Molecular epidemiology of high-level aminoglycoside-resistant enterococci isolated from patients in a university hospital in southern Italy

Raffaele Zarrilli1*, Marie-Francoise Tripodi2, Anna Di Popolo1, Rosaria Fortunato2, Maria Bagattini1, Margherita Crispino1, Anna Florio3, Maria Triassi1 and Riccardo Utili4

1Dipartimento di Scienze Mediche Preventive, Università di Napoli ‘Federico II’, Naples, Italy; 2Medicina Interna ed Epatologia, Dipartimento di Geriatria, Gerontologia e Malattie del Metabolismo, Seconda Università di Napoli, Naples, Italy; 3Cattedra di Chirurgia Vascolare, Dipartimento di Internistica Clinica e Sperimentale ‘F. Magrassi’, Seconda Università di Napoli, Naples, Italy; 4Unità di Medicina Infettivologica e dei Trapianti, Dipartimento di Scienze Cardiotoraciche e Respiratorie, Seconda Università di Napoli, Naples, Italy

Received 8 June 2005; returned 10 July 2005; revised 17 August 2005; accepted 2 September 2005

Objectives: We evaluated the genetic and molecular basis of high-level resistance to gentamicin and amikacin in 91 clinical isolates of Enterococcus faecalis and Enterococcus faecium in a university hospital in southern Italy from 1987 to 2003.

Methods: Antibiotic susceptibility was evaluated by disc diffusion and microdilution methods. Genotyping was performed by PFGE and dendrogram analysis. Aminoglycoside resistance genes were analysed by multiplex PCR. Aminoglycoside resistance gene transfer was performed by filter mating.

Results: In our strain collection, 44% of E. faecalis and 52% of E. faecium were high-level-resistant to gentamicin. Fifty-two PFGE profiles were identified for E. faecalis and 15 for E. faecium. Although the majority of PFGE patterns were single isolates, four patterns (two for E. faecalis and two for E. faecium) were isolated each in 8 and 4, and 6 and 4 different patients, respectively. The aac(6')-Ie-aph(2'-Ia) gene was responsible for high-level resistance to gentamicin and amikacin in E. faecalis and E. faecium; the aph(2'-Id) gene responsible for resistance to gentamicin was also isolated in E. faecium; the aph(3')-IId and ant(4')-Ia genes responsible for resistance to amikacin were also isolated in E. faecalis and E. faecium. High-level resistance to gentamicin, along with the aac(6')-Ie-aph(2'-Ia) gene, was transferred at a frequency of about 10^-5 to 10^-8 per recipient cell in 14 of 17 E. faecalis and 3 of 4 E. faecium different genotypes.

Conclusions: The spread of the aac(6')-Ie-aph(2'-Ia) gene was responsible for high-level resistance to gentamicin and amikacin among enterococci isolated from patients in our geographical area.

Keywords: antimicrobial resistance genes, pulsed-field gel electrophoresis, multiplex-PCR, mating

Introduction

Enterococci have increasingly emerged as a cause of serious nosocomial and community-acquired infections, including bacteraemia and endocarditis.1–8 The treatment of choice for such infections is usually the synergic combination of a penicillin or a glycopeptide with an aminoglycoside, most commonly gentamicin.5,6,11 The efficacy of the above combinations has been compromised by the emergence of enterococcal strains displaying multiple antibiotic resistance, including high-level resistance to aminoglycosides, and resistance to penicillins or glycopeptides.12 Enterococci with high-level gentamicin resistance were first described in the 1980s in the hospital setting5 and have been subsequently identified with increasing frequencies from clinical samples of hospitalized patients,5,6,8 and in the intestinal tract of humans2,4,13 and animals.14 High-level aminoglycoside resistance in enterococci is mediated by aminoglycoside-modifying enzymes (AMEs), which eliminate the synergic bactericidal effect between...
cell wall active agents, such as β-lactams or glycopeptides and virtually all commercially available aminoglycosides, including gentamicin, tobramycin, netilmicin, kanamycin and amikacin. The most common AMEs in Enterococcus spp. are the AAC(6’)-APH(2’), which inactivates gentamicin, kanamycin, tobramycin, netilmicin and amikacin; APH(3’), which inactivates kanamycin and amikacin; ANT(4’), which inactivates kanamycin, amikacin and tobramycin; and ANT(6’), which inactivates streptomycin. Moreover, all Enterococcus faecium strains produce a chromosomally encoded aminoglycoside acetyltransferase, AAC(6’)-II, which inactivates tobramycin, kanamycin, netilmicin and sisomicin.12

High-level gentamicin resistance (MIC ≥ 500 mg/L) is usually mediated by the aac(6’)-Ie-aph(2’)-Ia gene, which encodes the bifunctional enzyme AAC(6’)-APH(2’). In three recent new aminoglycoside resistance genes [aph(2’)-Ib, aph(2’)-Ic and aph(2’)-Id] that also mediate resistance to gentamicin, have been detected in enterococci. Other genes such as the aph(3’)-IIIa and ant(4’)-Ia, that encode the APH(3’) and the ANT(4’) AMEs and confer resistance to various aminoglycosides but not to gentamicin, have also been identified. The types and distribution of aminoglycoside-modifying enzymes and genes in enterococci vary in different geographical regions12–14 and have not yet been reported in southern Italy.

The aim of this study was to investigate the genetic and molecular basis of high-level resistance to gentamicin and amikacin in Enterococcus spp. isolated in a university hospital in the Campania region from 1987 to 2003. To address this issue, enterococci isolates in our geographical area were typed by pulsed-field gel electrophoresis (PFGE) and analysed for high-level aminoglycoside resistance gene content. The conjugative transfer of high-level aminoglycoside resistance from selected clones of enterococci was also examined.

Materials and methods

Bacterial strains

Ninety-one enterococcal strains selected for their antimicrobial resistance pattern were included in the study. Strains were isolated from urine (26), blood (23), wound (23), ulcer (11) and other sources (8) in patients from the internal medicine (48 strains), vascular surgery (27 strains) and cardiac surgery (16 strains) of the university hospital of the Second University of Naples over a period of 17 years (1987–2003). Written informed consent to participate in this study was obtained from the patients. Only one strain for each patient was selected. All strains were grown in blood-agar plates at 37°C and stored at −70°C in BHI broth plus 10% glycerol. Bacteria were identified by conventional methods (Gram stain, catalase test) and biochemical tests using API 20 Strep (bioMérieux, France). Two E. casseliflavus strains (isolates 909 and 923), that showed ambiguous identification with API 20 Strep, were further identified as E. faecium by amplification and sequencing of the 16S ribosomal DNA performed as previously described.15

Antimicrobial susceptibility testing

Antibiotic susceptibility studies were performed first by disc diffusion method (Kirby–Bauer) according to NCCLS guidelines16 for the following antibiotics: ampicillin, gentamicin, streptomycin, vancomycin, teicoplanin. Additionally, for all high-level gentamicin-resistant strains, the disc diffusion test was performed for linezolid, rifampicin, erythromycin, and tetracycline. All strains were subsequently evaluated by a microdilution method for MIC determination according to NCCLS guidelines17 for the following antibiotics: ampicillin, vancomycin, teicoplanin, ciprofloxacin, gentamicin, amikacin, streptomycin.

Preparation of chromosomal DNA and PFGE analysis

The preparation of chromosomal DNA for pulsed-field gel electrophoresis was performed as previously described.18 The agarose discs were equilibrated in restriction enzyme buffer for 30 min and digested with 40 U/disc of Smal (New England BioLabs, Beverly, MA, USA) at 25°C for 6 h.

Genomic DNA fragments were separated through 1% agarose gels by a clamped-homogeneous field electrophoresis apparatus (CHEF-DRII system, Bio-Rad, Hemel Hempstead, UK). The electrophoresis conditions were as follows: pulse times ranged from 5 to 35 s over 26 h at 6-0 V/cm and at 12°C. Lambda ladders of 48.5 kb concatamers (New England BioLabs, Beverly, MA, UK) were run as molecular weight markers. After staining of the gels with ethidium bromide (0.5 μg/mL), DNA bands were visualized by UV and photographed.

Dendrogram analysis

PFGE profiles obtained were converted to TIFF files and subjected to cluster analysis using the GelCompar II version 3.5 software package (AppliedMaths, Sint-Martens-Latem, Belgium). The molecular sizes of the bands of each PFGE profile were calculated by comparing their migration to those of lambda molecular weight marker run on a standard gel. The standard lambda molecular weight marker adopted as reference pattern in one gel was used to normalize subsequent gels containing lanes with the same reference pattern. Clustering was based on the unweighted pair group method with arithmetic averages (UPGMA). The Dice correlation coefficient was used to analyse the similarities of the banding patterns.19 The tolerance position was 1%. Interpretation of chromosomal DNA restriction patterns was based on the criteria of Tenover et al.20 Briefly, strains showing more than three DNA fragment variations and a similarity of <85% at dendrogram analysis were considered to represent different PFGE types, while one to three-fragment differences and a similarity of >85% upon dendrogram analysis were considered to represent PFGE pattern subtypes.

Multiplex PCR

The preparation of genomic DNA for PCR and multiple PCRs were performed as previously described21 with some modifications. PCR primers were synthesized by MWG-Biotech AG, Ebersberg, Germany. PCR was performed in a Perkin-Elmer GeneAmp 2400 thermal cycler with an initial lysis step of 2 min at 94°C; 35 cycles of 40 s at 94°C, 60 s at 55°C, and 80 s at 72°C; and a final extension step of 5 min at 72°C. PCR products were analysed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

Plasmid DNA preparation

Plasmid DNA preparation was performed following the method described by Werner et al.22 for the isolation of large conjugative plasmids in enterococci.

Mating experiments

Filter mating procedures were performed as previously described.23 At least one donor isolate for each different PFGE type was selected among Enterococcus faecalis and E. faecium showing high-level resistance to gentamicin. E. faecalis strain 285 and E. faecium strain 818 were selected as recipients because they were susceptible to gentamicin and resistant to rifampicin or tetracycline independent selectable markers. Cultures of donor and recipient were grown in 5 mL of BHI (brain heart infusion broth; bioMérieux, France)
overnight at 37°C. Filter mating was performed with a donor/recipient ratio of 1:10, 100 μL of donor was added to 900 μL of recipient, cells were collected by centrifugation (8000 rpm, 5 min) and suspended to a final volume of 400 μL. The bacteria were scraped from the filter and suspended in 400 μL of BH broth. Two hundred microlitres of the suspension were plated on BH agar in the presence of a single selective antibiotic, gentamicin for donor cells, tetracycline or rifampicin for recipient cells. The frequency of transfer was calculated as the number of transconjugants divided by the number of surviving recipients. The potential transconjugants were purified on BH agar and stored at –70°C for further phenotypic and genotypic analysis.

Results

Distribution of high-level gentamicin and amikacin resistance in E. faecalis and E. faecium strain collection

Ninety-one single clinical isolates of enterococci collected over a period of 17 years (1987–2003) were investigated. Among the strains analysed, 68 were E. faecalis, and 23 E. faecium. Twenty-three (25.3%) strains (20 E. faecalis and 3 E. faecium) of those selected for the study were isolated from bacteraemic patients.

MIC determinations of gentamicin and amikacin for E. faecalis and E. faecium are reported in Tables 1 and 2. High-level resistance to gentamicin or amikacin was detected in 30 and 34 E. faecalis strains, and in 12 and 13 E. faecium strains, respectively. All enterococci strains with high-level resistance to gentamicin showed an MIC ≥ 2000 mg/L. Forty-four E. faecalis strains and 18 E. faecium strains also showed high-level streptomycin resistance (MIC ≥ 2000 mg/L).

The susceptibility to other antimicrobials was also evaluated for all high-level gentamicin-resistant strains. Among the 30 E. faecalis with high-level resistance to gentamicin, one strain was resistant to ampicillin, two strains were resistant to vancomycin, one of which was also resistant to teicoplanin, and 15 strains were resistant to ciprofloxacin. Among the 12 E. faecium with high-level resistance to gentamicin, nine strains were resistant to ampicillin and five to ciprofloxacin; none of the strains were resistant to glycopeptides. All high-level gentamicin-resistant enterococci were susceptible to linezolid. Of the 30 high-level gentamicin-resistant E. faecalis isolates, 29 of 30 were resistant to tetracycline, 22 to erythromycin and one to rifampicin. All 13 high-level gentamicin-resistant E. faecium were resistant to erythromycin, three were resistant to tetracycline, and five were resistant to rifampicin.

Molecular epidemiology of high-level gentamicin and amikacin resistances in E. faecalis isolates

To study the clonal relatedness between enterococci isolates, all strains were genotyped by Smal digestion and PFGE analysis and two independent dendrograms were generated for E. faecalis and E. faecium strains.
E. faecium. As shown in Figure 1, 52 major PFGE types, named from 1 to 52, were identified for E. faecalis. Of these, PFGE types 9, 10, 11, 19, 23, 25 and 39 could be further classified into 4, 1, 1, 2, 1, 1 and 2 subtypes. The majority of PFGE patterns were single isolates, whereas PFGE types 9, 10, 11, 19, 23, 25 and 39 were observed in 8, 2, 2, 3, 2, 2 and 4 different patients, respectively. Interestingly, five strains of PFGE type 9 and two strains of PFGE type 10 were isolated from patients in the vascular surgery ward during a 6 or 1 month period, respectively; all four strains of PFGE type 10 were isolated from patients in the internal medicine ward (Figure 1).

Multiple PCR analysis was performed to study the molecular epidemiology of aminoglycoside resistance genes. As shown in Figures 1 and 2(a) and in Table 1, the aac(6')-Ie-aph(2')-Ia gene, which codes for high-level resistance to gentamicin and all other aminoglycosides with the exception of streptomycin, was identified in 30 E. faecalis strains, while the aph(2')-Ib, the aph(2')-Ic, and the aph(2')-Id genes, that are also known to mediate resistance to gentamicin in enterococci, were not amplified in any of the E. faecalis strains. Sixty-three of 68 E. faecalis strains also showed genes that code for resistance to amikacin, 53 strains having the aph(3')-IIIa gene, two of the ant(4')-Ia gene and eight both the aph(3')-IIIa and the ant(4')-Ia genes. Of the 34 high-level amikacin-resistant E. faecalis strains, two strains showed the presence of the aac(6')-Ie-aph(2')-Ia gene alone, 21 strains the aac(6')-Ie-aph(2')-Ia and the aph(3')-IIIa genes, six strains the aac(6')-Ie-aph(2')-Ia, the ant(4')-Ia and the aph(3')-IIIa genes, two strains the aph(3')-IIIa and the ant(4')-Ia genes, and three strains the aph(3')-IIIa gene alone. On the other hand, 29 E. faecalis strains having low to intermediate levels of amikacin resistance showed only the aph(3')-IIIa gene (Figure 1 and Table 1). Strains with an identical PFGE type showed an identical aminoglycoside resistance gene profile. Nevertheless, the two strains of PFGE profile 25 and 25a, that showed a similarity of >85% at dendrogram analysis, were susceptible and resistant to high-level gentamicin, respectively, the aac(6')-Ie-aph(2')-Ia gene being present in strains of PFGE type 25a, but absent in those of PFGE type 25. Also, the aph(3')-IIIa gene was amplified in all strains of PFGE type 9, but not in strains of PFGE type 9d (Figure 1).

Molecular epidemiology of high-level gentamicin and amikacin resistances in E. faecalis isolates

Genotypic analysis of E. faecalis strains identified 15 major PFGE patterns, named from I to XV. Of these, PFGE types VIII could be further classified into one additional subtype, VIIIa, that showed a two-fragment variation in the macrorestriction pattern and a similarity of >90% at dendrogram analysis. Although the majority of PFGE patterns were single isolates, PFGE types VIII and XIII were observed in 6 and 4 different patients, respectively. Interestingly, all four strains of PFGE type XIII were isolated from patients in the vascular surgery ward during a 5 month period, two strains each of PFGE type VIII were isolated 1 month apart from patients in the vascular surgery and internal medicine wards, respectively (Figure 3).

Molecular analysis of aminoglycoside resistance genes identified the aac(6')-le-aph(2')-Ia and aph(2')-Id genes and the aac(6')-Ie-aph(2')-Ia gene alone in 10 and two E. faecium strains showing high-level resistance to gentamicin, while the aph(2')-Ib and the aph(2')-Ic genes were not amplified in any of the E. faecium strains. Twenty strains also showed genes that code for resistance to amikacin, 14 strains the aph(3')-IIIa gene and 6 both the aph(3')-IIIa and the ant(4')-Ia genes (Figures 2b and 3 and Table 2). Of the 13 high-level amikacin-resistant E. faecium strains, six strains showed the presence of the aac(6')-Ie-aph(2')-Ia and the aph(3')-IIIa genes, six strains the aac(6')-Ie-aph(2')-Ia, the ant(4')-Ia and the aph(3')-IIIa genes, and one strain the aph(3')-IIIa gene alone. On the other hand, E. faecium strains having low to intermediate levels of amikacin resistance showed only the aph(3')-IIIa gene (Table 2). Strains with identical PFGE types showed identical aminoglycoside resistance gene profiles (Figure 3).

Conjugative transfer of high-level gentamicin resistance in E. faecalis and E. faecium strains of different PFGE types

The transfer of high-level gentamicin resistance from E. faecalis and E. faecium strains of different PFGE types was examined by filter mating experiments. Fourteen of 17 E. faecalis strains of different PFGE types transferred high-level gentamicin resistance at frequencies ranging from 3.1 × 10⁻⁷ to 7.9 × 10⁻⁹ cfu/recipient cells. All transconjugants were resistant to rifampicin, and to high levels of gentamicin and amikacin (MIC > 2000 mg/L). Moreover, all the transconjugants showed a PFGE profile identical with that of the recipient strain (Table 3). Multiple PCR analysis of aminoglycoside resistance genes demonstrated the presence of the aac(6')-le-aph(2')-Ia gene in 14 E. faecalis transconjugants with high-level gentamicin resistance and the presence of the aph(3')-IIIa, but not the ant(4')-Ia, gene in nine E. faecalis transconjugants (Table 3).

Similarly, three of four E. faecium strains of different PFGE types transferred high-level gentamicin resistance at frequencies ranging from 2.3 × 10⁻⁸ to 1.1 × 10⁻⁷ cfu/recipient cell. The three E. faecium transconjugants were resistant to tetracycline, and to high levels of gentamicin and amikacin (MIC > 2000 mg/L) and had the same PFGE profile as the recipient strain (Table 3). Multiple PCR analysis of aminoglycoside resistance genes demonstrated the presence of the aac(6')-le-aph(2')-Ia, but not the aph(2')-Id, gene in all three E. faecium transconjugants (Table 3). None of four colonies from each of the E. faecalis and E. faecium parents and transconjugants analysed for plasmid content by agarose gel electrophoresis yielded detectable plasmid DNA.

Discussion

In this study, we have evaluated the molecular epidemiology of high-level aminoglycoside resistance in a large group of enterococci isolated during a 17 year period from patients in a university hospital in southern Italy. Twenty-five percent of the strains included in the study were isolated from bacteremic patients.

Figure 1. Genotype analysis of PFGE profiles and aminoglycoside resistance genes of E. faecalis strains. The dendrogram was constructed with PFGE data by similarity and clustering analysis using the Dice coefficient and UPGMA with GelCompar II version 3.5 software. A percent genetic similarity scale is shown above the dendrogram. Sizes in kilobases (kb) of lambda DNA molecular mass markers are indicated above the PFGE profiles. Strain number, month/year and ward of isolation is shown on the right of each profile. Genotypes are shown in Arabic numerals, subgenotypes in letters. The profile of resistance genes to gentamicin and amikacin is shown on the right. m, month; y, year; w, ward; IM, internal medicine; VS, vascular surgery; CS, cardiac surgery.

831
Figure 2. (a) Multiplex PCR analysis of aminoglycoside resistance genes in representative *E. faecalis* strains. Lanes: 1–3, DNA from strains 1319, 1070 and 1106, respectively; 4, negative control (no DNA); M, 1 kb DNA ladder molecular mass marker. Migrations of aminoglycoside resistance genes are indicated on the left of each panel. Sizes in base pairs (bp) of 1 kb DNA ladder molecular mass markers are indicated on the right of each panel.

Figure 3. Genotype analysis of PFGE profiles and aminoglycoside resistance genes of *E. faecium* strains. The dendrogram was constructed with PFGE data by similarity and clustering analysis using the Dice coefficient and UPGMA with GelCompar II version 3.5 software. A percent genetic similarity scale is shown above the dendrogram. Sizes in kilobases (kb) of lambda DNA molecular mass markers are indicated above the PFGE profiles. Strain number, month/year and ward of isolation is shown on the right of each profile. Genotypes are shown in Roman numerals, subgenotypes in letters. The profile of resistance genes to gentamicin and amikacin is shown on the right. m, month; y, year; w, ward; IM, internal medicine; VS, vascular surgery; CS, cardiac surgery.

An elevated percentage of high-level aminoglycoside resistance was found in our strain collection, high-level resistance to gentamicin being detected in 44% and 52% of *E. faecalis* and *E. faecium* strains, respectively, and high-level resistance to amikacin in 50% and 56% of *E. faecalis* and *E. faecium* strains, respectively. The emergence and spread of high-level resistance to aminoglycosides, in particular to gentamicin, is diffused worldwide.1,2,4 Also, high-level aminoglycoside resistance has been more frequently found in *E. faecium* than in *E. faecalis* in Europe,9,24 as in USA.21

Molecular typing of enterococci included in this study showed a high variability between strains, 52 and 15 major PFGE patterns being identified for *E. faecalis* and *E. faecium*, respectively. The wide diversity of the enterococci isolated from our patient populations may suggest the selection of sporadic clones from the endogenous intestinal microflora. Similarly, it has been recently reported that high-level aminoglycoside-resistant enterococci isolated from faecal samples from healthy volunteers and food handlers in Spain13 or from patients in Kuwait hospitals6 show unrelated PFGE patterns. Although the majority of PFGE patterns reported herein were single isolates, PFGE types 9 and 39 for *E. faecalis* and VIII and XIII for *E. faecium* predominated, being isolated in 8, 4, 6 and 4 different patients, respectively.
High-level aminoglycoside-resistant enterococci

Table 3. Conjugative transfer of aminoglycoside resistance genes in *E. faecalis* and *E. faecium* strains of different PFGE types

<table>
<thead>
<tr>
<th>Transconjugant strain numbers</th>
<th>Donor PFGE type</th>
<th>Transconjugant PFGE type</th>
<th>Frequency of transfer</th>
<th>Aminoglycoside resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1070:285</td>
<td>3</td>
<td>36</td>
<td>$10^{-6}$,a</td>
<td>aac(6')-Ie-aph(2')-Ia, aph(3')-IIIa</td>
</tr>
<tr>
<td>86:285</td>
<td>6</td>
<td>36</td>
<td>$10^{-8}$,a</td>
<td>+</td>
</tr>
<tr>
<td>1319:285</td>
<td>7</td>
<td>36</td>
<td>$10^{-7}$,a</td>
<td>+</td>
</tr>
<tr>
<td>135:285</td>
<td>9</td>
<td>36</td>
<td>$10^{-7}$,a</td>
<td>–</td>
</tr>
<tr>
<td>308:285</td>
<td>11</td>
<td>36</td>
<td>$10^{-8}$,a</td>
<td>+</td>
</tr>
<tr>
<td>1106:285</td>
<td>14</td>
<td>36</td>
<td>$10^{-8}$,a</td>
<td>–</td>
</tr>
<tr>
<td>855:285</td>
<td>16</td>
<td>36</td>
<td>$10^{-7}$,a</td>
<td>+</td>
</tr>
<tr>
<td>183:285</td>
<td>20</td>
<td>36</td>
<td>$10^{-6}$,a</td>
<td>+</td>
</tr>
<tr>
<td>1421:285</td>
<td>23</td>
<td>36</td>
<td>$10^{-6}$,a</td>
<td>+</td>
</tr>
<tr>
<td>1423:285</td>
<td>23a</td>
<td>36</td>
<td>$10^{-7}$,a</td>
<td>–</td>
</tr>
<tr>
<td>842:285</td>
<td>25a</td>
<td>36</td>
<td>$10^{-9}$,a</td>
<td>+</td>
</tr>
<tr>
<td>653:285</td>
<td>26</td>
<td>36</td>
<td>$10^{-8}$,a</td>
<td>+</td>
</tr>
<tr>
<td>713:285</td>
<td>38</td>
<td>36</td>
<td>$10^{-8}$,a</td>
<td>+</td>
</tr>
<tr>
<td>1233:285</td>
<td>39a</td>
<td>36</td>
<td>$10^{-7}$,a</td>
<td>+</td>
</tr>
<tr>
<td>1238:285</td>
<td>40</td>
<td>36</td>
<td>$10^{-7}$,a</td>
<td>+</td>
</tr>
<tr>
<td>285</td>
<td>36</td>
<td>36</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>923:818</td>
<td>VIII</td>
<td>XI</td>
<td>$10^{-6}$,b</td>
<td>+</td>
</tr>
<tr>
<td>889:818</td>
<td>XII</td>
<td>XI</td>
<td>$10^{-7}$,b</td>
<td>+</td>
</tr>
<tr>
<td>718:818</td>
<td>XIII</td>
<td>XI</td>
<td>$10^{-6}$,b</td>
<td>+</td>
</tr>
<tr>
<td>818</td>
<td>XI</td>
<td>XI</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Mating rate was calculated as frequency of gentamicin-resistant (GENR) transconjugants per recipient cell resistant to rifampicin (RIFR) or tetracycline (TETR) in filter mating experiments.

*GENR*RIFR,

*GENR*TETR.

Interestingly, these four epidemic clones and three other *E. faecalis* clones, each with similar PFGE profiles, that were isolated in two different patients, showed high-level resistance to gentamicin and amikacin, thus suggesting that high-level aminoglycoside resistance might have been responsible for their selection. Intra-hospital spread and persistence in different wards were observed for *E. faecalis* and *E. faecium* strains of epidemic PFGE type 9 and VIII, respectively, that were isolated during time intervals of 6 and 3 years, respectively. On the other hand, *E. faecalis* and *E. faecium* strains of epidemic PFGE type 39 and XIII, respectively, were each isolated in the same ward in short time intervals, thus suggesting the spread from one patient to another. In agreement with our data, it has been previously reported that the increase in the incidence of high-level gentamicin-resistant enterococci in a Dutch university hospital has been due to both clonal expansion and emergence of unique high-level gentamicin-resistant enterococci.5

Additional epidemiological information was provided by molecular analysis of high-level aminoglycoside resistance genes in enterococci isolates. The aac(6')-le-aph(2')-Ia gene was identified in all high-level gentamicin-resistant *E. faecalis* and *E. faecium* strains; 10 of 14 high-level gentamicin-resistant *E. faecium* strains also showed the aph(3')-Ia gene. This is in agreement with previous data showing that the aac(6')-le-aph(2')-Ia gene is the most prevalent gene among the gentamicin-resistant enterococci isolated from humans, food and farm animals.6,13,21 Our data also confirm that either the aac(6')-le-aph(2')-Ia alone, or the combination of aph(3')-IIIa and ant(4')-Ia genes were responsible for high-level resistance to amikacin in enterococci strains. In fact, high-level amikacin-resistant *E. faecalis* and *E. faecium* strains showed the presence of either the aac(6')-le-aph(2')-Ia gene alone, or the aac(6')-le-aph(2')-Ia and the aph(3')-IIIa genes, or the aph(3')-IIIa and the ant(4')-Ia genes. On the other hand, enterococci strains having only the aph(3')-IIIa gene showed low to intermediate levels of amikacin resistance. This is in agreement with previous data showing that both aph(3')-IIIa and ant(4')-Ia genes mediate an amikacin MIC of only 64–256 mg/L in enterococci and confer resistance to ampicillin–amikacin synergy.12

High-level aminoglycoside resistance genes in enterococci strains might have been acquired either through horizontal gene transfer or selection of novel resistant clones. The data reported here show that strains of identical PFGE types show identical aminoglycoside resistance gene profiles. This supports the hypothesis that aminoglycoside resistance has been acquired through the selection of independent resistant clones. However, the majority of high-level aminoglycoside-resistant enterococci have different PFGE profiles, suggesting the horizontal transfer of antimicrobial resistance genes. In further support of this hypothesis, *E. faecalis* strains of PFGE profiles 25 and 25a, that showed a similarity of >85% at dendrogram analysis, were found to be susceptible and resistant to high-level gentamicin, respectively, the aac(6')-le-aph(2')-Ia gene being present in strains of PFGE type 25a,
but absent in those of PFGE type 25. Also, the aph(3′)-Ila gene was amplified in all strains of PFGE type 9, but not in strains of PFGE type 9d. In agreement with our data, it has been previously shown that identical aminoglycoside resistance genes are found in enterococci strains of different PFGE profiles isolated from humans.6,11 Several studies demonstrate that high-level aminoglycoside resistance genes in enterococci are encoded on plasmid or chromosomally located conjugative elements, the most prevalent of which are the conjugative transposons, that mediate the horizontal transfer of resistance determinants.3,25–28 The data reported here show that high-level resistance to gentamicin, along with the aac(6′)-Ile-aph(2′″)-Ia gene, was transferred at a frequency of about $10^{-9}$ to $10^{-8}$ per recipient cell by filter mating in 14 of 17 E. faecalis and 3 of 4 E. faecium different genotypes. This is in agreement with previous data showing that high-level gentamicin resistance is transferred at a frequency of about $10^{-7}$ to $10^{-6}$ per donor cell by filter mating experiments in enterococci isolated from humans. However, other studies have shown that high-level gentamicin resistance can be transferred by filter mating at a frequency of about $10^{-7}$ to $10^{-5}$ per donor cell.3,25–26 One possible explanation for this discrepancy may be the presence of pheromone responsive plasmids that would increase the frequency of conjugal transfer.3,26 In fact, enterococci strains that transfer aminoglycoside resistances at high frequency harboured conjugative gentamicin resistance plasmids,3,26,27 while no conjugative plasmids have been detected in parental and transconjugant enterococci analysed in this study or in that others that transfer aminoglycoside resistance at low frequency.6 However, frequencies of $10^{-5}$ to $10^{-7}$ have been reported for plasmids transferring high-level gentamicin resistance in E. faecium.27,28 Also, the aac(6′)-Ile-aph(2′″)-Ia gene, which confers high-level gentamicin resistance, has been identified in very large conjugative plasmids (>147 kb).28 Therefore, we cannot completely rule out the possibility that low copy high molecular weight conjugative plasmids might mediate high-level aminoglycoside resistance in enterococci analysed herein. Another explanation for the low rate of transfer of high-level aminoglycoside resistance might be dependent on differences in the structure or localization of conjugative elements that carry resistance determinants. In further support of this, it has been previously reported that chromosomally located transposon Tn5385 transfers resistance determinants at a frequency 2 to 3 log lower than conjugative plasmids.25 Further studies are required to identify the genetic elements responsible for conjugative transfer of high-level aminoglycoside resistances in our isolates.

In conclusion, we show here that both clonal expansion and the emergence of unique high-level aminoglycoside-resistant strains have contributed to the selection of high-level aminoglycoside resistance in enterococci isolated from our patient populations. The aac(6′)-Ile-aph(2′″)-Ia gene was identified in all high-level gentamicin and amikacin-resistant E. faecalis and E. faecium strains and was transferred through conjugation by the majority of the strains. Based on these findings, we postulate that high-level resistance to gentamicin and amikacin among enterococci isolated from patients in our geographical area may also depend on the spread of the aac(6′)-Ile-aph(2′″)-Ia gene.

Acknowledgements

We thank Maria Grazia Catenacci for the excellent artwork and Dr Domenico Vitale from CEINGE, Naples, Italy, for technical support in DNA sequencing. This work was supported by grants from Ministero dell’Istruzione, dell’Universit`e and della Ricerca Scientifica e Tecnologica, Italy (PRIN 2003 to R. U. and PRIN 2004 to M.-F. T. and R. Z.) and from Centro di Eccellenza per lo Studio delle Malattie Cardiovascolari della Seconda Universit`a di Napoli, Naples, Italy.

Transparency declarations

No declarations were made by the authors of this paper.

References