in the study had previously had macrolide therapy, since this did not disqualify them

<table>
<thead>
<tr>
<th>Donor</th>
<th>Genes carried</th>
<th>MIC (mg/L)</th>
<th>Transconjugants</th>
<th>MIC&lt;sup&gt;5&lt;/sup&gt; (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATM</td>
<td>ERY</td>
<td>species</td>
</tr>
<tr>
<td>CHH002 erm(F), mef(A)</td>
<td>64</td>
<td>128</td>
<td>H. influenzae</td>
<td>2.5 × 10&lt;sup&gt;-10&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH025 mef(A)</td>
<td>4</td>
<td>16</td>
<td>H. influenzae</td>
<td>3.7 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH063 mef(A)</td>
<td>4</td>
<td>8</td>
<td>H. influenzae</td>
<td>7.4 × 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH073 mef(A)</td>
<td>16</td>
<td>32</td>
<td>H. influenzae</td>
<td>3.5 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH048 erm(F)</td>
<td>16</td>
<td>128</td>
<td>H. influenzae</td>
<td>3.5 × 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH078 erm(B), mef(A)</td>
<td>6</td>
<td>12</td>
<td>H. influenzae</td>
<td>5.3 × 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH078 erm(B), mef(A)</td>
<td>6</td>
<td>12</td>
<td>E. faecalis</td>
<td>1.1 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH036 erm(F), mef(A)</td>
<td>32</td>
<td>256</td>
<td>E. faecalis</td>
<td>1.2 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH082 erm(B), mef(A)</td>
<td>8</td>
<td>8</td>
<td>E. faecalis</td>
<td>2.4 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH028 erm(B), mef(A)</td>
<td>4</td>
<td>8</td>
<td>E. faecalis</td>
<td>1.3 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH090 erm(B), mef(A)</td>
<td>4</td>
<td>8</td>
<td>E. faecalis</td>
<td>1.0 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH134 erm(C), mef(A)</td>
<td>4</td>
<td>8</td>
<td>E. faecalis</td>
<td>1.6 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH039 erm(B)</td>
<td>3</td>
<td>4</td>
<td>E. faecalis</td>
<td>2.6 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH080 erm(A)</td>
<td>&lt;2</td>
<td>4</td>
<td>E. faecalis</td>
<td>1.8 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH012 erm(C)</td>
<td>8</td>
<td>16</td>
<td>E. faecalis</td>
<td>5.8 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH132 erm(C)</td>
<td>4</td>
<td>4</td>
<td>E. faecalis</td>
<td>7.2 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH049 erm(C)</td>
<td>3</td>
<td>3</td>
<td>E. faecalis</td>
<td>4.3 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH122 erm(C)</td>
<td>1</td>
<td>4</td>
<td>E. faecalis</td>
<td>1.7 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHH089 erm(C)</td>
<td>&lt;2</td>
<td>4</td>
<td>E. faecalis</td>
<td>9.4 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ATM, azithromycin; ERY, erythromycin; ND, not determined.

aTransconjugants/number of recipient bacteria.

bMultiple transconjugants tested.

Discussion

CF is a genetic disease that is characterized by recurrent pulmonary infections that may damage the lungs. In addition, CF patients are often colonized with a variety of bacteria, including H. influenzae.5 Since the 1980s the beneficial effect of long-term low-dose treatment with macrolides has been reported, and this treatment has led to improvements in pulmonary functions.2,3,8-11 In the current study, 25.5% of the H. influenzae were Azii Erm<sup>+</sup> and 73.6% were Azii Erm<sup>-</sup>, while 100% carried one or more macrolide resistance gene(s). The Azii Erm<sup>-</sup> isolates were identified throughout the 16 months of collection but it is unknown how many of the isolates were from the children in the azithromycin treatment group.

There are a number of possible reasons why the CF H. influenzae in the current study carried macrolide resistance genes while the earlier study did not find acquired macrolide resistance genes.7 In the previous study the H. influenzae were isolated from an adult population while the current study looked at CF children, of whom half were on azithromycin therapy. These two populations have different microbiota and it is likely that all the CF children in the study had previously had macrolide therapy, since this did not disqualify them...
macrolide resistance and the number of macrolide genes carried. However, a larger number of isolates will need to be characterized to verify this hypothesis.

Twenty-five randomly selected \textit{H. influenzae} were able to transfer their macrolide resistance gene(s) to Gram-positive and/or Gram-negative recipients, resulting in Erm\textsuperscript{r} Azi\textsuperscript{r} or Erm\textsuperscript{r} Azi\textsuperscript{r} \textit{H. influenzae} transconjugants and Erm\textsuperscript{r} \textit{E. faecalis} transconjugants. Since all of these macrolide resistance genes have been identified in oral bacteria and in \textit{P. aeruginosa} from healthy children, it is tempting to speculate that these same genes could be transferred \textit{in vivo} between bacteria within the lungs or oral cavity of CF patients and \textit{H. influenzae} could act as a donor or recipient in gene exchange. Further work is needed to determine whether bacteria from CF patients are able to exchange these macrolide resistance genes \textit{in vivo} and whether current azithromycin therapies increase gene exchange \textit{in vivo}. Additional work is needed to determine whether continued azithromycin therapy in CF patients routinely increases carriage of macrolide-resistant bacteria and whether this will ultimately reduce the effectiveness of continued or repeated macrolide therapy.

Acknowledgements
We thank Dr Jane Burns and Anne Marie Buccat from the CF laboratory at Children’s Hospital, Seattle, for providing the strains.

Funding
The CF study was supported in part by the Cystic Fibrosis Foundation Therapeutics.

Transparency declarations
None to declare.

References
5 Tazumi A, Maeda Y, Goldsmith CE et al. Molecular characterization of macrolide resistance determinants \textit{erm(B)} and \textit{erm(A)} in \textit{Streptococcus pneumoniae} and viridans group streptococci (VGS) isolated from adult patients with cystic fibrosis (CF). \textit{J Antimicrob Chemother} 2009; \textbf{64}: 1–6.