Species prevalence and antibacterial resistance of enterococci isolated in Kuwait hospitals

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This study investigated the species prevalence and antibacterial resistance among enterococci isolated in Kuwait hospitals. They consisted of 415 isolates of *Enterococcus faecalis* (85.3 %), *Enterococcus faecium* (7.7 %), *Enterococcus casseliflavus* (4.0 %), *Enterococcus avium* (1.2 %), *Enterococcus durans* (1.0 %), *Enterococcus gallinarum* (0.5 %) and *Enterococcus bovis* (0.2 %) isolated from urine (36.6 %), blood (10.4 %), wound swabs (11.0 %), stool samples (12.0 %), high vaginal swabs (9.0 %), endocervical swabs (3.0 %) and miscellaneous sources (18.0 %). All of them were susceptible to linezolid. Fifty-two (12.5 %) isolates were ampicillin resistant but none of them produced β -lactamase. They were resistant to erythromycin (63.3 %), tetracycline (60.5 %), ciprofloxacin (40.0 %), chloramphenicol (28.0 %), vancomycin (2.6 %), and teicoplanin (2.6 %). Fourteen, 19 and 20 % of them expressed high-level resistance to gentamicin, kanamycin and streptomycin, respectively. All of the vancomycin-resistant strains carried the *vanA* phenotype and genotype. There was no evidence of clonal spread of the vancomycin-resistant isolates.

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INTRODUCTION

Enterococci are members of the normal flora in the gut of humans and animals but have become increasingly important as hospital-acquired pathogens. They have been associated with infections of the urinary tract, post-surgical wounds, septicaemia, endocarditis and meningitis (Cetinkaya *et al.*, 2000; Jones *et al.*, 1997; Woodford *et al.*, 1993; Huycke *et al.*, 1998). They have a remarkable ability to adapt to exposure to antibacterials maintaining intrinsic resistance to penicillins and low-level resistance to aminoglycosides and a tremendous capacity to acquire resistance to other antibacterials including high-level resistance to aminoglycosides and glycopeptides (Quale *et al.*, 1996; Donskey *et al.*, 1999; Nelson *et al.*, 2000). Vancomycin-resistant enterococci (VRE) have caused outbreaks in many centres in the USA (Cetinkaya *et al.*, 2000; Donskey *et al.*, 1999; Quale *et al.*, 1996) and are recognized as an important resistant pathogen in Europe (Reinert *et al.*, 1999; Schouten *et al.*, 2000; Woodford *et al.*, 1993) and (Toutouza *et al.*, 2001). Infections caused by VRE are associated with severe adverse outcomes such as extended length of hospital stay, increased cost and increased mortality due to the reduction in the choices of antibacterials available to treat such infections (Linden *et al.*, 1996; Huycke *et al.*, 1998). Despite the increasing reports of VRE in different countries, the prevalence of VRE in Kuwait hospitals was unknown. A recent study of antibacterial resistance of enterococci isolated in a teaching hospital revealed a low prevalence of glycopeptide

resistance (E. E. Udo, unpublished data). However, the situation in other hospitals remained uncertain. This paper reports on a study conducted to investigate the prevalence of antibacterial resistance in enterococci isolated from clinical samples in five hospitals in Kuwait.

METHODS

Enterococcus isolates. A total of 415 isolates was obtained from different clinical samples between January 1999 and 31 December 2001 in Mubarak Al-Kabeer (206), Amiri (63), Adan (28), Ibn Sina (46) and Maternity (74) hospitals in Kuwait. They were cultured from urine, wound swabs, blood, endotracheal secretions, cervical swabs, high vaginal swabs and ear swabs. Stool samples were inoculated into enterococcal broth, incubated overnight at 35 °C and subcultured onto brain-heart infusion agar (BHIA) containing 6 μ g vancomycin ml⁻¹ (Jordens *et al.*, 1994; Ieven *et al.*, 1999) and onto BHIA without vancomycin to recover vancomycin-susceptible isolates. The isolates were identified to the genus and species level by cultural characteristics, Gram's stain, catalase test, bile aesculin reactions and by biochemical tests using API Strep (bioMérieux).

Susceptibility testing. The disk diffusion method on Mueller–Hinton agar (National Committee for Clinical Laboratory Standards, 2000) was used to detect resistance to tetracycline, erythromycin, chloramphenicol, ampicillin, ciprofloxacin, gentamicin, kanamycin, streptomycin, vancomycin and teicoplanin. Detection of high-level aminoglycoside resistance was performed with disks containing gentamicin (120 μ g), kanamycin (200 μ g) and streptomycin (300 μ g). Results were read after incubation at 35 °C for 24 h and after 48 h for streptomycin. MICs for vancomycin and teicoplanin were determined using E-test strips (AB Biodisk). MICs for gentamicin, kanamycin, streptomycin, tobramycin, amikacin and linezolid were determined by

Abbreviation: VRE, vancomycin-resistant enterococci.

the agar dilution method. *Enterococcus faecalis* strain ATCC 29212 was used as a sensitive control. Linezolid powder was a gift from Pharmacia.

Detection of β -lactamase production. β -Lactamase production was detected with nitrocefin (Oxoid) according to the manufacturer's instructions. Nitrocefin solution (5 μ l) was dropped onto a loopful of pure overnight growth placed on a filter membrane. The development of a red colour within 60 s indicated a positive result. *Staphylococcus aureus* strain ATCC 29213 was used as a positive control.

Detection of vancomycin-resistance determinants. Genes encoding the vancomycin-resistance determinants *vanA* and *vanB* were investigated by PCR using specific primers as published in the literature (Dutka-Malen *et al.*, 1995; Miele *et al.*, 1995). The primers were *VanA* (VA1, 5'-GGG AAA ACG ACA ATT GC-3' and VA2, 5'-GTA CAA TGC GGC CGT TA-3'), *VanB* (VA3, 5'-CCC GAA TTT CAA ATG ATT GAA AA-3', and VA4, 5'-CGC CAT CCT CCT GCA AAA-3'). Amplification was performed using a kit from Gibco-BRL. DNA for PCR was isolated as described previously (Udo & Dashti, 2000) except that lysozyme (1 mg ml⁻¹) was used instead of lysostaphin in the lysis solution. The amplification mixture consisted of 45 μ l Supermix (22 mM Tris/HCl, pH 8.4, 55 mM KCl, 1.65 mM MgCl₂, 220 μ M each dNTP, 22 U recombinant Taq DNA polymerase ml⁻¹, 3 μ l bacterial DNA and 2 μ l primer solution in a total reaction volume of 50 μ l. A Perkin Elmer 9600 thermocycler was programmed for 32 cycles with the following parameters: denaturation at 94 °C for 3 min, annealing at 60 °C for 45 s, extension at 72 °C for 1 min and final extension at 72 °C for 2 min. Amplified products were detected by agarose gel electrophoresis using 2.0 % agarose (w/v) in TAE buffer for 2 h at 70 V. *E. faecalis* strain E206 (*vanA*) and *E. faecium* E2781 (*vanB*) kindly provided by Dr N. Woodford were used as controls in the PCR experiments.

PFGE of vancomycin-resistant enterococci. Cell preparation for PFGE was performed as described previously (Morrison *et al.*, 1999). Each of the blocks was digested with 20 U of *Sma*I restriction endonuclease (New England Biolabs) and incubated at 25 °C for 4 h. Electrophoresis was performed with the CHEF DR-III system (Bio-Rad) with the following run parameters: initial pulse, 5 s; final pulse, 40 s; voltage, 6 V cm⁻¹; time, 20 h; and temperature, 10 °C. The gel was stained in ethidium bromide solution (0.5 μ g ml⁻¹) and visualized with UV transillumination. Banding patterns were compared visually and isolates that had the same number of fragments of the same size were regarded as being the same strain.

Transfer of vancomycin resistance. Donor strains were mixed with *E. faecalis* recipient strain JH2-2 on filter membranes as described previously (Woodford *et al.*, 1991) and transconjugants were selected on BHI agar containing 100 μ g rifampicin, 32 μ g fusidic acid, 32 μ g vancomycin or 15 μ g erythromycin. Transconjugants were screened for the co-transfer of unselected markers by replica plating. Representative colonies were analysed for the carriage of plasmid DNA as described previously (Werner *et al.*, 1999).

RESULTS

A total of 415 enterococcal isolates were obtained from different clinical samples in five hospitals in Kuwait. The majority of them were obtained from urine (36.6 %), stool (11.8 %), wound swabs (11.0 %), blood (10.3 %), high vaginal swabs (9.1 %), cervical swabs (3.1 %) and miscellaneous sources, including cervical swabs, catheter tips, endotracheal secretions and ear swabs (18.0 %). The stool samples were used to screen for faecal carriage of VRE in Intensive Care Unit patients who had been on prolonged therapy with vancomycin and were suspected to suffer from

Clostridium difficile associated diarrhoea. The isolates were identified as *E. faecalis* (85.3 %), *Enterococcus faecium* (7.7 %), *Enterococcus casseliflavus* (4.0 %), *Enterococcus avium* (1.2 %), *Enterococcus durans* (1.0 %), *Enterococcus gallinarum* (0.5 %) and '*Enterococcus bovis*' (0.2 %).

Antibacterial resistance

The distribution of antibacterial resistance according to species is presented in Table 1. They were resistant to erythromycin (63.3 %), tetracycline (60.5 %), ciprofloxacin (40.0 %) and chloramphenicol (28.0 %). High-level resistance to gentamicin (MIC $\geq$ 500 μ g ml⁻¹), kanamycin (MIC $\geq$ 1000 μ g ml⁻¹) and streptomycin (MIC $\geq$ 2000 μ g ml⁻¹) was detected in 13.9, 18.8 % and 20.0 % of the isolates respectively. In addition, 52 (12.5 %) of the isolates were resistant to ampicillin (MIC $\geq$ 16 μ g ml⁻¹). However, none of them produced β -lactamase.

The isolates were tested for their susceptibility to linezolid, a new oxazolidinone antibacterial that has been reported to have activity against Gram-positive cocci, including methicillin-resistant *S. aureus* and VRE. They were all susceptible to linezolid (MIC range: 0.5–4 μ g ml⁻¹).

Vancomycin and teicoplanin resistance were detected in 11 (2.6 %) isolates consisting of four *E. faecium* and seven *E. faecalis* (Table 1). Six of the seven vancomycin-resistant *E. faecalis* and all four vancomycin-resistant *E. faecium* isolates had MIC values $>$ 256 μ g ml⁻¹ for vancomycin (Table 2). Three *E. faecalis* and two *E. faecium* had MIC values $>$ 256 μ g ml⁻¹ for teicoplanin. All of the vancomycin-resistant isolates expressed low MIC values for linezolid (MIC $\leq$ 2 μ g ml⁻¹). One *E. casseliflavus* and two *E. gallinarum* isolates had MIC 8 μ g ml⁻¹ for vancomycin (Table 2). The vancomycin-resistant isolates were also resistant to erythromycin, ciprofloxacin and high-level streptomycin. Six of them were resistant to ampicillin, nine isolates expressed high-level gentamicin resistance, four were resistant to tetracycline and one was resistant to chloramphenicol (Table 2).

Detection of *van* genotypes

The 11 vancomycin-resistant enterococci were investigated for their vancomycin-resistance genotypes by PCR. All of them gave positive results for the presence of the *vanA* genotype (Fig. 1a and Table 2). A 732 bp PCR product (Dutka-Malen *et al.*, 1995) was obtained in all the positive isolates. No *vanB* products were detected in any of the isolates (Fig. 1b).

Transfer of vancomycin resistance

Four vancomycin-resistant isolates, *E. faecium* E166 and E194 and *E. faecalis* E90 and E178, were selected on the basis of their resistance patterns and used as donors in separate filter mating experiments. Transconjugants were obtained only with *E. faecalis* E90 as the donor. Transconjugants were obtained on vancomycin as well as on erythromycin selection plates at frequencies of 6.0×10^{-6} c.f.u. ml⁻¹. The trans-

Table 1. Antibacterial resistance of enterococci by disk diffusion tests

Antibacterial	MIC breakpoint ($\mu\text{g ml}^{-1}$)	Resistant isolates (%)			
		<i>E. faecalis</i> (<i>n</i> = 354)	<i>E. faecium</i> (<i>n</i> = 32)	Other species* (<i>n</i> = 29)	Total (<i>n</i> = 415)
Ampicillin	> 16	29 (8.2)	15 (46.8)	8 (27.6)	52 (12.5)
Gentamicin	> 500	52 (14.7)	5 (15.6)	1 (3.4)	58 (13.9)
Kanamycin	> 1000	72 (20.3)	5 (15.6)	1 (3.4)	78 (18.8)
Streptomycin	> 2000	76 (21.5)	6 (18.7)	—	83 (20.0)
Erythromycin	ND	235 (66.4)	16 (50.0)	12 (41.3)	263 (63.3)
Tetracycline	ND	229 (64.6)	13 (40.6)	5 (17.2)	251 (60.5)
Ciprofloxacin	ND	140 (39.5)	16 (50.0)	10 (34.4)	166 (40.0)
Chloramphenicol	ND	106 (29.9)	7 (21.8)	3 (10.3)	116 (27.9)
Vancomycin	> 32	7 (1.9)	4 (12.5)	—	11 (2.6)
Teicoplanin	> 32	7 (1.9)	4 (12.5)	—	11 (2.6)

ND, MIC not determined.

*Other species consisted of *E. casseliflavus* (*n* = 17), *E. avium* (5), *E. durans* (4), *E. gallinarium* (2) and '*E. bovis*' (1).

Table 2. Antibacterial-resistance profiles and *van* genotypes of VRE isolates

Abbreviations: Amp; ampicillin, Cm, chloramphenicol, Cip, ciprofloxacin, Em, erythromycin, Gm, gentamicin (high-level), Sm, streptomycin (high-level), Tc, tetracycline. +, Positive, —, negative. Isolates E166, E194, E246 and E249 were from one hospital while the other isolates were from another hospital. Strains XU11 and XU15 were control strains for *vanA* and *vanB* genes, respectively, kindly provided by Dr N. Woodford.

Isolate	Source	Species	MIC ($\mu\text{g ml}^{-1}$)		Genotype		Resistance pattern
			Van	Tec	<i>vanA</i>	<i>vanB</i>	
E165	Ascitic fluid	<i>E. faecium</i>	256	32	+	—	Em, Cip, Tc
E166	Urine	<i>E. faecium</i>	> 256	> 256	+	—	Amp, Em, Cip, Gm, Sm
E194	Urine	<i>E. faecium</i>	> 256	> 256	+	—	Em, Cip, Gm, Sm, Cm
V90	Urine	<i>E. faecalis</i>	> 256	128	+	—	Em, Cip, Gm, Sm
E178	Urine	<i>E. faecalis</i>	128	16	+	—	Amp, Em, Cip, Sm, Tc
E232	Wound	<i>E. faecalis</i>	> 256	256	+	—	Em, m, Cip, Gm, Sm
E246	Urine	<i>E. faecalis</i>	> 256	32	+	—	Amp, Em, Cip, Gm, Sm, Tc
E249	Wound	<i>E. faecalis</i>	> 256	> 256	+	—	Em, Cip, Gm, Sm, Tc
V254	Wound	<i>E. faecium</i>	> 256	24	+	—	Amp, Em, Cip, Gm, Sm
V261	Blood	<i>E. faecalis</i>	> 256	> 256	+	—	Amp, Em, Cip, Gm, Sm
V296	Catheter tip	<i>E. faecalis</i>	> 256	64	+	—	Amp, Em, Cip, Gm, Sm
E80	Urine	<i>E. casseliflavus</i>	8	2	—	—	Susceptible
E144	Ascitic fluid	<i>E. gallinarium</i>	8	4	—	—	Em, Tc
E251	Urine	<i>E. gallinarium</i>	8	1	—	—	Km, Sm, Em, Tc, Cm
XU11		<i>E. faecalis</i> E206	> 256	> 256	+	—	ND
XU15		<i>E. faecium</i> E2781	64	1.5	—	+	ND

conjugants from both selections were all resistant to vancomycin, teicoplanin, erythromycin, kanamycin and streptomycin, but no detectable plasmid was demonstrated in any of the transconjugants or the parental strain. The *vanA* product of the same size as that in the parent strain was obtained when genomic DNA from representative transconjugants was tested by PCR (Fig. 1a).

PFGE of vancomycin-resistant isolates

The vancomycin-resistant isolates were typed by PFGE after *Sma*I digestion of their genomic DNA. Two of the seven *E. faecalis* isolates had identical PFGE patterns as shown in Fig. 2. These isolates were obtained from different patients who had been admitted to different wards in the same hospital.

Fig. 1. PCR amplification of (a) *vanA* and (b) *vanB* genes. (a) Lanes: 1, molecular size markers; 2–8; vancomycin-resistant *E. faecalis* isolates V90, E178, E232, E246, E249, V261 and V296; 9–12; vancomycin-resistant *E. faecium* isolates, E165, E166, E194 and V254; 13–14, representatives of E90 vancomycin-resistant transconjugants; 15, *E. faecalis* E206, *vanA* positive control strain; 16; *E. faecalis* ATCC 29212, vancomycin-susceptible strain. All of the vancomycin-resistant isolates generated a 732 bp PCR product. (b) Lanes: 1, Molecular size markers; 2–6; representatives of vancomycin-resistant strains; 7, *E. faecium* E2781, *vanB* positive control strain.

Fig. 2. PFGE patterns of seven vancomycin-resistant *E. faecalis* isolates digested with *Sma*I restriction endonuclease. Lanes: 1, E246; 2, E249; 3, V90; 4, E178; 5, E232; 6, V261; 7, V296.

One isolate was from a urine sample and the other was from a diabetic foot wound swab. The remaining five *E. faecalis* isolates had different PFGE patterns (Fig. 2). Similarly, the four *E. faecium* isolates had different PFGE patterns (not shown).

DISCUSSION

This study investigated the species prevalence and antibacterial-resistance patterns of enterococci isolated from clinical samples in Kuwait hospitals. The majority of the isolates were either *E. faecalis* (85.3 %) or *E. faecium* (7.7 %), while *E. casseliflavus*, *E. avium*, *E. gallinarum*, *E. durans* and '*E. bovis*' accounted for only 7.0 % of the isolates, which was comparable to the distribution of enterococcal species in other studies (Jones *et al.*, 1997; Perlada *et al.*, 1997; Zouain & Araj, 2001). The prevalence of *E. faecium* in this study (7.7 %) was lower than a 29 % prevalence of *E. faecium* reported in Cincinnati, OH, USA (Perlada *et al.*, 1997). *E. faecium* has also been reported as the most common species in other studies (Woodford *et al.*, 1993; Linden *et al.*, 1996). No VRE were isolated from any of the stool samples submitted for the detection of *C. difficile* toxin in this study in contrast to the detection of VRE in 19.8 % of stool samples screened for *C. difficile* toxin by Leber *et al.* (2001).

Of the 415 isolates, 63, 60 and 40 % were resistant to erythromycin, tetracycline and chloramphenicol, respectively which were similar to levels reported for these antibacterials among enterococci isolated in the UK (Woodford *et al.*, 1993) and Germany (Reinert *et al.*, 1999). It was reassuring that 98 and 91.8 % of the *E. faecalis* in this study were vancomycin- and ampicillin-susceptible, respectively, contrary to the situation in most hospitals in the USA (Jones *et al.*, 1997; Quale *et al.*, 1996; Perlada *et al.*, 1997) and Europe (Nelson *et al.*, 2000; Schouten *et al.*, 2000) where high prevalence of vancomycin and ampicillin resistance are common. The low prevalence of vancomycin resistance

among the isolates in this study indicated that vancomycin retains its therapeutic efficacy against the majority of enterococci isolated from patients in Kuwait hospitals.

Although 85.3 and 84.5 % of the isolates were susceptible to gentamicin and streptomycin, suggesting that gentamicin should maintain a synergistic effect when combined with cell-wall-active agents such as vancomycin and ampicillin in the treatment of enterococcal infections, the detection of high-level gentamicin resistance in 14.7 % of *E. faecalis* and 15.6 % of *E. faecium* isolates is cause for concern, as it may signify the beginning of a major resistance problem.

Vancomycin-resistance phenotypes in enterococci have been classified as *vanA*, *vanB*, *vanC*, *vanD* and *vanE* based on levels of resistance, cross-resistance to teicoplanin and inducible or constitutive nature of the resistance (Arthur & Courvalin, 1993). Two of these, *vanA* (vancomycin MIC $\leq 64 \mu\text{g ml}^{-1}$; teicoplanin MIC $\geq 16 \mu\text{g ml}^{-1}$) and *vanB* (vancomycin MIC 4–1024 $\mu\text{g ml}^{-1}$, teicoplanin MIC $\leq 0.5 \mu\text{g ml}^{-1}$) determinants have been described primarily in *E. faecalis* and *E. faecium*. The *vanA* determinant is carried on transposon Tn1546 or close relatives that are transferable in conjugation experiments (Arthur & Courvalin, 1993). All of the 11 vancomycin-resistant isolates in this study expressed vancomycin-resistance patterns compatible with the *vanA* phenotype and all gave positive results in PCR experiments for the *vanA* genotype. One of the four isolates tested transferred vancomycin resistance in conjugation experiments but the transconjugants contained no detectable plasmids, suggesting that the vancomycin-resistance determinant of this isolate may be carried on a conjugative transposon (Arthur & Courvalin, 1993). The transfer of vancomycin resistance without the transfer of detectable plasmids has been reported previously (Shlaes *et al.*, 1989; Leclercq *et al.*, 1989).

PFGE revealed that only two *E. faecalis* isolates were related (Fig. 2). The rest were unrelated, suggesting that the strains acquired vancomycin resistance independently, possibly, by horizontal transfer of the vancomycin-resistance determinants. The absence of a dominant clone among the vancomycin-resistant isolates is consistent with the fact that there has been no evidence of an outbreak of VRE in any of the hospitals from where the isolates were obtained. Furthermore, VRE have been isolated irregularly in these hospitals.

Although the prevalence of glycopeptide resistance was low among the isolates studied, their presence together with high-level aminoglycoside resistance calls for regular surveillance of antibacterial susceptibilities to detect emerging resistance and prevent the establishment and spread of multiply antibacterial-resistant strains.

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