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High-level vancomycin-resistant enterococci causing hospital infections

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SUMMARY

Nosocomial infection or colonization due to enterococci with high-level resistance to vancomycin (minimal inhibitory concentrations [MICs] between 64 and > 2000 mg/L) has occurred in 41 patients with renal disease. These vancomycin-resistant enterococci were cultured from many sources including blood. All but one strain contained one or more plasmids ranging in molecular weight from 1·0 to 40 Megadaltons (MDa). Vancomycin resistance was transferable by conjugation to a susceptible recipient strain of *Enterococcus faecalis* but this was not always associated with plasmid DNA. The emergence of transferable high-level vancomycin resistance in enterococci causing significant clinical infections is of particular importance since vancomycin is widely regarded as a reserve drug for the management of infections with multi-resistant Gram-positive organisms.

INTRODUCTION

Enterococci are opportunistic pathogens which may invade the tissues of compromised hosts from their normal habitat in the bowel. The incidence of nosocomial enterococcal infection may be rising in the UK and the USA and appears to be associated with increasing use of broad spectrum β-lactam antibiotics (1–4) and invasive surgical devices (5).

Serious infection including endocarditis with *Enterococcus faecalis* and especially *E. faecium* may be difficult to treat; the usual options are ampicillin (if the organism is susceptible) or vancomycin, often in combination with an aminoglycoside. Vancomycin resistance among Gram-positive cocci is rare (6,7) but there have been three recent reports of vancomycin resistance in strains of *E. faecium* (8–10), another report of a strain of *E. gallinarum* having low-level resistance to vancomycin (minimal inhibitory concentration (MIC) of 16 mg/L (11)) and two earlier reports of enterococci with MICs of vancomycin > 100 mg/L (12,13). We report here, further to our letter (14), clinical and microbiological

findings in a cluster of hospital infections among Renal Unit patients due to strains of *E. faecium*, *E. faecalis* and *E. avium* having high-level resistance to vancomycin.

PATIENTS AND METHODS

The Hospital and Renal Unit

The Dulwich Hospital has 32 designated renal beds located on five wards. During 1987, 203 patients required in-patient nephrological investigation and/or treatment. A further 76 cases of acute renal or multiple organ failure were admitted and managed by continuous arterio-venous haemofiltration dialysis. Patients were admitted to any one of the five wards, the intensive care unit (ICU) and/or the haemodialysis unit.

Clinical records of patients infected or colonized with vancomycin-resistant enterococci (VRE) were examined for details of age, sex, underlying disease, management and antimicrobial therapy in the previous 6 months and a similar study was made of eight Renal Unit patients who had bacteraemia due to vancomycin-susceptible enterococci (VSE) (data available on request). A spot survey was made to determine faecal carriage of VRE in 15 of 18 patients on one ward.

Primary isolation and susceptibility testing

Colonies isolated from routine clinical specimens and resembling enterococci were confirmed as such by Gram's stain and the criteria of Sherman (15). They were identified to species and biotype level with the API-Strep system (API, Basingstoke, UK). Lancefield groups were determined by the Streptex method (Wellcome Diagnostics, Dartford, UK). Antimicrobial disc susceptibility tests were performed on Isosensitest agar (Oxoid, Basingstoke, UK) with 7% lysed blood using a modified Stokes' method (16). The Oxford staphylococcus NCTC 6571 was used as a control culture. Identifications were confirmed and serotyping (17) done at the Streptococcus Reference Laboratory.

Minimal inhibitory concentration evaluations

MICs were determined against a range of antimicrobial agents: serial twofold dilutions were incorporated in Isosensitest, Diagnostic Sensitivity Test (Oxoid) and Wellcotest (Wellcome) agars containing 2, 5 and 7% lysed blood respectively. A multipoint inoculator (Mast, Bootle, UK) was used and inocula were standardized to contain 10^4–10^5 colony forming units (cfu) per inoculum spot. End points were read as complete inhibition of growth. High-level vancomycin resistance was defined as a MIC $\geqslant$ 64 mg/L.

Plasmid analysis and transfer studies

Brain Heart Infusion (BHI) agar (Oxoid) with 2% added blood was used for strain maintenance and BHI broth (Oxoid) to suspend and dilute cells after conjugation matings and for plasmid curing experiments. Plasmid DNA for agarose gel electrophoresis was extracted as described by Maniatis *et al.* (18) except that suspensions were incubated with lysozyme (10 mg/ml) in 10 mM Tris, 1 mM EDTA, pH 8·0 containing 25% (w/v) sucrose at 37 °C for 1 h before lysis with alkaline sodium dodecyl sulphate. Approximate molecular weights (MWts) of

plasmids were determined by comparison with plasmids from control strains *Escherichia coli* 39R861 (19) and V517 (20).

Two strains each of *Enterococcus faecalis* and *E. faecium* were studied for transfer of antimicrobial resistance. The recipient strain used was *E. faecalis* JH2-2 (21) which is plasmid-free and resistant to rifampicin (MIC > 1000 mg/L) and fusidic acid (MIC > 256 mg/L). Transfer of erythromycin resistance was studied on 25 mm, 0·45 μm membrane filters (Millipore Corp.). Donor and recipient cultures were grown by shaking in BHI broth at 37 °C for 6 h, equal volumes (3 ml) were then mixed and drawn through the filter which was incubated on BHI agar at 37 °C overnight. Growth on the filter was resuspended in 5 ml of BHI broth from which viable counts were made and selective antimicrobial agar plates containing both rifampicin (100 mg/L) and erythromycin (25 mg/L) were inoculated. Resulting transconjugants were retested for growth on agar containing fusidic acid (100 mg/L) and subsequently tested for susceptibility to vancomycin and other antimicrobial agents. Curing of erythromycin resistance in a strain of *E. faecium* was attempted by growing the culture at 42 °C overnight in BHI broth containing ethidium bromide (5 mg/L).

Antimicrobial inactivating activity

The method of Orberg & Sandine (22) was used to test for vancomycin-inactivating activity.

RESULTS

Between November 1986 and May 1988, 39 Renal Unit patients developed serious or persistent infection including bacteraemia (8), biliary tract infection (1), infected vascular access site (1), intra-abdominal infection (6), osteomyelitis (1), peritonitis (3), pneumonia (2), urinary tract infection (UTI) (16) and wound infection (1) due to VRE. Two patients were colonized. In 34 patients (83%) VRE were recovered in pure culture. The patients had been nursed on five different wards, the haemodialysis unit and/or the ICU and were frequently transferred among these wards and units. Thirty-nine patients (95·1%) had received one or more antimicrobial agents in the 6 months before isolation of their VRE. Thirty-eight (92·7%) had received a cephalosporin; 16 of these had also received a penicillin. Twenty-two (56%) were prescribed vancomycin; one patient received only vancomycin. Two had no antimicrobial agents in the preceding 6 months. In the same period the mean number of different antimicrobial agents administered to the 41 patients was 3·3 (range 0–7). Eight Renal Unit patients matched for age, sex, in-patient stay and management who had bacteraemia due to VSE had been exposed to closely similar antimicrobial therapy.

In 39 patients, specific therapy for infection with VRE was indicated and selected according to results of *in vitro* susceptibility testing. The majority received ciprofloxacin. Twenty-three patients were clinically and bacteriologically cured. In six patients UTI persisted for many months. One patient who had an apparently successfully treated bacteraemia and UTI developed vertebral osteomyelitis due to VRE, one had a strain of *E. faecium* which developed resistance to ciprofloxacin during therapy for a UTI (MIC increased from 2 to 8

Table 1. *Antimicrobial susceptibility of vancomycin-resistant enterococci*

Antimicrobial agent	Susceptibility defined by MIC < = (mg/L)	Number of strains showing susceptibility at the stated level (one strain of each species per patient) *E. faecium* (N = 27)	*E. faecalis* (N = 15)	*E. avium* (N = 3)
Ampicillin	8	0	15	1
Chloramphenicol	8	2	2	3
Ciprofloxacin	4	21	14	3
Clindamycin	1	0	0	2
Erythromycin	1	1	0	2
Fusidic acid	4	6	3	0
Daptomycin	4	27	15	3
Rifampicin	2	4	12	2
Teicoplanin	4	0	0	0
Tetracycline	2	2	0	1
Trimethoprim	2	2	0	2
Gentamicin (high-level)	128	26	2	3

Table 2. *Characterization of donor enterococci used in resistance transfer experiments*

Strain	Serotype	Source	Relevant resistance markers	Plasmid content (MDa)
E. faecalis 1	9	shunt site	Em^R Cm^R Gm^R Vc^R	40
E. faecalis 10	9/19	wound	Em^R Cm^R Gm^R Vc^R	35
E. faecium 7	—	CVP site	Em^R Cm^R Vc^R	35, 24, 3·4
E. faecium 14	—	blood	Em^R Cm^R Vc^R	35, 24, 3·4, 2·4

Em, erythromycin; Cm, chloramphenicol; Gm, gentamicin; Vc, vancomycin; R, resistance.

mg/L) and one was lost to follow-up. Seven patients died while infected with VRE.

In the spot survey of intestinal VRE-carriage, 4 of 15 patients were faecal carriers, 3 of whom were already infected with VRE. The fourth patient had been in the ward for 3 weeks following renal transplantation but did not become infected. None of the 11 patients who had been in hospital less than 3 weeks had faecal carriage of VRE. Three weeks prior to this, environmental and hand washing studies in the ICU when it contained infected patients failed to reveal VRE.

Forty-five strains of VRE were biotyped and serotyped. Fifteen were characterized as *E. faecalis* biotype II; 14 were serotype 9, one was serotype 9/19. Twenty-seven were *E. faecium* biotype II and three were *E. avium*. Five patients were infected with more than one species of VRE. Results of antimicrobial susceptibility testing of one strain of each species per patient are given in Table 1. All strains resistant to vancomycin on disc testing had MICs between 64 and > 2000 mg/L. Results using the three different susceptibility test media were within a twofold dilution of each other.

Table 3. *Transfer of erythromycin resistance, other unselected markers and plasmids from selected* E. faecalis *and* E. faecium *to* E. faecalis *JH2-2*

Donor	Transfer frequency of Em^R per donor cell	Plasmids transferred	Markers transferred with Em^R
E. faecalis 1	2×10^{-4}	None detected	Vc^R
E. faecalis 10	2×10^{-6}	35 MDa	Gm^R
E. faecium 7	1×10^{-8}	24 MDa	Cm^R, Vc^R
E. faecium 14	7×10^{-8}	24 MDa	Cm^R, Vc^R

Em, erythromycin; Cm, chloramphenicol; Gm, gentamicin; Vc, vancomycin; R, resistance.

Table 4. *Resistance phenotype of enterococcal strains, their transconjugants and the 'cured' variant*

Strains	Antimicrobial agent and minimal inhibitory concentration (mg/L)						
	Chloramphenicol	Gentamicin	Erythromycin	Vancomycin	Teicoplanin	Rifampicin	Fusidic acid
JH2-2 recipient	32	16	< = 0·5	2	2	> 1024	> 1024
E. faecalis 1	128	> 1024	> 1024	> 1024	256	2	32
Transconjugant	32	4	> 1024	> 1024	512	> 1024	> 1024
E. faecalis 10	128	> 1024	> 1024	> 1024	512	1	2
Transconjugant	32	> 1024	> 1024	2	2	> 1024	> 1024
E. faecium 7	64	32	> 1024	> 1024	256	16	16
Transconjugant	128	16	> 1024	> 1024	> 1024	> 1024	> 1024
E. faecium 14	64	64	> 1024	> 1024	256	4	16
Cured variant	16	64	1	2	2	4	16
Transconjugant	128	4	> 1024	> 1024	256	> 1024	> 1024

All strains, except one of *E. faecium*, contained one or more plasmids. Each of the 15 strains of *E. faecalis* contained a large plasmid of mol wt 35–40 MDa with two also having a 3 MDa plasmid. Ten strains of *E. faecium* contained three plasmids of mol wts approximately 40, 24 and 3·4 MDa. Sixteen strains were heterogeneous with respect to their plasmid profiles, although all contained a common plasmid of 24 MDa. The three strains of *E. avium* each contained a single 24 MDa plasmid.

Conjugation matings were attempted to transfer erythromycin resistance to *E. faecalis* JH2-2 from the four donor strains described in Table 2. As the majority of strains contained large plasmids and erythromycin resistance is known to be plasmid mediated in enterococci (23), transfer of this marker was sought in the first instance. The frequency of transfer was markedly higher in the two strains of *E. faecalis* than in the two strains of *E. faecium* (Table 3). Agarose gel electrophoresis of donors and transconjugants revealed that the 35 MDa plasmid had been transferred from *E. faecalis* 10 and the 24 MDa plasmid from both *E. faecium* 7 and 14. In contrast, no plasmid DNA was detected in transconjugants from *E. faecalis* 1 (Table 3), even though erythromycin resistance was transferred. Antimicrobial susceptibility tests showed that the erythromycin-resistant

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transconjugants derived from the two strains of *E. faecium* had also acquired resistance to vancomycin and chloramphenicol, while those from *E. faecalis* 1 acquired resistance to vancomycin alone. Transconjugants from *E. faecalis* 10 displayed high-level resistance to gentamicin but not to vancomycin (Table 3). MICs of donor strains, their transconjugants and the cured variant (*vide infra*) are given in Table 4.

Loss of erythromycin resistance from *E. faecium* 14 was demonstrated in one colony of 726 tested. The cured variant simultaneously lost resistance to chloramphenicol and vancomycin (Table 4) although it retained the full plasmid content of the parent strain. *E. faecalis* 10 reverted to vancomycin susceptibility (MIC = 2 mg/L) on subculture during laboratory tests. Plasmid analysis of the vancomycin-resistant parent culture and the susceptible derivative revealed the plasmid of mol wt 35 MDa in both. There was no simultaneous loss of other resistance markers.

Inactivation of vancomycin was not detected in any of our strains.

DISCUSSION

Nosocomial infection due to three species of enterococci having high-level vancomycin resistance has not previously been reported. Infection/colonization with VRE has been confined to Renal Unit patients although they share wards, the ICU and nurses with patients who have other acute medical or surgical problems. However, specific risk factor(s) associated with acquisition of VRE by Renal Unit patients have not been identified. The immune-incompetence which accompanies uraemia and end state renal failure (24) may render these patients susceptible to colonization/superinfection as may the widespread use of broad spectrum antimicrobial agents, in particular cephalosporins. Prior exposure to vancomycin or other antimicrobial agents was not a prerequisite for infection with VRE or VSE. Prolonged hospital stay may encourage colonization. Eleven patients in hospital less than 3 weeks had not become faecal carriers. In spite of failure to demonstrate VRE or VSE in the ICU environment or on the hands of ICU nurses and the involvement of three species of VRE, the geographical and temporal clustering of infected patients suggests cross-infection. Serotyping and biotyping of VRE strains were not sufficiently discriminatory to provide laboratory confirmation of this.

The antimicrobial therapy of infections due to the VRE could be difficult, six patients having persisting UTI and one developing vertebral osteomyelitis despite apparently successful treatment of a bacteraemia and UTI. The limited therapeutic options for management of these infections was highlighted by development of resistance to ciprofloxacin by one strain of *E. faecium* during therapy. In the 23 patients cured of infection with VRE the relative contributions of antimicrobial therapy, removal of invasive surgical devices and drainage of pus were difficult to assess. Seven patients died while infected with VRE. In view of their complex pathology it is impossible to attribute with confidence infection with VRE as a cause of death. However the isolation of VRE in pure culture from 34 patients with clinical evidence of sepsis demonstrates their potential for pathogenicity in suitable hosts.

In these VRE there may be both plasmid and chromosomal loci for the vancomycin resistance determinant. *E. faecium* 7 and 14 transferred a plasmid of mol wt 24 MDa in association with the genes for resistance to erythromycin, chloramphenicol and vancomycin. This indicated linkage between the three resistance determinants in these strains. However, while curing of erythromycin resistance from *E. faecium* 14 resulted in simultaneous loss of resistance to chloramphenicol and vancomycin, all plasmids present in the parent strain remained in the cured variant. Furthermore, resistance to vancomycin and erythromycin was co-transferred from *E. faecalis* 1 although six transconjugants tested contained no detectable plasmid DNA (data not shown). The transfer of antimicrobial resistance, in particular erythromycin resistance in the absence of plasmid DNA, has been reported in streptococci (23,25). In *E. faecalis* 10 there was no transfer of vancomycin resistance when erythromycin and high-level gentamicin resistance were co-transferred together with the plasmid of molecular weight 35Mda. Spontaneous loss of vancomycin resistance from this strain was not associated with loss of other resistance markers or the plasmid of molecular weight 35Mda. In one strain of *E. faecium*, no plasmid DNA was detected. This strain was sensitive to both erythromycin and chloramphenicol. There was therefore no linkage of erythromycin or chloramphenicol resistance with vancomycin resistance in this strain or in *E. faecalis* 10. Presumably the vancomycin resistance determinant was chromosomal. Conjugative transposons are documented in streptococci (26,27). Translocation of such an element between chromosome and plasmid would account for the different loci of the vancomycin resistance determinant. Excision of such an element can occur from plasmid or chromosome (27). Further genetic analysis is required to elucidate the nature of such a transferable element.

The biochemical basis of vancomycin resistance has not been explained. Our strains and those of Leclercq *et al.* (8) did not have detectable vancomycin inactivation. However, Wu *et al.* (28) performed polyacrylamide gel electrophoresis of membrane proteins of one of our vancomycin-resistant *E. faecium* strains and its resistant transconjugant and showed an additional major protein band of apparent molecular weight 39 Kilodaltons when the strain was grown in the presence of vancomycin. This band was not seen in membrane preparations from cells grown in the absence of vancomycin or in membranes isolated from a cured vancomycin-susceptible derivative. These findings suggest that the high-level vancomycin resistance may be inducible.

All of our VRE and their vancomycin-resistant transconjugants were resistant to the other clinically useful glycopeptide, teicoplanin. Conversely, spontaneous loss or curing of vancomycin resistance was accompanied by loss of teicoplanin resistance (data not shown). Our strains and their transconjugants were susceptible *in vitro* to the lipopeptide daptomycin (LY 146032) and the lipopeptolide MDL 62198 (data not shown) but the therapeutic value of these agents has not yet been established. Leclercq *et al.* (8) were able to select daptomycin-resistant mutants from their two VRE and we have obtained a similar resistant mutant from one strain of six VRE tested using the same techniques (MIC increased to 8 mg/L from 0·125 mg/L). Transferable high-level vancomycin resistance among enterococci, which has also been reported by

Leclercq *et al.* (29), is a potentially more serious development than the possibly mutational change to low-level vancomycin resistance reported in *Staphylococcus haemolyticus* (30) and *E. gallinarum* (11).

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Addendum

The strains of *E. faecalis* and *E. faecium* examined in detail have been deposited with the National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, England. The NCTC accession numbers are 12201-12204. Since preparation of this article, a further 21 Renal Unit patients have been infected or colonized with vancomycin-resistant enterococci.

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