Introduction

Despite the increasing use of vancomycin, both orally and parenterally, in human medicine since the mid-1980s, and the use of avoparcin as a growth promoter in animals, the first reports in the late 1980s of enterococci with acquired high-level resistance to vancomycin were met with surprise. Resistance is known to arise where use of antimicrobial agents is high and conditions favour the easy spread of bacteria. Five vancomycin-resistant enterococci (VRE; sometimes also called glycopeptide-resistant enterococci) phenotypes, VanA–VanE, have been distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin. Until mid-1999, reports of VRE in New Zealand had indicated that vancomycin resistance was rare, with one confirmed isolation of low-level resistant Enterococcus faecium (MIC 8 mg/L), one high-level resistant Enterococcus faecalis (MIC ≥256 mg/L) and several low-level resistant Enterococcus gallinarum and Enterococcus casseli-flavus. The identification of these isolates was based on simple biochemical tests and typing of the strains was not performed. In September 1999, three isolates of E. faecalis containing the vanA gene were recovered from the urine of a debilitated elderly patient with a chronic urinary tract infection. Two of the isolates were indistinguishable using molecular typing techniques, while the third was different and showed high-level gentamicin resistance. The present study was carried out to determine the prevalence of faecal carriage of VRE in hospitalized patients with an increased risk of infection or colonization with VRE. In addition, attempts were made to type the strains of enterococci present using DNA fingerprinting.

Materials and methods

Patients and isolation of VRE

One hundred and seventy-six stool samples from 127 individuals with antibiotic-associated diarrhoea and/or Clostridium difficile colitis were obtained from patients at the Dunedin, Wakari and Oamaru hospitals during two 3 month surveillance periods from June 1997 to November 1998. From each faecal sample, a pea-sized amount was emulsified in 10 mL Streptococcus faecalis medium (Bacto SF Medium; Difco Laboratories, Detroit, MI, USA)

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supplemented with vancomycin 6 mg/L and clindamycin 8 mg/L. After incubation for 48 h at 35°C, a 10 µL loopful was streaked on to: (i) 5% (v/v) sheep blood agar; (ii) bile aeruginosa azide (BAA) agar containing vancomycin 6 mg/L and clindamycin 8 mg/L (a VRE-selective medium); and (iii) BAA agar containing clindamycin 8 mg/L only (an enterococcus-selective medium). All plates were incubated for 48 h at 35°C. The inherent ability of enterococci to grow in the presence of bile and to hydrolyse aesculin to form black colonies was used for their selection, as was their inherent resistance to clindamycin (MIC >50 mg/L). This antimicrobial agent obviated the need to perform numerous Gram’s stains to differentiate between enterococci and the common contaminating lactobacilli (MIC of clindamycin 0.5 mg/L).

Identification of VRE

Any black colonies growing on BAA agar with vancomycin and clindamycin were assumed to be possible VRE. Any aesculin-positive colonies growing on BAA agar with clindamycin only were assumed to be vancomycin-resistant and -susceptible enterococci. All isolates were subcultured on to sheep blood agar and Gram-stained after overnight incubation at 35°C. All Gram-positive cocci were then tested according to the identification standards of Dunedin hospital. This involved testing for the production of pyrrolidonyl arylamidase (PYRase). Motility and pigment tests were performed as described previously. E. faecalis and E. faecium were differentiated by sorbitol fermentation. Any Gram-positive, bile- and aesculin-positive, PYRase-positive cocci were assumed to be enterococci. Any that showed resistance to vancomycin (see below) were referred to Dunedin hospital for species identification. This involved testing for the production of vancomycin resistance. The PCR products were purified using a High Pure PCR product purification kit (Boehringer Mannheim) according to the manufacturer’s instructions. The PCR products were sequenced directly using the vanC forward and reverse primers and the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer Applied Biosystems, Scoresby, Victoria, Australia). Sequence reaction mixtures were electrophoresed using a model ABI 377 automated DNA sequencer (Perkin Elmer Applied Biosystems).

PCR amplification and sequencing of 16S rDNA

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out using procedures described by Rainey et al. Purified PCR products were sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems) as directed by the manufacturer’s protocol. An Applied Biosystems 310 DNA Genetic Analyzer was used for the electrophoresis of the sequence reaction products.

Testing susceptibility to antimicrobial agents

The MICs of vancomycin, gentamicin and ampicillin were determined using Etests (AB Biodisk, Solna, Sweden). A saline suspension, equal to a 0.5 McFarland turbidity standard of each strain, was swabbed on to Kirby–Bauer Iso-sensitest agar (Oxoid, Basingstoke, UK) in a manner that would achieve confluent growth, and allowed to dry for 10 min. Two Etest strips were applied to each plate, one containing vancomycin (0.016–256 mg/L), and the other ampicillin (0.016–256 mg/L). For gentamicin (0.016–256 mg/L), one Etest strip was applied to the plate. All plates were incubated at 35°C for 24 h, after which the MIC was read. For ampicillin, isolates with an MIC of ≥16 mg/L were considered resistant. For vancomycin, the categories for susceptible, intermediate and resistant were MICs of ≤4, 8–16 and ≥32 mg/L, respectively. For the purposes of this paper, all isolates with vancomycin MICs of ≥8 mg/L were regarded as VRE. Control strains included in the study were E. faecalis ATCC 29212 (vancomycin susceptible) and E. gallinarum BM 4174 (vanC-positive control).

Genomic DNA preparation and pulsed-field gel electrophoresis

Cultures of each isolate were grown to an optical density at 650 nm of 0.6 in 10 mL of Todd–Hewitt broth (Difco Laboratories). Enterococcal genomic DNA embedded in agarose plugs was prepared essentially as described by Keis et al. for the preparation of clostridial genomic DNA, with
the exception that the lysis step was not carried out anaerobically. Enterococcal DNA was digested with the restriction endonuclease *Sma*I by equilibrating slices of DNA plugs (10 mm × 2 mm) three times for 15 min in 100 µL of the restriction enzyme buffer recommended by the manufacturer, before adding 10 U of the enzyme and preincubating overnight at 4°C. The plugs were then digested at 25°C for 4 h. Digested DNA plugs were equilibrated three times in 0.5 × TBE buffer (45 mM Tris–borate, 1 mM EDTA) for 15 min before electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed by contour-clamped homogeneous electric field (CHEF) electrophoresis using the CHEF-DRIII system (Bio-Rad Laboratories, Richmond, CA, USA) at 6 V/cm and 14°C. The agarose gel concentration, ramped pulse times and running time are noted in the Figure legend. The Low Range PFG Marker (New England Biolabs, Beverly, MA, USA) containing phage λ concatemers and *Hind*III-digested phage λ fragments was used as a size standard. Separated DNA fragments were stained with ethidium bromide and visualized with a UV transilluminator.

**Results**

**Isolation and characterization of VRE isolates**

Between 1997 and 1998, 176 stool specimens were obtained from 127 patients with suspected *C. difficile* colitis and/or patients with antibiotic-associated diarrhoea from Dunedin, Wakari and Oamaru hospitals. Sixty-six (38%) of these samples contained enterococci as judged using the enrichment procedures employed. Of these 66 enterococcal isolates, 11 (17%) were identified as *E. faecium*, 51 (77%) as *E. faecalis* and the remaining four as *E. gallinarum*. These isolates were identified using simple biochemical tests for the identification of enterococci and automated Gram-positive identification (GPI Vitek card).

Of the 66 isolates, only six had MICs in the vancomycin-resistant category (≥8 mg/L) (Table). Automated Gram-positive identification (GPI Vitek card), motility and pigment production tests indicated that four of these six strains were *E. gallinarum* (Table). Two isolates, numbers 1 and 4, were non-motile and non-pigmented, and therefore were identified as *E. faecium*. These six isolates were from four patients; three of them were from a patient in Oamaru hospital, representing three separate specimen samples, two were from two different patients in Dunedin hospital, and one was from a patient in Wakari hospital. The patients from whom these VRE were isolated were all elderly people in the long-term care wards of the respective hospital.

Testing the susceptibility to antimicrobial agents revealed that all six VRE strains had intermediate vancomycin MICs (8–12 mg/L), teicoplanin MICs of 0.5 mg/L, ampicillin MICs of 0.75 mg/L and gentamicin MICs of 1–6 mg/L (Table).

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Hospital</th>
<th>MIC (µg/L)</th>
<th>Phenotypic Glycopeptide</th>
<th>Glycopeptide genotype</th>
<th>Species identification</th>
<th>Species identification</th>
<th>PFGE pattern</th>
</tr>
</thead>
</table>
| 1              | Oamaru         | 8          | 0.75                    | vanC                  | *E. faecium*           | *E. gallinarum*       | 4b
| 2              | Oamaru         | 8          | 0.75                    | vanC                  | *E. gallinarum*       | *E. gallinarum*       | 3b
| 3              | Dunedin        | 8          | 0.75                    | vanC                  | *E. faecium*           | *E. gallinarum*       | 3a
| 4              | Oamaru         | 8          | 0.75                    | vanC                  | *E. faecium*           | *E. gallinarum*       | 4a
| 5              | Dunedin        | 8          | 0.75                    | vanC                  | *E. gallinarum*       | *E. gallinarum*       | 1
| 6              | Wakari         | 12         | 1                       | vanC                  | *E. gallinarum*       | *E. gallinarum*       | 2

Conventional identification tests included Gram’s stain (positive), growth in 7.5% salt, growth on bile, PYRase activity, motility, pigment production and hydrolysis of aesculin.
Genotype of vancomycin resistance

All six VRE isolated during this study (Table) produced a PCR product of 822 bp, a size consistent with the reported size of the vanC gene based on the primers used. DNA sequences obtained from the PCR amplification products of all isolates indicated strong similarity (93–99%) to the previously described vanC-1 gene sequences found in E. gallinarum15–16 (results not shown).

MDG test and 16S rDNA analysis

The vanC gene has not been reported previously in E. faecium. We therefore carried out further tests—the MDG test (which has been shown to be superior to motility testing in differentiating E. faecium from E. gallinarum11) and 16S rDNA analysis (which can be used to differentiate E. faecium and E. gallinarum15)—to determine the species of isolates 1 and 4. Isolates 1 and 4 (and the other four isolates) were MDG positive (Table), suggesting that isolates 1 and 4 were not E. faecium, but E. gallinarum. The 16S rDNA gene sequence of all six VRE isolates showed the greatest similarity (99.9%) to E. gallinarum, indicating that isolates 1 and 4 were non-motile strains of E. gallinarum (Table).

DNA fingerprinting of enterococci resistant to antimicrobial agents

DNA fingerprinting of the six VRE isolates was carried out by comparing the SmaI digestion patterns obtained after PFGE. Using the criteria described by Tenover et al.18 for interpreting chromosomal DNA restriction patterns produced by PFGE, the six VRE isolates had four distinct patterns (Figure). Strains that had been isolated in 1997 (isolates 1–5) had three distinct PFGE patterns (Figure). Isolates 1, 2 and 4 (Figure) were isolated from three separate specimens from a single patient. Isolates 1 and 4 were non-motile species of E. gallinarum and were closely related, but different types based on an additional band at 60 kb in isolate 4, while the bands in the region 48–60 kb were not of the same intensity in the two isolates. Isolates 2 and 3 appeared to be closely related, but bands in the 23–70 kb region were poorly resolved for isolate 2, making it difficult to tell if there were significant band differences in this region. Isolates 5 and 6 were distinct from the other isolates and shared very few common bands; they were therefore considered unrelated.

Discussion

The occurrence of vancomycin resistance in enterococci in both the USA and Europe has been increasing in recent years.19–21 The reasons for this increase seem to differ between these two regions. In the USA, studies have shown that VRE are spread mainly by nosocomial transmission and are infrequently found in non-hospitalized patients.2,19,22 Emergence and subsequent spread of VRE in this setting has been attributed to the intensive clinical use of vancomycin in both parenteral and oral forms, against a background of high-level cephalosporin and metronidazole use which promotes enterococcal superinfection.21,23–25 In Europe, VRE (predominantly of the vanA genotype) are frequently isolated from non-hospitalized patients, animals and environmental sources, and are rare in the hospital setting.21,26 This suggests that these bacteria come from a source in the community; they could be part of the normal flora in animals and be passed down the food chain.2,27–29 Enterococci of the VanB phenotype have not yet been documented from animals. Data linking this community source of VRE to the extensive use of avoparcin, a glycopeptide used as an animal feed supplement, resulted in a ban on avoparcin in Denmark and Germany in the mid-1990s.30,31 This has been followed by a European Union-wide ban.32 Avoparcin is still used in New Zealand as an animal growth promoter, so animals may be a potential source of VRE.

Previous reports of VRE in New Zealand have indicated that vancomycin resistance is very rare.7–9 The results of this study confirm these observations. In this study, VRE were recovered from the faeces of four hospitalized patients. Two of the patients were elderly, in long-term care facilities and were being given oral vancomycin for probable C. difficile colitis, with one patient having received vancomycin treatment for 20 continuous days. In light of this, it is somewhat surprising that VanA or VanB phenotypes were not recovered. All vancomycin-resistant isolates had the vanC-1 resistance gene. The VanC phenotype has been found in E. gallinarum (vanC-1), E. casseliflavus and E. faecium (vanC-2) genes.

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Figure. PFGE SmaI patterns of VRE isolates. DNA restriction fragments were resolved in 1.5% molecular biology grade agarose (Bio-Rad) in 0.5 × TBE buffer at pulse times of 5–15 s (running time, 24 h). Lane 1, isolate 5 (pattern 1); lane 2, isolate 6 (pattern 2); lane 3, isolate 3 (pattern 3a); lane 4, isolate 4 (pattern 4a), lane 5, isolate 1 (pattern 4b); lane 6, isolate 2 (pattern 3b); lane 7, Low Range PFG Marker (New England Biolabs). The sizes of the fragments are in kilobases.
Vancomycin-resistant enterococci in New Zealand

E. gallinarum and Enterococcus flavescens (vanC-2) and is characterized by low-level (4–8 mg/L) intrinsic resistance to vancomycin. VanC-1 has also been reported in E. faecium, but based on phenotypic analysis only. DNA fingerprint patterns from PFGE indicated that one patient (Oamaru hospital) was colonized with three different strains of VRE. One of these strains was closely related to a VRE strain isolated from a patient in Dunedin hospital.

Our results using an enrichment broth containing vancomycin 6 mg/L resulted in a significant number of vancomycin-susceptible enterococci growing, or at least surviving, in this broth until they were plated on BAA agar containing clindamycin. Reasons for this are unknown, although it is possible that the faecal specimens may provide bacterial protection from vancomycin. However, the ability of vancomycin-susceptible enterococci to grow on media containing supposedly inhibitory concentrations of vancomycin is not a new finding.

Phenotypically, differentiation between E. faecium, E. gallinarum and E. casseliflavus is based on a few physiological tests, the most discriminating being motility at 30°C and production of a yellow pigment. However, certain strains of E. casseliflavus are not pigmented and E. gallinarum can be non-motile. Using 16Sr RNA sequence analysis and MDG testing, two of our E. faecium VRE isolates were further identified as non-motive species of E. gallinarum. This identification is consistent with the intrinsic, low-level vanC-1-mediated resistance associated with this species. Non-motive E. gallinarum has been described previously.

This work and other studies clearly highlight the problems of identifying enterococci at a species level using conventional (and accepted) laboratory techniques, and reinforce the apparent absence of vancomycin-resistant E. faecium, and rarity of vancomycin-resistant E. faecalis, in New Zealand. How long this situation will remain is unknown. Recent results from Australia demonstrate how rapidly things can change. Not unexpectedly, strains of vancomycin are present, albeit at low levels, in New Zealand. Vancomycin-resistant enterococci in New Zealand are used very sparingly. To the best of our knowledge, no glycopeptide-resistant Staphylococcus aureus have been recovered from clinical specimens in New Zealand. In the past, oral vancomycin was used to treat possible C. difficile-associated diarrhoea, but in recent years has been replaced by metronidazole. While avoparcin is available, it is not used widely as an animal growth promoter. Glycopeptide use is rare in New Zealand, and this may help explain the low level of VRE presently found there.

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