Molecular epidemiology of vancomycin-resistant *Enterococcus faecium* strains isolated from haematological malignancy patients in a research hospital in Turkey

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Infections and outbreaks of vancomycin-resistant enterococci (VRE) still appear to be rare in Turkey. In the present study, VRE strains isolated during an outbreak in a haematology unit of a training and research hospital in Turkey were typed and their antimicrobial-resistance patterns were characterized by molecular methods. Twelve vancomycin-resistant *Enterococcus faecium* strains isolated from patients with haematological malignancies were investigated by PCR for the presence of genes encoding resistance to vancomycin, tetracycline, chloramphenicol, gentamicin and erythromycin. Their clonal relationship was evaluated by PFGE and multilocus sequence typing. All strains were resistant to vancomycin and erythromycin, and had the *vanA* and *ermB* genes, respectively. PFGE was used to determine the presence of two pulsotypes and determine their subtypes. Pulsotype A belonged to sequence type (ST) 17 and pulsotype B belonged to ST 78. All strains with the *vanA* gene were not the same clone, indicating multiple acquisitions of resistant isolates, even over such a short time period.

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INTRODUCTION

Enterococci are part of the normal flora in humans and animals (Huh *et al.*, 2006). These opportunistic pathogens have recently emerged as nosocomial infection agents, especially in patients with haemato-oncological disease and those in intensive care units (Vagnerova *et al.*, 2006). The first vancomycin-resistant enterococci (VRE) were reported from France in 1986 (Leclercq *et al.*, 1988). Reports on VRE colonization and infections in hospitalized patients have rapidly increased in many countries worldwide (Kosack *et al.*, 2009; Huh *et al.*, 2006; Vagnerova *et al.*, 2006; Vilela *et al.*, 2006). Solid-organ-transplant recipients, patients in intensive care units, haematology units or long-term care facilities are all at high risk of VRE infection and colonization (Chlebicki & Kurup, 2008). In the USA in particular, a remarkable rise in the frequency of VRE has been documented in the intensive care units of hospitals. However, the incidence of vancomycin resistance in enterococci from European hospitals remained at low

levels during the 1990s and the early 2000s. Recently, a significant increase in the frequency of glycopeptide-resistant enterococci (especially *Enterococcus faecium*) has been reported from a number of European countries such as Germany, Italy, Portugal, Greece and the UK (Klare *et al.*, 2005). Infections and outbreaks with VRE still appear to be rare and information on the prevalence of VRE is limited in Turkey (Colak *et al.*, 2002; Fisgin *et al.*, 2006; Kilic *et al.*, 2006). After the first VRE-infected patient was reported from Ankara, transmission of one epidemic strain has been described in north-western Turkey very recently (Comert *et al.*, 2007).

To date seven different glycopeptide resistance genotypes (VanA–VanE, VanG and VanL) have been described in enterococci (Boyd *et al.*, 2008; Cetinkaya *et al.*, 2000). The VanA and the VanB types are the most commonly encountered forms of acquired glycopeptide resistance (Quintiliani & Courvalin, 1996), and have the same basic mechanism of resistance (Evers & Courvalin, 1996). VanA and VanB type strains show inducible resistance, but the level of vancomycin and teicoplanin resistance differs: VanA types have high-level vancomycin and moderate-level teicoplanin resistance; however, VanB strains show

Abbreviations: CC, clonal complex; MLST, multilocus sequence typing; NEQAS, National External Quality Assessment Scheme for Microbiology; ST, sequence type; VRE, vancomycin-resistant enterococci.

various levels of vancomycin resistance but remain susceptible to teicoplanin (Dahl *et al.*, 1999). In the present study, we report the existence of multiple VRE clones with different pulsotypes and subtypes during an outbreak in a haematology unit of a training and research hospital in Turkey between May and October 2006.

METHODS

Study population and bacterial strains. Atatürk Training and Research Hospital, Izmir, Turkey, is a 1100 bed hospital, with a 15 bed haematology unit. All VRE were isolated from patients with haematological malignancies between May and October 2006. The first documented VRE was isolated in May 2006. After the isolation of five VRE strains from blood samples, a screening programme was initiated. Rectal swab specimens from patients and staff, and environmental samples, were taken. Rectal swab screening was performed three times at weekly intervals. Strains were identified using conventional methods such as Gram staining, the absence of catalase production, resistance to 6.5% sodium chloride and growth on bile aesculin agar. All VRE strains were confirmed as *E. faecium* with Phoenix automated microbiology system (BD Diagnostics).

Testing susceptibility to antimicrobials. The susceptibility of the strains to antimicrobials was screened by the disc diffusion method on Mueller–Hinton agar according to Clinical and Laboratory Standard Institute 2005 guidelines for the following antimicrobial agents: penicillin, vancomycin, teicoplanin, ampicillin, ciprofloxacin, gentamicin, erythromycin, tetracycline and chloramphenicol (CLSI, 2005). The antibiotic discs used were from Oxoid. MICs were examined by Phoenix automated microbiology system (BD Diagnostics). *E. faecium* 8565 (NEQAS) strain was used as an external quality control strain.

DNA isolation and PCR for analysis of the mechanism of resistance. Bacterial DNA was extracted by the boiling method. A colony of bacteria was suspended in 50 µl water, boiled for 10 min, placed on ice until chilled and centrifuged at 16000 g for 1 min to pellet cell debris. The supernatant was used as a template for PCR (Register & Yersin, 2005). The presence of *vanA*, *vanB* and *vanC* genes was studied using PCR primers described by Dutka-Malen *et al.* (1995). The resulting amplification fragments were separated on 2% agarose gel in 0.5 × TBE buffer (5.4 g Tris base, 2.75 g boric acid, 2 ml 0.5 M EDTA, in 1 L). *tetM*, *tetK* (Warsa *et al.*, 1996), *cat*, *aac-aph* (Kao *et al.*, 2000), *ermA*, *ermB* and *ermC* (Sutcliffe *et al.*, 1996) genes were screened with specific primers.

PFGE. The clonal relationship between vancomycin-resistant strains was studied using PFGE of the genomic DNA (Antonishyn *et al.*, 2000). DNA restricted by *SmaI* enzyme was separated on an agarose gel using a CHEF DR III apparatus (Bio-Rad laboratories). The running conditions were 6 V cm⁻¹, with pulses ranging from 2 to 15 s for 18 h at 14 °C. DNA banding patterns were visualized under UV light after staining with ethidium bromide (0.5 µg ml⁻¹). The similarity between isolates was determined by visual comparison of isolate band patterns. The interpretation of PFGE results was carried out by eye according to the criteria described by Tenover *et al.* (1995).

Multilocus sequence typing (MLST). MLST was used to analyse the genetic types of two *E. faecium* isolates with different pulsotypes. For each isolate from the two pulsotypes, seven housekeeping genes (*adh*, *atpA*, *ddl*, *gdh*, *gyd*, *purK* and *pstS*) were amplified by PCR and all amplicons were sequenced as described by Homan *et al.* (2002). The sequences were compared with those of the alleles recorded in the database available at the MLST website (<http://efaecium.mlst.net>) and sequence types (STs) were determined.

RESULTS AND DISCUSSION

Patients and bacteria

This study describes an outbreak of VRE strains in a haematology unit of a research hospital. A total of seven patients, aged between 32 and 62 years, were included in this study. The mean age was 45 ± 11.9 years. Of the seven patients five were female and two were male. All the VRE strains were isolated from patients with haematological malignancies, all of whom had been hospitalized in the haematology unit. Among the patients with infection, the first VRE strain was identified from a blood culture of a 56-year-old female patient at the beginning of the outbreak.

After the isolation of five VRE strains from blood samples, all patients in the haematology unit were screened using rectal swabs and seven more VRE were isolated. Twelve strains were isolated from a total of only seven patients: five patients each contributed two strains from a blood culture and a rectal swab, and two patients each contributed one strain from a rectal swab (Table 1). All strains were confirmed to be *E. faecium*. The swab specimens of environmental sites and screened staff were not positive based on culturing. No patients died during this outbreak.

Susceptibility pattern and the mechanism of antimicrobial resistance

The results of testing *in vitro* susceptibility to antimicrobials for 12 VRE are shown in Table 1. High-level resistance to both glycopeptides (vancomycin and teicoplanin) (with a vancomycin MIC >16 mg l⁻¹) was a feature of all 12 *E. faecium* isolates (VanA phenotype) and this was consistent with the identification of *vanA* gene by PCR. Testing of *vanB* and *vanC* genes was negative for the 12 VRE strains. Our results were similar to those of other European studies (Ergani-Ozcan *et al.*, 2008; Willems *et al.*, 2005; Schmidt-Hieber *et al.*, 2007; Sample *et al.*, 2002). In most Australian states, several sporadic and outbreak-related strains have been reported. The majority were of the *vanB* genotype, but *E. faecium* carrying *vanA* has also been reported (Bartley *et al.*, 2001; Bell *et al.*, 1998; Worth *et al.*, 2007; Christiansen *et al.*, 2004; Paterson *et al.*, 1996).

All the *E. faecium* isolates were resistant to most of the drugs tested. All the strains were sensitive to chloramphenicol and tetracycline except one; this one strain was resistant to tetracycline and had the *tetM* gene. The 12 *E. faecium* had intermediate resistance to chloramphenicol and they were negative for the presence of the *cat* gene. All *E. faecium* isolates were resistant to erythromycin and presence of *ermB* gene was shown by PCR. The gentamicin-resistant strains did not carry an *aac-aph* gene. The pattern of sensitivity to antibiotics for all the *E. faecium* in the present study is consistent with the notion that resistance to vancomycin is usually accompanied by resistance to other antimicrobial agents, such as penicillin, ampicillin, teicoplanin, erythromycin, ciprofloxacin and gentamicin.

Table 1. Characteristics of vancomycin-resistant *E. faecium* cases

MICs (mg l⁻¹): teicoplanin, >16; vancomycin, >16; ciprofloxacin, >2; erythromycin, >4; gentamicin, >8; ampicillin, >8; penicillin, >8.

Case no.	Strain	Sample	Sex of patient	Age of patient (years)	TET (mg l ⁻¹)	High level GEN (mg l ⁻¹)	Genotype	Pulsotype	ST
1	1	Blood	F	56	≤0.5 S	>500 R	<i>vanA</i>	B	
	2	Rectal swab			≤0.5 S	>500 R	<i>vanA</i>	B	
2	3	Blood	F	34	≤0.5 S	>500 R	<i>vanA</i>	B1	
	4	Rectal swab			≤0.5 S	>500 R	<i>vanA</i>	B2	
3	5	Blood	F	62	≤0.5 S	≤500 S	<i>vanA</i>	A	17
	6	Rectal swab			≤0.5 S	>500 R	<i>vanA</i>	A	
4	7	Blood	M	35	≤0.5 S	>500 R	<i>vanA</i>	A1	
	8	Rectal swab			≤0.5 S	>500 R	<i>vanA</i>	A2	
5	9	Blood	F	44	≤0.5 S	>500 R	<i>vanA</i>	B	78
	10	Rectal swab			>4	>500 R	<i>vanA</i>	B	
7	11	Rectal swab	F	32	≤0.5 S	>500 R	<i>vanA</i>	A2	
8	12	Rectal swab	M	52	≤0.5 S	>500 R	<i>vanA</i>	A	

F, Female; GEN, gentamicin; M, male; R, resistant; S, sensitive; TET, tetracycline.

All strains, except one, remained sensitive to chloramphenicol and tetracycline as has been reported in other studies.

PFGE and MLST

In this outbreak, a main pulsotype A and minor pulsotype B with their subtypes were determined among 12 VRE isolates by PFGE. Six isolates belonged to pulsotype group A (3A, 1A1 and 2A2 subtypes) and other isolates were from group B (4B, 1B1 and 1B2 subtypes) (Fig. 1).

MLST was performed for two *E. faecium* isolates with different pulsotypes. The allelic profile of pulsotype A was: *atpA* 1, *ddl* 1, *gdh* 1, *purK* 1, *gyd* 1, *pstS* 1, *adk* 1, thus corresponding to ST 17. Pulsotype B belonged to ST 78 (*atpA* 15, *ddl* 1, *gdh* 1, *purK* 1, *gyd* 1, *pstS* 1, *adk* 1). Both ST 17 and ST 78 belong to the clonal complex-17 (CC-17) lineage, which is the cause of most of the nosocomial VRE outbreaks worldwide. The hospital-adapted CC-17 has rapidly spread globally during the last two decades. There is a large complex of genetically related STs: ST 22 is the primary founder and within ST 22, ST 17 represents an important secondary founder of a distinct branch designated CC-17 (Ergani-Ozcan *et al.*, 2008). Nosocomial outbreaks of ST 78, belonging to CC-17, have been described in Korea and in Europe, including in Germany and Italy (Klare *et al.*, 2005). Ergani-Ozcan *et al.* (2008) found *E. faecium* isolates, with the *vanA* genotype, a main pulsotype (A) and three minor pulsotypes (B, C, D) in a nosocomial outbreak in a paediatric unit at a Turkish university hospital. The epidemic strain A was ST 31, and the minor strain D was ST 18. ST 31 and ST 18 are both in CC-17. We found that all VRE isolates of the present outbreak were similar to the CC-17 designated by Ergani-Ozcan *et al.* (2008). Rapid diagnosis of CC-17 strains is very important as it may help to control spreading. In many European countries, a relatively large community

reservoir of VRE exists as a result of the massive use of the antimicrobial drug avoparcin as a growth promoter, while the prevalence of hospital-adapted (CC-17) VRE is, in general, much lower (Willems *et al.*, 2005).

To control this outbreak, the patients and carriers were screened, and all hygiene measures recommended were taken according to international guidelines. Staff education and strict implementation were monitored by the Hospital Infection Control Committee (Ataturk Training and Research Hospital). In the haematological unit in our study

Fig. 1. PFGE of *Smal*-digested genomic DNA from vancomycin-resistant *E. faecium* isolates from different patients. Lanes 1–12, isolates from patients: lanes 5, 6 and 12, pulsotype A; lane 7, pulsotype A1; lanes 8 and 11, pulsotype A2; lanes 1, 2, 9 and 10, pulsotype B; lane 3, pulsotype B1; lane 4, pulsotype B2. M, Marker.

all patients were separated and treated with linezolid, and the outbreak was controlled by limiting new admissions to the unit. The presence of multiple antimicrobial resistance *E. faecium* obligates the clinician to choose appropriate treatment. In conclusion, our data indicated that all strains with the *vanA* gene were not the same clone indicating multiple acquisitions of resistant isolates, even over such a short time period, and the strains belonged to an internationally disseminated lineage.

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