ANTIMICROBIAL RESISTANCE

Incidence and detection of multi-drug-resistant enterococci in Dublin hospitals

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In February 1994, an outbreak of vancomycin-resistant Enterococcus faecium (VREM) occurred in the oncology unit of a Dublin hospital. Between February and July 1994, VREM was isolated from 18 patients, one staff member and 14 environmental sites within the unit. The isolates also had high-level aminoglycoside and penicillin resistance. Three pulsed-field gel electrophoresis (PFGE) types were identified, two of them from multiple patients and environmental sites. Plasmid typing allowed subdivision of PFGE types. A retrospective study of enterococci isolated from blood cultures between January 1991 and January 1994 showed that, before the outbreak, fewer than 2% of isolates were vancomycin-resistant but that the incidence of high-level gentamicin resistance had increased from 17% to 60% and ampicillin resistance from 22% to 51%. Among clinically significant non-blood-culture enterococci isolated between September and December 1993, fewer than 1% were vancomycin-resistant, 13% were ampicillin-resistant and 44% highly gentamicin-resistant. None produced β-lactamase. High-content gentamicin disks (120 μg) and low-content vancomycin disks (5 μg) allowed simple, reliable detection of resistant enterococci. MICs of vancomycin and teicoplanin determined by agar dilution and E-test agreed well, but values tended to be slightly lower by E-test.

Introduction

Enterococci have emerged as important nosocomial pathogens, requiring bactericidal antimicrobial therapy. Successful treatment regimens have used an aminoglycoside combined with a β-lactam or a glycopeptide, but resistance to these antibiotics is increasingly frequent, precluding use of such combinations, and resulting in serious therapeutic difficulties [1-4].

Enterococci typically have low-level resistance to aminoglycosides, with MICs from 8 to 256 mg/L according to the compound. High-level gentamicin resistance (HLGR, MIC > 1000 mg/L) was first reported in Enterococcus faecalis in 1979 and is associated with a bifunctional enzyme that has 6’-acetyltransferase and 2”-phosphotransferase activities [5]. This enzyme confers resistance to all clinically useful aminoglycosides except streptomycin, and causes synergy with cell-wall active agents to be lost.

Enterococci are inherently more resistant to β-lactam agents than other streptococci, with E. faecium more resistant than E. faecalis [6], typical MICs of ampicillin being 4–8 mg/L and 0.5–2.0 mg/L, respectively. High-level ampicillin resistance (MIC > 100 mg/L) may result from the production of a low affinity penicillin-binding protein in E. faecium [7] or from β-lactamase production in E. faecalis [2, 3].

Since first reports in 1988 [8], three classes of glycopeptide resistance have been recognised in enterococci: generally, VanA gives inducible high-level resistance to both vancomycin (MIC > 128 mg/L) and teicoplanin (MIC > 8 mg/L); VanB gives inducible moderate resistance to vancomycin (MIC 32–128 mg/L), but producers appear susceptible to teicoplanin (MIC 0.5–2.0 mg/L); and VanC gives low-level resistance to vancomycin (MIC 8–16 mg/L) but not to teicoplanin.

We report the emergence and epidemiology of multidrug-resistant enterococci in St James’s Hospital (SJH) and the Federated Dublin Voluntary Hospitals (FDVH) (Hospitals A, B), and present an evaluation of laboratory tests for their detection.
Materials and methods

Bacterial isolates

Three populations of enterococci from patients at SJH and the FDVH were investigated. The first comprised all enterococci isolated from blood cultures between January 1991 and January 1994 (n = 158). The second comprised all clinically significant non-blood-culture enterococci (one isolate/patient site) isolated from September to December 1993 (230). The third consisted of vancomycin-resistant enterococci (VRE) isolated between February and July 1994 (47). Forty-two of the last group were from an outbreak in the oncology unit of Hospital A, isolated from stool cultures from patients (34), hand and rectal swabs from staff (43) and environmental swabs (44). Five VRE from four patients in the paediatric oncology unit of Hospital B comprised the remainder of the third population.

Enterococci were identified on the criteria that they were catalase-negative gram-positive cocci, bile-aesculin- and pyrrolidonylarylamidase-positive, grew on MacConkey agar and in NaCl 6.5% broth, and bound anti-group D antiserum. Vancomycin-resistant isolates were speciated with API 20Strep kits (bioMérieux, Marcy L’Etoile, France), and identification was confirmed by the Laboratory of Hospital Infection, Central Public Health Laboratory, London. Isolates were stored at -70°C on cryoprotective beads (Protect, Technical Service Consultants Ltd, Heywood, Lancs).

Susceptibility tests

Diffusion tests were performed on Diagnostic Sensitivity Test agar (DST, Unipath, Basingstoke, UK), by the modified Stokes’ method [9], with disks (Unipath) as follows: vancomycin 5 μg and 30 μg; teicoplanin 30 μg; ampicillin 10 μg and gentamicin 120 μg (Mast Diagnostics, Merseyside). E. faecalis ATCC 29212 was the control. Agar dilution, with breakpoint concentrations of 1000 mg/L, was used to detect HLGR and MICs for 20 glycopeptide-resistant isolates. For all except teicoplanin, which was a gift from Marion Diagnostics, Merseyside), was also used to determine MICs of vancomycin and teicoplanin were determined a modification of a method described previously [12]. A single colony was inoculated into 20 ml of nutrient broth containing glucose 0.5%, and incubated overnight at 37°C. The culture was centrifuged and the cells were resuspended in washing buffer (0.01 M Tris base (Sigma), 1 M NaCl, 0.1 M EDTA) to 4 × 10⁹ cfu/ml. This suspension was mixed with an equal volume of low melting point agarose (Gibco BRL), 1.6%, at 50°C, pipetted into a block mould (6 × 20 × 1 mm) and solidified at 4°C. Cells were lysed at 37°C overnight by gentle shaking in lysis mixture (lysozyme 1 mg/ml; RNAase 25 μg/ml; 6 mM Tris base; 100 mM EDTA; 1 M NaCl; Brij 58 0.5%; sodium deoxycholate, 0.2%; lauroyl sarcosine 0.5% and 1 mM MgCl₂) followed by a further overnight incubation at 50°C in proteolysis mixture (proteinase K 100 μg/ml; lauroyl sarcosine, 1% in 0.5 M EDTA). The blocks were washed three times for 10 min each at 4°C in TE buffer (10 mM Tris base, 1 mM EDTA), equilibrated in restriction enzyme buffer for 30 min and digested with Smal (40 units per half block) for 3–4 h, then washed in TE buffer for 60 min at 37°C and loaded into the wells of an agarose.

β-Lactamase production was examined with nitrocefin disks (BBL, Cockeysville, MD, USA) with S. aureus ATCC 29213 and E. faecalis CDC N15 (Centers for Disease Control, Atlanta, GA, USA) as positive controls and Haemophilus influenzae ATCC 10211 as a negative control.

Plasmid analysis

Plasmid DNA was extracted by a modification of the alkaline lysis technique of Birnboim and Doly [11]. Overnight cultures in BHI broth (1.5 ml) were harvested by centrifugation at 14 000 g for 3 min at room temperature, then resuspended in 100 μl of 25 mM Tris HCl, 10 mM EDTA, 50 mM glucose, pH 8.0, containing lysozyme 20 mg/ml and incubated for 1 h at 37°C. Cells were lysed by addition of freshly prepared 0.2 N NaOH containing sodium dodecyl sulphate 1%, mixed by gentle inversion and stood on crushed ice for 5 min. Chilled 5 M potassium acetate, pH 4.8 (150 μl) was added and the preparations were mixed by inversion, then stood on crushed ice for a further 15 min. After centrifugation at 14 000 g for 5 min at 4°C, the supernates were transferred to new microcentrifuge tubes. DNA was extracted with phenol chloroform, precipitated with ice-cold absolute ethanol and washed once with ethanol 70%. After centrifugation, the supernates were discarded, and the extracted DNA was dried and dissolved in 50 μl of 50 mM Tris HCl, 1 mM EDTA, pH 8.0. The resulting extracts were electrophoresed in agarose 0.6% w/v gels, at 70 V for 2 h, stained with ethidium bromide and visualised with UV illumination. Isolates with identical plasmid profiles were considered to belong to the same plasmid type. S. aureus NCTC 50580 was used as a control.

Chromosomal typing

PFGE typing of Smal-digested DNA was performed by a modification of a method described previously [12]. A single colony was inoculated into 20 ml of nutrient broth containing glucose 0.5%, and incubated overnight at 37°C. The culture was centrifuged and the cells were resuspended in washing buffer (0.01 M Tris base (Sigma), 1 M NaCl, 0.1 M EDTA) to 4 × 10⁹ cfu/ml. This suspension was mixed with an equal volume of low melting point agarose (Gibco BRL), 1.6%, at 50°C, pipetted into a block mould (6 × 20 × 1 mm) and solidified at 4°C. Cells were lysed at 37°C overnight by gentle shaking in lysis mixture (lysozyme 1 mg/ml; RNAase 25 μg/ml; 6 mM Tris base; 100 mM EDTA; 1 M NaCl; Brij 58 0.5%; sodium deoxycholate, 0.2%; lauroyl sarcosine 0.5% and 1 mM MgCl₂) followed by a further overnight incubation at 50°C in proteolysis mixture (proteinase K 100 μg/ml; lauroyl sarcosine, 1% in 0.5 M EDTA). The blocks were washed three times for 10 min each at 4°C in TE buffer (10 mM Tris base, 1 mM EDTA), equilibrated in restriction enzyme buffer for 30 min and digested with Smal (40 units per half block) for 3–4 h, then washed in TE buffer for 60 min at 37°C and loaded into the wells of an agarose.
1.2% gel (Molecular Biology Certified Agarose, BioRad Laboratories) in 0.5 × TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA). Electrophoresis was performed with a contour-clamped homogeneous electric field with a CHEF-DRII Drive Module (BioRad). DNA fragments were separated with pulse times of 1–10 s for 30 h followed by 10–30 s for 15 h. The gels were stained with ethidium bromide and photographed. DNA banding patterns were examined visually and all loci were scored for the presence or absence of bands. The percentage similarity of banding patterns was estimated by the Dice coefficient [13], with cluster analysis performed by the unweighted pair-group average linkage method (UPGMA) [14] with the multivariate statistical computer package (MVSP) program (Kovach Computing Services, Pentraeth, Wales) [15]. E. faecium with banding patterns > 84% similar were taken to represent single PFGE types [16].

Results

Changes in resistance over time

The numbers of enterococci that appeared resistant by disk diffusion to vancomycin, teicoplanin, gentamicin and ampicillin are shown in Table 1. Prior to 1994, 2% of enterococci had vancomycin resistance but the proportions exhibiting ampicillin and high-level gentamicin resistance (HLGR) increased from 22% to 51% (p < 0.01) and 17% to 60% (p < 0.001), respectively. Among non-blood-culture isolates, < 1% were vancomycin-resistant, 13% were ampicillin-resistant and 44% highly gentamicin-resistant. No isolates produced β-lactamase.

Detection of aminoglycoside resistance

Of 388 enterococci screened for HLGR with gentamicin 120-μg disks, 159 were classified as highly resistant. Only three of these failed to grow at the breakpoint concentration of gentamicin (1000 mg/L), whereas no isolates that were inhibited by the 120-μg disk (zone diameter > 10 mm) grew at the breakpoint concentration. All except 20% of the enterococci exhibiting HLGR also had HLSR. In two cases, one of HLGR and one of HLSR, resistance was detected in agar dilution tests only after extending incubation to 48 h.

Detection of glycopeptide resistance

To detect low-level glycopeptide resistance, enterococci were screened with both 5-μg and 30-μg disks, but no isolates exhibiting resistance to the 5-μg disk and susceptibility to the 30-μg disk were detected. When the MICs of vancomycin and teicoplanin were compared by agar dilution and E-test methods, agreement (± one dilution) was 100% for vancomycin and 90% for teicoplanin (Table 2). However, there was a bias towards lower MICs with the E-test. Most E-tests gave a well-defined intersection of the zone edge with the strip, but, in a few cases, numerous small colonies were observed within the inhibition ellipse. As recommended by the manufacturer, the MIC was recorded as the antibiotic concentration giving complete inhibition of growth.

Outbreak of vancomycin-resistant enterococci

In February 1994, an outbreak of vancomycin-resistant E. faecium (VREM) occurred in the oncology unit of

Table 1. Antibiotic resistance among enterococci isolated between January 1991 and January 1994 in St James's Hospital and the Federated Dublin Voluntary Hospitals

<table>
<thead>
<tr>
<th>Population of isolates</th>
<th>Year</th>
<th>Number of isolates</th>
<th>Vancomycin</th>
<th>Teicoplanin</th>
<th>HL Gentamicin</th>
<th>Ampicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood isolates</td>
<td>1991</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>6 (17%)</td>
<td>8 (22%)</td>
</tr>
<tr>
<td></td>
<td>1992</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>14 (24%)</td>
<td>22 (37%)</td>
</tr>
<tr>
<td></td>
<td>1993</td>
<td>63</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
<td>38 (60%)</td>
<td>32 (51%)</td>
</tr>
<tr>
<td>Other clinical isolates</td>
<td>1993</td>
<td>230</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>101 (44%)</td>
<td>31 (13%)</td>
</tr>
</tbody>
</table>

HL gentamicin, high-level gentamicin.
*Resistance was determined according to Stokes' criteria [9].
†Isolates collected between September and December 1993.

Table 2. Comparison of E-test and agar dilution MICs for vancomycin and teicoplanin for 20 enterococci

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>Agreement</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>65</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>5</td>
<td>5</td>
<td>55</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Percentage ±1 doubling dilution.
†p values were obtained from the Wilcoxon signed-rank test.
Hospital A. Between February and July, there were 42 VREM isolates from the unit: 27 from 18 patients, one from hands of a staff member and 14 from environmental sites including office computers, toilets, showers, pumps and bed-pan washers. Six patients developed bacteraemia due to VREM, but there was no directly attributable mortality. MICs of vancomycin and teicoplanin were 256–512 mg/L and 32–64 mg/L, respectively. All the VREM had HLGR (MIC > 1000 mg/L), HLSR (MIC > 1000 mg/L), and the MICs of penicillin exceeded 100 mg/L.

Thirty-two of the 42 VREM were typed by PFGE and plasmid analysis. PFGE distinguished 14 unique patterns, with an average of 17 bands (range 14–18) (Fig. 1), and cluster analysis divided these into three distinct groups, designated PFGE types 1, 2 and 3. The PFGE patterns within each cluster were 84% similar, and differed by seven bands or fewer (Fig. 2). Isolates of PFGE types 1 and 2 were isolated from six and eight patients, respectively, and from many environmental sites. All the VREM isolates had five to eight plasmids and plasmid-profile analysis distinguished seven types, designated P1–P7. Types P1–P4 were most frequent (Fig. 3). Plasmid profiles were stable for up to 86 days and allowed subdivision of PFGE types. Fig. 4 shows the PFGE types and plasmid types isolated from patients (blood and faeces), staff and the environment. The four common

![Fig. 1](image-url) 
**Fig. 1.** Representative PFGE banding patterns of Smal-digested DNA of vancomycin-resistant *E. faecium* (VREM) from Hospital A. Lane 1, PFGE type 1, oncology unit outbreak (1994); 2, PFGE type 2, oncology unit outbreak (1994); 3, VREM isolated on ICU 11 months prior to oncology unit outbreak; 4, PFGE type 3, oncology unit outbreak (1994).

![Fig. 2](image-url) 
**Fig. 2.** Cluster analysis (DICE coefficient and unweighted pair-group average linkage) of DNA banding patterns of vancomycin-resistant *E. faecium* from the oncology unit outbreak at Hospital A. PFGE subtypes represent unique banding patterns observed within each PFGE type, and are indicated by lower case letters.
PFGE/plasmid types isolated from faeces were also isolated from blood cultures. Multiple isolates were obtained from six patients: the same PFGE/plasmid type was isolated from blood and faecal samples of three of these patients, whereas two had different PFGE/plasmid types in blood and faecal samples and another had two PFGE/plasmid types in a single faecal sample. One VREM isolate from a blood culture of an intensive care unit (ICU) patient in March 1993, almost a year before the oncology unit outbreak, had a PFGE banding pattern 83% similar to PFGE type 2 (Fig. 1) differing by two to eight bands from the other isolates in this cluster. However, its plasmid profile type differed from those of the outbreak isolates. A PFGE type 1, plasmid type P2 isolate was cultured from the hands of a staff member.

Five VRE isolates were obtained from four patients in the paediatric oncology unit of Hospital B. Three species were identified: *E. faecium* (3), *E. raffinosus* (1) and *E. avium* (1). Two of the three *E. faecium* isolates were of the same PFGE/plasmid type but all differed from those isolated at Hospital A.

**Discussion**

This study demonstrated an increased presence of enterococci with high-level resistance to aminoglycosides, penicillin and glycopeptides among patients in SJH and the FDVH. Between January 1991 and January 1994 the incidence of HLGR in enterococci from blood cultures increased from 17% to 60% (p < 0.001). The incidence of HLGR reported by other centres worldwide ranges from < 2% to 55% [1, 17)]. Detection of HLGR by disk diffusion testing showed excellent agreement with agar dilution, as found by others [6, 18], and is a simple means of predicting if synergy with a cell-wall-active agent is likely.

Ampicillin resistance in SJH and the FDVH rose from 22% to 51% (p < 0.01) among blood culture enterococci over 3 years and, as reported by others, was commoner in these isolates than among isolates from other significant sites [19] (Table 1). One explanation may be the greater proportion of *E. faecium* among blood culture isolates [3, 19]. Ampicillin resistance (MIC ≥ 16 mg/L) rates from 22% to 59% have been reported in *E. faecium* isolates from the USA, UK,
Fig. 4. PFGE and plasmid types of vancomycin-resistant *E. faecium* (VREM) isolated on the oncology unit at Hospital A between February and July 1994. □, patient VREM (B, blood isolates; F, faecal isolates); ■, environment VREM; ○, staff VREM.

Canada and Spain [7]. None of the present isolates produced *β*-lactamase and, to date, there have been no reports of *β*-lactamase-producing enterococci in Europe.

The only established alternative therapy to a penicillin/aminoglycoside combination for serious enterococcal infections is vancomycin plus an aminoglycoside. Since the first reports in 1988 [8], VRE have become a serious clinical problem in some centres [4, 20]. Few problems arise in detecting susceptible or highly resistant isolates, but low-level resistance is more difficult to detect and was missed by 50% of laboratories in a recent survey by the UK National External Quality Assurance Scheme [21]. Laboratories using 5-μg disks or a breakpoint of vancomycin of 4 mg/L were more likely to detect low-level resistance than those using 30-μg disks. In this study, no low-level resistance was observed.

On comparing MICs of vancomycin and teicoplanin by E-test and agar dilution, good correlation was found, although values tended to be slightly lower by E-test, as found by others [22, 23].

PFGE typing of VREM from the oncology unit of Hospital A distinguished three types, two of them from multiple patients. The presence of these same types in environmental sites in the unit suggested the possible role of the patients’ environment in transmission. Stosor *et al.* [24] demonstrated survival of VRE on various surfaces for up to 7 days, and other studies have also implicated environmental contamination in transmission [4, 20]. Screening for VRE also revealed one outbreak strain on the hands of a staff member. The role of this individual in transmission was not determined, but other studies have shown that staff may spread enterococci amongst patients [1, 2]. A VREM isolated from an ICU patient 11 months before the oncology unit outbreak was similar to one of the outbreak strains by PFGE, but differed in plasmid profile. This strain may have been the source of the present outbreak, having persisted undetected in the hospital. Such endemic strains have been reported previously [25].

Plasmid profiles were stable, permitted further discrimination of the PFGE types and provided greater insight into the route of spread within the unit. Several investigators have proposed that plasmid analysis should be used in conjunction with PFGE for enterococci, but there is no consensus view [26, 27]. Some investigators observed little or no variation in plasmid profiles over months and found good
correlation with chromosomal typing [2, 4, 28], while others found discrepancies between the methods for isolates obtained over periods as short as 3 months [29, 30].

This study suggests that there has been a significant spread.

References


