Molecular genetic analysis of high-level gentamicin resistance in
Enterococcus hirae

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High-level resistance to gentamicin was studied in seven clinical isolates of Enterococcus hirae. In common with other members of the genus Enterococcus, such resistance in E. hirae was associated with single, large, conjugative plasmids. Molecular genetic analysis revealed five distinct plasmid types amongst the seven isolates. The determinant mediating high-level gentamicin resistance in E. hirae was also shown to be homologous to that already characterized for other enterococcal species.

Introduction

During the last decade, enterococci have become more prevalent in hospital-acquired infections in the United Kingdom and the United States.\(^1,2\) In addition, the degree of multiple antibiotic resistance in enterococci has increased, with direct implications for the therapy of enterococcal infection. In a survey of antimicrobial susceptibilities of clinical isolates of enterococci in Ireland,\(^3\) we established that Enterococcus hirae accounted for 3% of isolates, 34% of which displayed high-level gentamicin resistance (HLGR; MIC > 1000 mg/L). This to our knowledge was the first report of HLGR for this enterococcal species.

HLGR was initially documented in two enterococcal species, E. faecalis\(^4\) and E. faecium.\(^5\) The bifunctional aminoglycoside-modifying enzyme A A C(6\(^\prime\))-A PH(2\(^\prime\)) responsible for HLGR is often plasmid mediated, and has been well-characterized in both species. There is marked heterogeneity in plasmids mediating HLGR among geographically diverse strains of E. faecalis\(^6\) whereas there is extensive homology among plasmids mediating HLGR in E. faecium.\(^7\) In addition, there are reports of HLGR occurring in other enterococcal species, specifically E. mundtii, E. gallinarum, E. raffinosus, E. caseliflavus, E. avium\(^8\) and E. hirae.\(^9\) A more recent study on a single E. hirae isolate showed that HLGR was associated with a single, large (90 kb) conjugative plasmid.\(^9\) In this study, we investigated the basis of HLGR among seven clinical isolates of E. hirae originating from a nationwide survey of enterococcal isolates.\(^3\)

Materials and methods

Bacterial strains and media

Enterococci were recovered from clinical material as part of a nationwide survey of antibiotic susceptibilities in enterococci.\(^3\) The isolates were identified on the basis of Gram-stain morphology, catalase-negativity, tolerance to bile, hydrolysis of aesculin, pyrrolidonyl peptidase activity (Murex Diagnostics Limited, Dartford, UK), and reactivity to Lancefield group D antiserum (Streptex latex suspension to group D, Murex Diagnostics, Ltd). Speciation was performed using the Ruoff \textit{et al.}\(^10\) modification of the method described by Facklam & Collins.\(^11\) The initial antibiotic susceptibility tests were performed using the breakpoint technique following the British Society for Antimicrobial Chemotherapy (BSAC) guideline\(^12\) using Oxoid Diagnostic Sensitivity Test agar (DST, Oxoid, Basingstoke, UK) supplemented with 5% lysed horse blood. The medium was inoculated with a multipoint inoculator delivering 10\(^5\) cfu/spot. Control strains of Staphylococcus aureus NCTC 6571 and enterococci with known MICs were incorporated in each batch for susceptibility testing. A number of antibiotics were tested using BSAC Working Party\(^12\) breakpoint concentrations (where given). High-level streptomycin resistance (HLSR) and HLGR were each assessed using a breakpoint concentration of 1000 mg/L. All isolates were tested for \(\beta\)-lactamase production (Cefinase discs 3165, BBL Becton Dickinson, Cockeysville, MD, USA). HLGR was evident in 7% of the isolates from 13 of the 23 hospitals surveyed, which were
spread throughout Ireland. Seven isolates of *E. hirae* were studied from three geographically dispersed hospitals. All isolates were β-lactamase negative. (see Table for source and susceptibilities). The sole septicemic patient had endocarditis, and made a full recovery after prolonged vancomycin and ciprofloxacin therapy.

The recipient strain for conjugation experiments was *E. faecalis* JH 2-2, which is resistant to both rifampicin and fusidic acid, does not exhibit HLGR or HLSR, and is susceptible to erythromycin, chloramphenicol, tetracycline and trimethoprim. Two *Escherichia coli* strains, V517 and 39R 861, both of which contain plasmids of known molecular weight, were employed as markers for plasmid analysis.

### Conjugation experiments and analysis of transconjugants

Enterococcal strains were grown in BHI broth, or on BHI agar plates with 1% agar. Conjugation experiments were carried out using a filter mating technique between the clinical isolates (all HLGR and all susceptible to rifampicin and fusidic acid) and *E. faecalis* JH 2-2 (fusidic acid and rifampicin resistant and gentamicin susceptible). A aliquots of 50 μL of overnight cultures of donor and recipient strains were grown in BHI broth, pooled and resuspended on 0.2 μm pore nitrocellulose filters (Sartorius, Dublin, Ireland), resting on BHI agar plates in the absence of selective pressures. After overnight incubation at 37°C, growth was resuspended in 2 mL BHI broth. Serial dilutions in BHI broth were made and 100 μL aliquots of each dilution were then plated on to selective BHI plates. HLGR transconjugants were isolated on BHI plates supplemented with gentamicin (1000 mg/L), rifampicin (15 mg/L) and fusidic acid (25 mg/L). For the purposes of this study, HLGR donors were labelled 1–7, and their respective transconjugants 1–7.

### Transconjugant antibiotic susceptibility testing

The antibiotic susceptibilities of the transconjugants were determined by the disc diffusion method on Iso-Sensitest A agar (Oxoid, Basingstoke, U K), in accordance with the **B**SA **C** Working Party guide to sensitivity testing. Plates were seeded with 10⁵ cfu/mL of the relevant cultures, and susceptibility was determined using the *S. aureus* strain as before.

The antibiotic concentrations per disc were: ampicillin (25 μg), erythromycin (30 μg), tetracycline (50 μg), trimethoprim (2.5 μg), chloramphenicol (10 μg), rifampicin (5 μg), vancomycin (5 μg), ciprofloxacin (5 μg) and amikacin (30 μg).

### Plasmid DNA extraction and analysis

Closed covalent circular marker DNA from *E. coli* strains V517 and 39R 817 was isolated by a modified alkaline lysis method, omitting the use of lysozyme. Enterococcal plasmid DNA was isolated by a modified alkaline lysis procedure. Bacteria were harvested directly from agar plates by aseptically spreading 5 mL of sterile water on the plate. Four 1 mL aliquots of resuspended growth were transferred to sterile microfuge tubes, which were then centrifuged at 1640g. The supernatant was removed, and the bacteria were resuspended in 1 mL SET (200 mM sodium chloride, 25 mM EDTA, 20 mM Tris; pH 8.0). The relatively strong concentration of EDTA that may be present either in the BHI or on the cells themselves, and which may inhibit effective lysis.

### Table. Sources and susceptibilities of HLGR *E. hirae* strains

<table>
<thead>
<tr>
<th>Antibiotic (breakpoint concentration, mg/L)</th>
<th>Source of <em>E. hirae</em> strains (1-7)</th>
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<tbody>
<tr>
<td></td>
<td>1 urine</td>
</tr>
<tr>
<td>Ampicillin (8)</td>
<td>R</td>
</tr>
<tr>
<td>Vancomycin (4)</td>
<td>S</td>
</tr>
<tr>
<td>Teicoplanin (4)</td>
<td>S</td>
</tr>
<tr>
<td>Ciprofloxacin (4)</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin (0.5)</td>
<td>R</td>
</tr>
<tr>
<td>Chloramphenicol (8)</td>
<td>R</td>
</tr>
<tr>
<td>Tetracycline (1)</td>
<td>R</td>
</tr>
<tr>
<td>Trimethoprim (2)</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin (1000)</td>
<td>R</td>
</tr>
<tr>
<td>Gentamicin (100)</td>
<td>R</td>
</tr>
</tbody>
</table>

R, resistant; S, susceptible.
HLGR in Enterococcus hirae

The resulting cell pellet was resuspended in 200 μL GET buffer (600 mM glucose, 10 mM EDTA, 25 mM Tris; pH 8.0), supplemented with 2 mg/mL lysozyme, and incubated at 37°C for 45 min. A 300 μL aliquot of a 3% sodium dodecyl sulphate and 0.3 M sodium hydroxide solution was used for alkaline lysis. The resulting solution was incubated at room temperature for 10 min, then 500 μL of 5 M potassium acetate, pH 4.8, was added and the lysate was incubated at 4°C for 30 min, and centrifuged at 12,400g. Plasmid DNA was recovered from the supernatant by purification on Nucleobond AX-100 columns (Machery-Nagel, Dueren, Germany) according to the manufacturer’s instructions.

Plasmid DNA isolated from all seven E. hirae strains was separated from electrophoresis on a 0.7% agarose gel using 1.0 × Tris-acetate-EDTA (TAE) buffer, run at 35 V, 50 mA, for 16–20 h. After staining with ethidium bromide (0.5 mg/L), fractionated plasmid DNA was visualized under UV light. The molecular weight of parental and transconjugant plasmid DNA was determined by comparing its position on the gel with that of plasmid DNA standards isolated from E. coli strains V517 and 39R817.

Restriction enzyme analysis of plasmid DNA

Purified transconjugant plasmid DNA was digested with restriction endonuclease HindIII and buffer as supplied by New England Biolabs (Beverly, MA, USA), and used in accordance with the manufacturer’s instructions. Conditions were as described previously.17

DNA amplification

Sequence information for DNA primers for both the aminoglycoside acetyltransferase activity [AAC(6')] and the aminoglycoside phosphotransferase activity [APH(2')] of the gentamicin resistance determinant were described by Kaufhold et al.18 Primers were obtained from the Biochemistry Department of The Queen’s University of Belfast (Belfast, UK). Polymerase chain reaction (PCR) analysis of transconjugant plasmid DNA was performed as outlined by Dutka-Malen et al.19 using a Hybaid ‘Omnigene’ PCR cycler.

PCR controls employed were HindIII-digested pSF815A, containing the AAC(6')-APH(2') determinant,20 which was used as a positive control, and HindIII-digested pBR32221 as a negative control.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Size (MDa)</th>
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<tbody>
<tr>
<td>A</td>
<td>98</td>
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<tr>
<td>B</td>
<td>42</td>
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<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>5</td>
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<tr>
<td>6</td>
<td>2.24</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1. A 0.7% TAE-agarose gel illustrating plasmid DNA from seven E. hirae parental isolates and E. faecalis transconjugants derived from parental isolates. Lanes A and B, marker plasmid DNA isolated from E. coli V517 and 39R817, respectively. Lanes 1–7, plasmid DNA isolated from the parental strains; lanes 1’–7’, transconjugants derived from these isolates. The position of DNA isolated from E. faecalis JH 2-2 is denoted by J.
Results and discussion

All seven HLGR E. hirae strains studied transferred HLGR to the E. faecalis JH 2-2 recipient, although at varying frequencies. Four strains transferred plasmid DNA with frequencies ranging from $6.0 \times 10^{-4}$ to $4.0 \times 10^{-4}$. Another strain transferred HLGR at a rate some two orders of magnitude higher, whereas the remaining strains transferred resistance with much lower frequencies of transfer ($6.0 \times 10^{-6}$). These rates of transfer, although varying, are considerably higher than that already reported for E. hirae.9

MIC analysis determined that transconjugants acquired both HLGR (MIC $> 1000$ mg/L) and HLSR (MIC $> 1000$ mg/L). In addition, resistances to erythromycin, chloramphenicol and trimethoprim were co-transferred in six of the seven HLGR transconjugants studied, and tetracycline resistance was co-transferred in five of the seven transconjugants. This again differs from the earlier report on HLGR in E. hirae, where resistances to these antibiotics were not apparently co-transferred.9 Some of these antibiotics are routinely used in the community and may provide a selective pressure for the dissemination of the HLGR determinant in enterococci.

Plasmid analysis of the transconjugant strains confirmed that the transfer of large plasmids was associated with acquisition of HLGR (Figure 1), as confirmed by MIC studies. Restriction profiles of gentamicin-resistant transconjugant plasmids revealed that they varied both in size and restriction pattern. Five plasmid sizes of 40, 42, 43, 46 and 52 kb corresponding to five restriction patterns of between 22 and 30 restriction fragments apiece were demonstrated using the restriction enzyme HindIII (Figure 2). Thus, in the E. hirae isolates studied here, five structurally distinct plasmid types were responsible for HLGR. Interestingly, these plasmids differed in size from that previously reported for E. hirae.9

Some of these antibiotics are routinely used in the community and may provide a selective pressure for the dissemination of the HLGR determinant in enterococci.

Of considerable interest in this study of HLGR in a small sample size, was the presence of five different plasmid types mediating HLGR. Whereas the structural HLGR determinants were undoubtedly homologous, as demonstrated by PCR experiments, the co-transferred resistances differed, as did the restriction enzyme digest patterns of the plasmids. This suggests a heterogeneity in the patterns of resistance transfer, and further implies the possibility of different conditions and times of origin, a fact to which other researchers have alluded.7,9

E. hirae is an uncommon causative agent in human infections. Although not widely considered to be as clinically significant an agent as other members of the genus, multiple resistance in E. hirae does pose therapeutic difficulties when this species is responsible for serious infection. The lack of routine speciation of clinical enterococcal isolates may conceal the actual number of infections caused by E. hirae, which may be higher than previously considered.3 A likewise E. hirae may act as a repository of antibiotic resistance determinants which could subsequently disseminate resistances irrespective of the capacity of this species to cause disease in humans.

Acknowledgements

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HLGR in Enterococcus hirae

![Figure 3](image)

**Figure 3.** A 1.0% TAE-agarose gel showing the amplification of a 1.47-kb fragment by PCR from the HindIII-digested plasmid DNA. Lane A, marker DNA (λ*+PstI*); lanes 1–7: amplified 1.47-kb fragment major PCR product from HindIII-digested transconjugants 1–7; lane 8: negative control for PCR, pBR322 digested with HindIII, showing no amplification; lane 9, positive control for PCR, pSF815A digested with HindIII, showing 1.47-kb fragment.

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**References**


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