Acquired macrolide resistance genes in *Haemophilus influenzae?*

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Objectives: The objective of this study was to determine the prevalence of specific acquired macrolide resistance genes previously reported as present in clinical isolates of *Haemophilus influenzae.*

Methods: A collection of 172 clinical respiratory isolates of *H. influenzae*, including 59 isolates from cystic fibrosis patients and 27 from non-cystic fibrosis bronchiectasis patients with significant prior macrolide use, was established. This collection was tested for azithromycin susceptibility using Etest and screened for the presence of *erm*(A), *erm*(B), *erm*(C), *erm*(F), *mef*(A) and *mef*(E) using locked nucleic acid dual-labelled hydrolysis probes.

Results: The azithromycin MICs ranged from 0.09 to >256 mg/L, with 2 (1.2%) isolates susceptible, 163 (94.8%) intermediate and 7 (4%) resistant according to EUCAST breakpoints (susceptible, ≤0.12 mg/L; resistant, >4 mg/L). None of the acquired macrolide resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(F), *mef*(A) or *mef*(E) was detected in any of the isolates.

Conclusions: The specific acquired macrolide resistance genes are not widespread in *H. influenzae* and the high prevalence of these genes previously reported might be unique to the specific circumstances of that study.

Keywords: *erm*, *mef*, PCR

Introduction

Macrolide antibiotics have limited activity against *Haemophilus influenzae* due to the presence of an intrinsic efflux pump (azithromycin MICs typically 0.25–4 mg/L) and additional high-level resistance (azithromycin MICs typically >64 mg/L) is also present in some isolates and associated with L4 and L22 ribosomal protein and 23S rRNA mutations.1,2 There is a large range of acquired macrolide resistance genes that are particularly common in, but not limited to, Gram-positive bacteria. These include various *erm* genes that code for rRNA methylases and inhibit macrolide binding at the 23S rRNA target and various *mef* or related genes that encode alternate efflux pumps.3

These acquired macrolide resistance genes have not been widely reported in *H. influenzae*, with the only significant report by Roberts et al.4 in 2011. In that study, 106 isolates of *H. influenzae* from cystic fibrosis (CF) patients in a placebo-controlled trial of azithromycin were tested for the presence of *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A) by molecular methods. All isolates, including those on placebo, were reported to have one or more of the acquired macrolide resistance genes, but there was no clear association between the presence of these genes and the azithromycin or erythromycin MICs. The only other report of an acquired macrolide resistance gene in *Haemophilus* species was *mef*(A) detected by microarray and sequencing in an isolate of *Haemophilus parainfluenzae*; in this case, macrolide MICs were >256 mg/L.5

The aim of our study was to determine whether the presence of these acquired macrolide resistance genes is widespread in the broader *H. influenzae* population.

Methods

Bacterial strains and susceptibility testing

Our study collection consisted of 172 clinical respiratory isolates of *H. influenzae* identified by colony morphology, X and V factor dependence and a positive PCR for either fucK or hpd as previously described.6 Of these, 59 were from CF patients, 27 from a cohort of non-CF bronchiectasis patients with significant prior macrolide use7 and the remaining 86 from a range of other patients. Azithromycin MICs were determined using Etest on Mueller–Hinton agar supplemented with 5% (v/v) defibrinated horse blood and 20 mg/L NAD and incubated at 37°C in 5% CO2 for 24 h using *H. influenzae* ATCC 49247 as a control. EUCAST breakpoints (susceptible, ≤0.12 mg/L; resistant, >4 mg/L) were used for interpretation.8

Detection of acquired macrolide resistance genes

Genomic DNA extracted using the ISOLATE II kit (Bioline, NSW, Australia) was used as template for PCR.
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Table 1. Probes and primers for detection of acquired macrolide resistance genes

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequencea</th>
<th>Positionb</th>
<th>GenBank accessionc</th>
</tr>
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<tbody>
<tr>
<td>16S probe</td>
<td>ttcCtcCacAtcTcCacTc</td>
<td>697–715</td>
<td>L42023</td>
</tr>
<tr>
<td>16S F</td>
<td>AATTACAGATGGTGACTAGAG</td>
<td>634–655</td>
<td></td>
</tr>
<tr>
<td>16S R</td>
<td>GTCAGTACATCAGCAGGG</td>
<td>735–753</td>
<td></td>
</tr>
<tr>
<td>ermA probe</td>
<td>tcACttGgGATGAgGt</td>
<td>184–202</td>
<td>X03216</td>
</tr>
<tr>
<td>ermA F</td>
<td>ACAAGACAACTGTAAGAACATCG</td>
<td>90–112</td>
<td></td>
</tr>
<tr>
<td>ermA R</td>
<td>CTTTATATCTAGAGGTTTAC</td>
<td>214–238</td>
<td></td>
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<tr>
<td>ermB probe</td>
<td>cCGCtCacAtcAgGat</td>
<td>528–543</td>
<td></td>
</tr>
<tr>
<td>ermB F</td>
<td>TCACTTAAACCAAAAGTAACAG</td>
<td>486–509</td>
<td></td>
</tr>
<tr>
<td>ermB R</td>
<td>TTAAGAAGATCGAAGATATTTC</td>
<td>599–623</td>
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<tr>
<td>ermC probe</td>
<td>agCGAacGCAACCAATTG</td>
<td>379–396</td>
<td></td>
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<tr>
<td>ermC F</td>
<td>AGTCAcGATATAACATCAGG</td>
<td>319–343</td>
<td></td>
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<tr>
<td>ermC R</td>
<td>ATGCCAATGAGCGTTTTG</td>
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<tr>
<td>ermF probe</td>
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<td>AF219231</td>
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<td>ermF F</td>
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<tr>
<td>ermF R</td>
<td>CTCTAAGAGATGAAAG</td>
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<td>mefA probe</td>
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<td>mefA F</td>
<td>GGAAGACATAGAAAGAG</td>
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<tr>
<td>mefA R</td>
<td>CAGTACGTCATATAGATGC</td>
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<tr>
<td>mefE probe</td>
<td>tccGcGcCacCacAuctct</td>
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<td>CCTAAGCTGGTATCAAGTG</td>
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<tr>
<td>mefE R</td>
<td>CTAAAGTGATAAAGGAAACATCC</td>
<td>658–682</td>
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</table>

a Sequences are given 5′ to 3′ and, for probes, capital letters indicate locked nucleotides.
b Numbering based on ORF, except for 16S, which is based on a 1539 bp sequence.
c Reference sequences used for primer design.

All isolates were initially tested for the presence of the genes using locked nucleic acid dual-labelled hydrolysis probes (Sigma–Aldrich, NSW, Australia) in two multiplex reactions for ermA(A), ermA(B) and ermA(C) in reaction 1 and ermA(F), mefA and mefE in reaction 2, with both reactions also including a 16S rRNA amplification control. Reaction conditions were as follows: iQ Multiplex Powermix (Bio-Rad, NSW, Australia) with probe and primers at final concentrations of 0.25 and 0.5 μM, respectively, over 30 cycles with an annealing temperature of 60°C. Primer sequences are given in Table 1.

Subsequently, the isolates were retested for the same genes [excluding mef(E)] using the primers and annealing temperatures described by Roberts et al.,2 but on a real-time PCR platform using SYBR Green (Bio-Rad, NSW, Australia). Positives were checked on an agarose gel and amplicons of an appropriate size were sequenced using the respective amplification primers. The primer sequences are given in Table S1 (available as Supplementary data at JAC Online).

The control material was as follows: clinical isolates for mef(E), mef(A), ermA(A) and ermA(B) with gene identity confirmed by sequencing; pE194 for ermA(C); and an erm(F) amplicon kindly provided by Zhongtang Yu (Department of Animal Sciences, Ohio State University, Columbus, OH, USA).

Results and discussion

The azithromycin MICs ranged from 0.09 to >256 mg/L, with MIC50 and MIC90 values of 1.5 and 3 mg/L, respectively. Two (1.2%) isolates were susceptible, 163 (94.8%) intermediate and 7 (4%) resistant according to EUCAST breakpoints (susceptible, ≤0.12 mg/L; resistant, >4 mg/L).9 Of the resistant isolates, five were low level (6–16 mg/L) and not further investigated, but two had azithromycin MICs of >256 mg/L and were further investigated by sequencing of the L4 and L22 ribosomal protein and 23S rRNA genes as previously described.2 One strain had an A2058 mutation in the 23S rRNA gene and the other had C2611T and R88P mutations in the 23S rRNA and L22 ribosomal protein genes, respectively, all of which have been previously associated with macrolide resistance.2,9,10

None of the acquired macrolide resistance genes ermA(A), ermA(B), ermA(C), ermA(F), mefA or mefE was detected in any of the isolates.

The absence of these acquired macrolide resistance genes in our isolates is in stark contrast to the findings of Roberts et al.,6 where at least one gene was detected in each of their 106 isolates, two different genes were detected in 28 isolates and three different genes were detected in 7 isolates. This difference cannot be readily explained. Admittedly, only a small proportion of our isolates (27/106) came from a known background of high cohort macrolide exposure, but the Roberts et al.6 study found the acquired macrolide resistance genes in isolates from both placebo and macrolide treatment groups, so recent macrolide use was not a prerequisite for the presence of the genes. Similarly, the isolates from the Roberts et al.6 study were more resistant than ours, with 27/106 (25%) resistant, but again the acquired macrolide resistance genes were detected across all isolates irrespective of MIC, including ermA(B) and mefA in the single susceptible isolate.

To investigate technical differences, we retested our isolates using the primers used in the Roberts et al.6 study and found 21 isolates to be presumptively positive for mefA and 6 for ermA(A). The azithromycin MIC range for these isolates was 0.125 to
These positive PCR results were found to be false positives. The amplicons from the mef(A) and erm(A) primers were indistinguishable from the 942 and 332 bp amplicons of the respective positive controls on gel electrophoresis and were subsequently sequenced and identified using a blastn search. The false positive mef(A) and erm(A) amplicons were found to span nucleotides 588,138–589,053 (916 bp) encompassing the transcription accessory protein and nucleotides 32,743–32,383 (361 bp) encompassing the nod shape-determining protein, respectively, in H. influenzae Rd (GenBank accession L42023) and similar sequences in other strains of H. influenzae. Close examination of the ends of these sequences revealed significant degrees of similarity to the primers, particularly at the 3′ end. These similarities are illustrated in Table S2.

These observations raise the question as to why all of our isolates did not give false positives with these PCRs, given that these genes are part of the core genome of H. influenzae. When we examined the relevant regions of GenBank sequences for these genes in other strains of H. influenzae, some sequence variation that could affect primer binding was evident. In addition, our false positive reactions had Ct values of between 24 and 27 cycles compared with 15–20 cycles for the positive controls, so it would not be unexpected for small variations in nucleotide sequence or DNA quality across our test isolates to result in inconsistent amplification of these chromosomal targets.

It is interesting to speculate as to whether some of the positive results in the Roberts et al.4 study, particularly for mef(A) and erm(A), could have been similar false positives. This might explain the large number of positives and the absence of associated raised macrolide MICs. However, although none of the amplicons in that study was confirmed by sequencing, they were confirmed using hybridization probes.

In conclusion, our study shows that acquired macrolide resistance genes are not widespread in H. influenzae and the high prevalence of these genes previously reported might be unique to the specific circumstances of that study. We also demonstrate that the use of primers in organisms other than those for which the primers were initially designed and evaluated can produce false positive amplification not easily detected without confirmatory tests such as sequencing.

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>256 mg/L with MIC50 and MIC90 values of 1.5 and 4 mg/L, respectively. In the additional investigation detailed below, these positive PCR results were found to be false positives.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**