Nosocomial outbreak of vancomycin-resistant Enterococcus faecium in a paediatric unit at a Turkish university hospital

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Background: Despite growing concern about vancomycin-resistant enterococci (VRE) as nosocomial pathogens, especially in the USA, they have been rarely isolated in Turkish hospitals. After initial description in 2001 of unrelated VRE isolates, we report now the molecular characterization of a nosocomial outbreak at the Akdeniz University Hospital, Antalya, Turkey.

Methods: VRE isolates were from either clinical or rectal swab specimens. Identification, susceptibility testing and molecular characterization were performed according to standard techniques. Virulence genes (encoding aggregation substance, gelatinase, cytolysin, enterococcal surface protein and hyaluronidase) were sought by PCR.

Results: Thirty-six VRE were isolated from 10 patients between June and October 2005 in the Department of Paediatrics. Six patients were only carriers, two had urinary tract infections and two had bloodstream infections. All isolates were Enterococcus faecium, of vanA genotype and belonged either to a main pulsotype (A) or to three minor pulsotypes (B, C and D). The epidemic strain A, found in eight patients, expressed high-level glycopeptide resistance (MIC of vancomycin 256 mg/L and MIC of teicoplanin 64 mg/L) and was of multilocus sequence typing sequence type (ST) 31, whereas the minor strain D, found in two patients, expressed heterogeneous glycopeptide resistance (MIC of vancomycin 8 to 256 mg/L) and was ST18. Strains B and C were only found in single patients either with strain A or alone. The two epidemic strains A and D were esp gene-positive. Their vanA genes were located on transposons similar to Tn1546, except for deletion of the transposition genes and the presence of IS1542, inserted upstream of the vanA operon, and IS1216, inserted at the 3’ end of the vanX gene. VRE outbreak was contained by early identification and implementation of measures for patient isolation and of stringent hand and environmental disinfection policies.

Conclusions: This work underlines the emergence in Turkey of epidemic VRE clones that belong to the clonal complex-17 (CC-17) and that are esp-positive.

Keywords: VRE, vanA, Tn1546, MLST

Introduction

Enterococcus faecium is an important nosocomial pathogen, creating serious limitations in treatment options because of cumulative resistance to antimicrobial agents.1 Since their initial discovery from patients in France and the UK in 1986,2,3 vancomycin-resistant enterococci (VRE) have been reported worldwide.3,4 During the 1990s, a dramatic increase in VRE infections was reported mostly in intensive care units in the USA (30% in 2006).5 Conversely, infections with VRE remained rare in many European hospitals.

Six different glycopeptide resistance phenotypes (VanA to VanE and VanG) have been described in enterococci.6 VanA and VanB are of greatest clinical relevance.5,7 The VanA

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phenotype is usually inducible by glycopeptides and determined by a highly conserved gene cluster, vanRSAX, located on Tn\text{1546}-type elements, which may be active transposons. These transposons are often carried by plasmids, which further facilitates their horizontal spread among enterococcal strains. Nosocomial VRE outbreaks, attributed to horizontal transfer of resistance determinants, to clonal dissemination of epidemic strains or both, have been reported.6,9

Widely disseminated VRE strains have been reported previously as the source of the high degree of vancomycin resistance resulting in increasing nosocomial infections in hospitals. As VRE infections are associated with higher mortality and costs than infections caused by vancomycin-susceptible strains, epidemiological data concerning occurrence and spread of these microorganisms have to be compiled and VRE isolates have to be epidemiologically investigated.6

Several molecular typing schemes have been developed to study epidemiology of VRE.11–14 Among these techniques, PFGE has been considered the gold standard because of its high degree of isolate differentiation.15,16 Multilocus sequence typing (MLST) is a sequence-based typing method used for strain characterization and long-term epidemiological investigations of epidemic \textit{E. faecium} isolates.13

After the first description of a VRE isolate in 2001 and of unrelated VRE isolates in our hospital, we report here a detailed molecular follow-up of an outbreak of VRE in a Paediatric Unit of Akdeniz University Hospital, between June and October 2005. The MLST-type and Tn\text{1546}-like structures were investigated for the main epidemic strains.

Materials and methods

Hospital setting, outbreak and surveillance

Akdeniz University Hospital is a 746-bed tertiary-care teaching hospital. The Paediatric Unit has 47 beds with 18 beds for its newborn unit. After the first isolation of VRE from the urine sample of a patient in the Paediatric Unit (index patient, June 2005), a prospective surveillance study was performed to determine the epidemiology of VRE, and infection control measures were strengthened according to the recommendations of the Centers for Disease Control and Prevention by the Infection Control Committee of the hospital. A screening programme was initiated. Contact patients of each VRE carrier were defined as patients who were in the same unit as the carrier at the same time, and these contact patients were also routinely screened. Rectal swab cultures were taken once a week from all patients hospitalized in the Paediatric Unit, from the contact patients and from all newly admitted patients. Screening was repeated weekly for as long as VRE-positive patients remained on the ward. In February 2006, i.e. 4 months after the last VRE case was diagnosed (October 2005), the epidemic was officially considered over and the surveillance programme returned to a normal operation level. Environmental screening was also performed in the affected ward around cases of cross-transmission.

Contact precautions were maintained for all VRE-positive patients until three rectal screenings at weekly intervals were negative according to the recommendations of the Centers for Disease Control and Prevention. In addition to contact precautions, stringent compliance with standard precautions, especially the use of alcohol-based hand-rub solutions, was strongly recommended for all patients in the ward regardless of their bacteriological status.

Bacterial strains and VRE isolation

VRE isolation from clinical specimens and rectal swabs were carried out by standard microbiological techniques, using bile aesculin broth and bile aesculin agar plates supplemented with 6 mg/L vancomycin (Oxoid, England). After 72 h of incubation, black-pigmented colonies on VRE agar were analysed.

Species identification was performed by using species-specific PCR for \textit{Enterococcus faecalis} and \textit{E. faecium} in addition to conventional tests and the Phoenix System (Becton–Dickinson, USA). Rifampicin- and fusidic acid-resistant \textit{E. faecium} strain BM76 was used as a recipient in conjugation experiments.12–20

Antimicrobial agents and MIC determinations

Routine antibiograms for enterococci, performed in the Phoenix System (Becton–Dickinson), were confirmed by a disc diffusion method on Mueller–Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the CLSI guidelines. A panel of 22 antimicrobial agents was tested: penicillin G, oxacillin, amoxicillin, piperacillin, imipenem, kanamycin, streptomycin, gentamicin, cefotaxime, erythromycin, clindamycin, pristinamycin, levofoxacin, rifampicin, fosfomycin, tetracycline, linezolid, chloramphenicol, nitrofurantoin, co-trimoxazole, vancomycin and teicoplanin. MICs of vancomycin and teicoplanin were determined by Etest (AB BIODISK, Solna, Sweden) on Mueller–Hinton agar according to the manufacturer’s instructions. All plates were incubated at 37°C for 18 h. MIC results were interpreted according to the CLSI criteria.21

Plasmid content and conjugation assays

Direct transfer of the vancomycin resistance determinant from \textit{E. faecium} clinical isolates to \textit{E. faecium} strain BM76 was attempted by solid mating-out assays at 37°C as described previously.22–24 Transconjugant selection was performed on Trypticase soy agar (bioMérieux, Marcy l’Étoile, France) containing rifampicin (60 mg/L; Aventis, Paris, France), fusidic acid (20 mg/L; Leo Pharma, St Quentin-en-Yvelines, France) and vancomycin (10 mg/L; DakoTA Pharm, Le Plessis-Robinson, France). Donors and recipients were plated on vancomycin- (10 mg/L) or rifampicin- (60 mg/L) and fusidic acid- (20 mg/L) containing plates, respectively, in order to determine a transfer frequency. Plasmid DNA of the parental \textit{E. faecium} strains and of transconjugants was prepared by an alkaline lysis or Kieser extraction protocol.21,22 Plasmid DNA was analysed by electrophoresis on 0.8% agarose gels containing 0.5 mg/L ethidium bromide. \textit{Escherichia coli} NCTC 50192, harbouring plasmids of 154, 66, 38 and 7 kb, was used as a reference strain.24

PCR and sequencing

DNA of clinical \textit{E. faecium} isolates and of selected transconjugants was extracted by boiling as described previously. The PCR conditions and primers used to detect \textit{vanA} genes have been described previously. Whole-cell DNA of isolates was purified as described previously. A total of 500 ng of whole-cell DNA was used in standard PCR mixtures in a GeneAmp 2700 thermal cycler (Applied Biosystems, Les Ulis, France). Tn\text{1546}-like elements were amplified using PCR primers as described previously. Amplicons were purified with a QIAquick PCR purification kit (Qiagen, Les Ulis, France).
VRE in a paediatric unit at a Turkish hospital

France) prior to sequence determination with an ABI Prism 3100 sequencer (Applied Biosystems). Nucleotide sequence alignments were carried out at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov). Genetic modifications in the van genes were studied by comparing the obtained sequences with those of prototype Tn1546.3 The virulence genes of E. faecium (encoding aggregation substance, gelatinase, cytolysin, enterococcal surface protein and hyaluronidase) were sought by multiplex PCR as described previously.27

MLST
MLST was performed according to the scheme described previously.13 Internal fragments of seven housekeeping genes (adk, atpA, ddi, gdh, gyd, purK and pstS) were amplified by PCR. Amplicons were purified by using a QIAquick PCR purification kit (Qiagen) prior to their sequence determination with an ABI Prism 3100 sequencer (Applied Biosystems). The allele number for each gene was assigned based on the E. faecium MLST database (http://www.mlst.net). The combination of the allelic sequences for the seven genes yielded the allelic profile [sequence type (ST)] for each isolate.

PFGE typing
Whole-cell DNAs of culture extracts from each isolate and of selected transconjugants embedded in 1% agarose plugs (Bio-Rad) were digested with Smal restriction enzyme (Amersham Pharmacia Biotech) and separated in a 0.5 mg/L ethidium bromide solution (Bio-Rad) by using a CHEF DRII system (Bio-Rad), as described previously.18 PFGE was run at 14°C, with a 6 V/cm current, a switch angle of 120° and switch times of 0.1–20 s for 20 h. After migration, gels were stained in a 0.5 mg/L ethidium bromide solution, and PFGE results were analysed by eye according to the criteria of Tenover et al.16

Results
Hospital setting, clinical isolates and surveillance study
The first VRE was isolated from the urine sample of a patient hospitalized in the Paediatric Unit in June 2005 (Table 1). From June until October, at least one colonized patient was always present in the ward. Patient-care equipment and environmental surfaces were also screened for VRE colonization. Two thousand four hundred and eighty-eight rectal swabs and 16 stool samples from 586 patients and 21 environmental cultures (10 bed side rails, 6 room door handles, 3 over bed tables, 1 patient-care table and 1 phymogramanometer) were collected. Meanwhile, 35 urine samples and 5 blood cultures were received from these patients during this period. From October 2005 until February 2006 (end of surveillance), no new cases of VRE were diagnosed throughout the hospital. Thirty-six VRE isolates were recovered at the end of the surveillance period (until February 2006) (Table 1).

Bacterial strains
Thirty-six VRE isolates were recovered from 10 patients (5 male and 5 female) aged between 0 and 17 years. All patients were hospitalized in the Department of Paediatrics. Among the 10 patients, 6 were carriers and 4 had true VRE infections: 2 bacteremias and 2 urinary tract infections (Table 1). The most frequent underlying diseases were haematological malignancy and solid organ tumours.

PFGE typing and antimicrobial agents susceptibility testing
The 36 VRE isolates were analysed by PFGE, and the results are shown in Table 1. The predominant pulsotype ‘A’ was present in eight patients, while four isolates (three from patient I and one from patient J) belonged to a second pulsotype D. In addition, two sporadic pulsotypes were also found, one in patient B (strain B) and one in patient H (strain C).

The antimicrobial susceptibility followed the clonal distribution observed in PFGE. Most of the E. faecium isolates (strains A, B and C) expressed high-level glycopeptide resistance (MIC of vancomycin: 256 mg/L and of teicoplanin: 64 mg/L) (Table 1). The isolates with a PFGE type A were also resistant to ampicillin, clindamycin and erythromycin, and displayed high-level resistance to gentamicin, kanamycin and streptomycin, but remained susceptible to chloramphenicol, fosfomycin, nitrofurantoin, levofloxacin, linezolid, pristinamycin, rifampicin, tetracycline and tigecycline. Isolates of strains B and C had a similar resistance profile, except they were susceptible to kanamycin and gentamicin. Differences were observed for isolates of strain D from patients I and J, where heterogeneous vancomycin–teicoplanin resistance expression and gentamicin susceptibility were observed (Table 1). Determination of actual MICs of vancomycin was difficult since these isolates had heterogeneous expression of resistance, illustrated by growth of colonies in the inhibition zone when performing disc diffusion susceptibility testing. All E. faecium isolates were resistant to ampicillin, but β-lactamase-negative according to cefinase-test.

Detection and sequencing of vanA genes
The vanA gene was detected by PCR in the isolates. The sequence of the 730 bp amplicon determined for all E. faecium isolates was identical to that of the prototype vanA gene (GenBank accession no. M97297).26 The strains were PCR-negative for vanB genes.

Amplification and sequencing of Tn1546-like transposons
The genetic backgrounds of two E. faecium isolates were investigated in detail: isolate 2 (patient A) and isolate 31 (patient I) represented strains A and D, respectively (Table 1). These isolates had a vanA genotype with a genetic organization similar to that described for Tn1546 (Figure 1), except for the deletion of the transposition genes, orf1 and orf2, and the presence of insertion sequences (ISs). Despite repeated attempts, PCR of orf1 and orf2 failed, suggesting they were absent. Sequencing of the orf2-vanR intergenic region in these isolates revealed an 8 bp direct repeat of CTATAATC, corresponding to nucleotides 3925–3932 of the published Tn1546 sequence, on both sides of an IS1542.26 Similarly, another IS, IS216, was located in the vanX-vanY intergenic region at position 8832, as described previously. A 288 bp deletion was found upstream of this IS (from 8544 to 8832), which extended into the 3’ end of the vanX gene: the predicted VanX protein would be truncated by 26 amino acids.
Table 1. Clinical strains used in this study

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<th>Sex</th>
<th>Age</th>
<th>Underlying disease</th>
<th>Isolate (#)</th>
<th>Date of isolation (dd.mm.yy)</th>
<th>Source</th>
<th>MIC (mg/L) of teicoplanin (Phoenix)</th>
<th>MIC (mg/L) of vancomycin (Etest)</th>
<th>PFGE type</th>
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<td>256c</td>
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aM, male; F, female.
bPFGE types were considered different when more then seven band differences were observed; according to the criteria of Tenover et al.16

cSince a heterogeneous expression of the glycopeptide resistance was observed, only the highest MICs observed are indicated. MICs of vancomycin varied from 8 to 256 mg/L and MICs of teicoplanin varied from 1 to 64 mg/L.
Plasmid extraction and mating experiments

Plasmid DNA extraction from isolates 2 and 31 revealed the presence of a similar sized plasmid (160 kb). Vancomycin-resistant transconjugants were obtained at a frequency of $10^5$ to $10^6$ (transconjugants/donor). No other antibiotic resistance determinant was co-transferred with the glycopeptide resistance determinant (data not shown). The vancomycin and teicoplanin MICs were similar to those for parental strains, and transconjugant Tc31 displayed heterogeneous expression of vancomycin resistance similar to its parental isolate 31, suggesting that this heterogeneous behaviour was plasmid encoded (data not shown).

Detection of virulence genes and MLST

All *E. faecium* isolates were negative for genes encoding aggregation substance, gelatinase, cytolysin and hyaluronidase, but strains A and D were positive for *esp*, which encodes enterococcal surface protein.

MLST was performed on two *E. faecium* isolates representative of the two predominant strains A and D. The allelic profile of strain A was AtpA1; Ddl3; Gdh1; PurK1; Gyd1; PstS1; Adk3, thus corresponding to MLST ST31. The strain D belonged to ST18 (allelic profile AtpA7; Ddl1; Gdh1; PurK1; Gyd5; PstS1; Adk1). Both STs belong to clonal complex-17 (CC-17) and are double locus variants of ST17.

Discussion

The prevalence of VRE remains low among nosocomial-acquired pathogens in Turkey. During a susceptibility survey performed in 2000 of enterococci isolated in European hospitals, no vancomycin-resistant *E. faecium* strains were detected in Turkey. The first VRE-infected patient was reported from Ankara. Then several VRE strains, isolated from different patients over 1 year, were described at the University Hospital of Antalya. Very recently, transmission of one epidemic strain has been described in north-western Turkey.

Here, we describe an outbreak of VRE belonging to ST31, which was initially found in Australia (www.mlst.net), and ST18, which is a common type in the UK. There is a large complex of genetically related STs: ST22 is the primary founder and within ST22, ST17 represents an important secondary founder of a distinct branch designated CC-17. ST18, which we have determined in our strains, is also in CC-17. Genetic population studies have shown that most of the nosocomial VRE outbreaks worldwide belong to the same CC-17 lineage. Its founder type ST17 was recovered in most hospital outbreaks and clinical VRE isolates. CC-17 strains are generally resistant to ampicillin and carry *esp* and *hyl* genes for virulence factors.

ST31 is also in the CC-17 complex but, while ST18 is located on the main branch of CC-17, ST31 is located on a separate branch of the CC-17 phylogenetic tree (http://www.mlst.net). Hospital outbreaks, ampicillin resistance and the presence of a pathogenicity island are found to be strongly associated with CC-17. Its founder type ST17 was recovered in most hospital outbreaks and clinical VRE isolates. CC-17 strains are generally resistant to ampicillin and carry *esp* and *hyl* genes for virulence factors. ST31 is also in the CC-17 complex but, while ST18 is located on the main branch of CC-17, ST31 is located on a separate branch of the CC-17 phylogenetic tree (http://www.mlst.net). Hospital outbreaks, ampicillin resistance and the presence of a pathogenicity island are found to be strongly associated with CC-17. In addition, the two epidemic strains were *esp*-positive, which was recently suggested to be one of the main virulence factors of epidemic strains and which is part of a pathogenicity island. The presence of the *esp* gene is strongly associated with hospital outbreak strains of vancomycin-resistant *E. faecium*, suggesting a role for *esp* in nosocomial transmission.

Colonization and infection with VRE primarily affect moderately to severely ill patients in acute-care hospitals. The patients involved in this outbreak had most of the known risk factors of which the severity of underlying illness was the most
important. Screening for carriers and implementation of re-enforced hygiene measures led to the control of the outbreak. Several investigations suggest that a monoclonal outbreak, if not brought under control, can evolve into polyclonal endemity. Early detection and implementation of control measures kept the epidemic as a clonal dissemination, as demonstrated by one major E. faecium PFGE type. In addition to PFGE type A, three distinct PFGE types were isolated from several patients suggesting either horizontal gene transfer or introduction of new strains from outside. The finding of similar sized plasmids carrying identical Tn1546 variants in isolates with different PFGE patterns in a single patient, or in two distinct patients (patients D and I), is strongly suggestive of horizontal gene transfer. Data presented here illustrate the complexity of the epidemiological situation concerning VRE (diffusion of a main strain together with several unrelated strains) that may occur in a single medical centre, or even within a single patient.

Although most isolates displayed a ‘true’ VanA phenotype (MIC of vancomycin 256 mg/L and MIC of teicoplanin 64 mg/L), isolates belonging to strain D had heterogeneous expression of glycopeptide resistance (MIC of vancomycin 8–256 mg/L and MIC of teicoplanin 1–64 mg/L) (Table 1). These MICs would fit with a VanB or VanD phenotype. PCR analysis detected a vanA genotype in this strain, demonstrating the value of molecular tools for the detailed investigation of such outbreaks. Interestingly, retesting these VRE isolates with an automated antimicrobial susceptibility test system (Phoenix, Becton–Dickinson) permitted their unambiguous identification as VRE.

VRE strains with the vanA genotype, but susceptibility to teicoplanin in vitro, i.e. a VanB or VanD phenotype, have been detected previously in Japan, Taiwan and Korea. Three point mutations in the putative sensor domain of the VanS protein could be responsible for this impaired teicoplanin resistance phenotype. However, in the present study, no point mutations were found in the vanA gene. Impairment of accessory proteins VanY, D-carboxypeptidase or VanZ has also been proposed as a possible explanation for impaired resistance to teicoplanin among VRE isolates possessing the vanA gene cluster. In our study, several IS-like elements have been found in the vanA gene cluster of the epidemic E. faecium strains. They are IS1542, which is inserted in the intergenic region of orf2-vanR and IS1216 in the intergenic region of vanX-vanY. Neither of these IS elements could explain the reduced glycopeptide resistance of strain D, since the same Tn1546-like element was found in strain A, in which a high level of expression was observed. These data further suggest that the occurrence of VanB/VanD phenotype–vanA genotype isolates is not due to a single genetic mechanism. Further investigations will be necessary to understand the molecular basis of this heterogeneous glycopeptide resistance expression.

As a relatively new pathogen, VRE acts as a sensitive marker for measuring the effectiveness of infection control programmes and the appropriate application of preventive measures. In the present study, all measures recommended in international guidelines were discussed during staff meetings and with the Infection Control Committee; the international recommendations were adapted to local conditions and implementation was monitored by the Infection Control Committee. This outbreak was controlled by limiting transmission in the affected ward. From October 2005 until December 2007, no new cases of VRE were diagnosed throughout the hospital.

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Transparency declarations

None to declare.

References

VRE in a paediatric unit at a Turkish hospital


