

ORIGINAL ARTICLE

The Epidemiological and Molecular Characterization of Vancomycin-Resistant Enterococci Isolated from Rectal Swab Samples of Hospitalized Patients in Turkey

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SUMMARY

Background: Vancomycin-resistant enterococci (VRE) are a serious problem all over the world. The present study was conducted to investigate antimicrobial resistance patterns, genotypes, clonal relationship, and virulence factors of VRE species isolated from rectal swab samples of hospitalized patients, patient's relatives, and medical staff at Istanbul University Cerrahpasa Medical School hospital.

Methods: The VRE isolates were typed with an automated VITEK system and their antibiotic sensibilities were analysed by disc diffusion and Etest® method. The molecular characterization and clonal relationships were performed using a PCR method and virulence genes by sequence typing.

Results: A total of 100 (10.3%) of the 971 patients were colonized with VRE. None of the investigated 25 patient's relatives and 45 medical staff carried VRE. All VRE strains were identified as *E. faecium*. They were *vanA* genotype and originated from a single clone. VRE strains exhibited multi-drug resistance. High-level gentamicin-resistance was 93%. However, lower resistance rates were found for linezolid (40%) and quinopristin-dalfopristin (11%). The enterococcal surface protein gene *esp* was found positive in 87 of 100 isolates, and four strains were positive for the *cytB* (cytolysin) gene.

Conclusions: The identification of VRE strains to the species level and detection of virulence genes will assist in infection control practices.

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KEY WORDS

Enterococcus faecium, VRE, *vanA*, virulence genes

INTRODUCTION

Enterococci are part of the normal intestinal flora of humans and animals and they can be found in nature, soil, water, and raw foods. In 1988, vancomycin-resistant enterococci (VRE) had been described in the United Kingdom and, over two decades later, these opportunistic human pathogens now have become important etiological

agents of nosocomial infections all over the world [1-3]. Vancomycin resistance in enterococci occurs by the acquisition of different van gene clusters (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*) [4-5]. Enterococcal virulence factors encoded by virulence genes contribute to VRE-infections. The virulence genes are enterococcal surface protein (*esp* gene), cytolysin (encoded by five genes: *cylL1*, *cylL2*, *cylM*, *cylB*, and *cylA*), hyaluronidase (*hyl* gene), etc. The *esp* gene is responsible for the enterococcal colonization, adherence, and biofilm formation as well as the high-level antibiotic resistance in *E. faecium* strains [6]. In addition, the *esp* gene has recently been identified as a marker of the highly prevalent vancomycin-resistant *E. faecium* clones among hospitalized patients. Another virulence factor, the production of *cyl* gene has been shown to significantly worsen the severity of endocarditis, endophthalmitis, and peritonitis in a number of animal models [7-9]. Geographical differences in phenotypic and molecular characterization of glycopeptide resistance and virulence genes present in *E. faecium* isolates have been described [10-12].

The present study was conducted to investigate the antimicrobial resistance patterns, betalactamase producing, genotypes, clonally relationships and the presence of virulence factors (*esp*, *hyl* and *cyl*) of VRE species isolated from rectal swab samples of hospitalized patients, patient's relatives, and medical staff at Istanbul University Cerrahpasa Medical School hospital.

MATERIALS AND METHODS

Between January 2009 and March 2013, rectal swab samples were collected from 971 patients, hospitalized in various clinics, within the first 72 hours after admission and also from patient's relatives and medical staff in our hospital. Samples were inoculated into BBL enterococcosel broth and incubated for 24 hours at 35°C to enrich for enterococci. Positive cultures were transferred onto BBL enterococcosel agar with and without 6 mg/L of vancomycin in order to separate the vancomycin-resistant isolates. Plates were incubated at 35°C for 48 hours. The identification of the microorganism at the species level was performed on an automated VITEK system (BioMerieux), supplemented with conventional microbiological tests (Gram-positive cocci, growth on bile-aesculin agar, catalase-negative, pyrrolidonyl arylamidase positive, salt tolerance). Beta-lactamase presence of enterococci was investigated by the chromogenic method (Nitrocefin strip, Oxoid). To control the quality of this test were used beta-lactamase positive *Staphylococcus aureus* (ATCC 29213) and beta-lactamase-negative *S. aureus* (ATCC 10211). Minimum Inhibitory Concentrations (MIC) of vancomycin, teicoplanin, and high-level gentamicin resistance (HLGR) was carried out using the Etest® method (BioMerieux). The antibiotic resistance of VRE strains was determined using the Kirby-Bauer disk diffusion meth-

od according to CLSI (the Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) criteria [13,14]. Validation of minimum inhibitory concentration (MIC) values was performed by concurrent testing of CLSI-recommended quality control reference strains (*Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212, and ATCC 51299).

Molecular characterization of VRE strains was performed using a polymerase chain reaction (PCR) method. Genomic DNA was isolated using the High Pure DNA isolation kit (Roche) according to the manufacturer's recommendations, and the quality and quantity of the DNA samples were analyzed by NanoDrop ND-1000 spectrophotometry. Analyses of vancomycin resistance genes (*vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanD*, *vanE* and *vanG*) were performed by real time PCR method [15]. The primers used for *vanA*-gene detection were 5'-AATACTGTTTGGGGTTGCTC-3' (forward) and 5'-CTTTTCCGGCTCGACTTCCT-3' (reverse), 5'-GCGGGGAGGATGGTGCGATACAG-3' (forward) and 5'-GGAAGATACCGTGGCTCAAAC-3' (reverse) for *vanB*-gene detection, 5'-TTGACCCGCTGAAATATGAAGTAA-3' (forward) and 5'-TAGAACCGTAAGCAAAAGCAGTCG-3' (reverse) for *vanC1*-gene detection, 5'-GCATGGCAAATACGGGAAGAT-3' (forward) and 5'-CATGGCAGGATAGCGGGAGTGA-3' (reverse) for *vanC2/C3*-gene detection, 5'-TTTGTAAGCCTGCCGTTTC-3' (forward) and 5'-CCAAGTAYCCGGTAAATCTTC-3' (reverse) for *vanD*-gene detection, 5'-AAATAATGCTCCATCAATTTGCTGA-3' (forward) and 5'-ATAGTCGAAAAAGCCATCCACAAG-3' (reverse) for *vanE*-gene detection, 5'-TTGGAGGCAATTCAACAGAGT-3' (forward) and 5'-TCGCAGCCAACAACAGGTATT-3' (reverse) for *vanG*-gene detection. The detection of virulence genes (*esp*, *cylA*, *cylB* and *hyl*) were also determined by using real time PCR assay. The primers used for *esp*-gene detection were 5'-TGATTCTTGTTGTCGGATACG-3' (forward) and 5'-TGGTTGCGTTGCTTCTTCG-3' (reverse), 5'-TTGCTGGAGTAATAGACACGATTG-3' (forward) and 5'-TTGTAATTGTACCATATCCTCCTG-3' (reverse) for *cylA*-gene detection, 5'-CGCAAGGAGAGCATAGTGAATG-3' (forward) and 5'-AACTTATTACAGGAGTCGGAAGATC-3' (reverse) for *cylB*-gene detection, 5'-ACAGAAGAGCTGCAGGAAATG-3' (forward) and 5'-GACTGACGTCCAAGTTTCCAA-3' (reverse) for *hyl*-gene detection [4,15,16]. The Real Time PCR assay was optimized for each tested gene, and the specificity of the primers was confirmed by DNA sequencing of amplified products. Standard real time PCR conditions were used on a LightCycler 1.5 platform (Roche). The positive and negative control strains, *E. faecium*

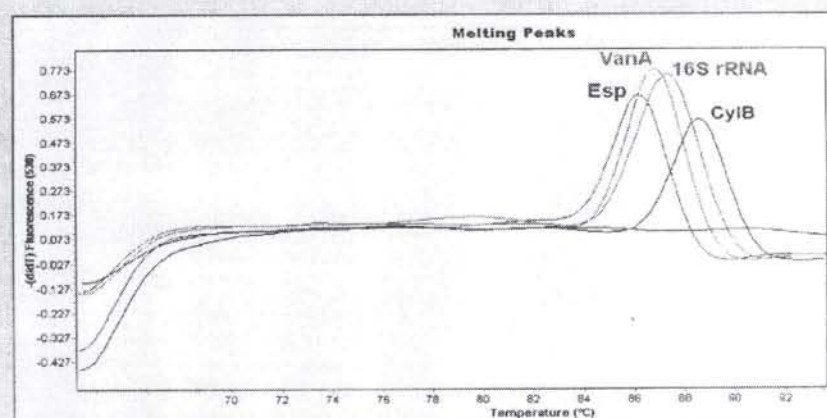


Figure 1. Real Time PCR assay was optimized for each tested gene.

ATCC 51559, *E. faecalis* ATCC 51299, and *E. faecalis* ATCC 29212 were included in each assay.

The clonal relationships of the VRE strains were examined by DNA sequencing of the 16S rRNA gene. DNA sequences were compared using a web-based CLUSTALW program (<http://workbench.sdsc.edu/>) and BLAST program (<http://blast.ncbi.nlm.nih.gov/>).

RESULTS

During the 15-month study period, a total of 1041 rectal samples from 971 patients hospitalized in various clinics, 25 patient's relatives, and 45 medical staff were examined. A total of 100 VRE strains were isolated from the patients' rectal samples. VRE was not detected in any of the investigated patients' relatives and medical staff. At one-week intervals, a total of three more rectal swab samples were collected from the patients who were VRE culture-negative. Follow-up of the patients was stopped, if VRE was still negative at the end of this period. During the weekly scans, patients who were colonized or infected with VRE were always positive for VRE in the control cultures. Forty-three pediatric patients staying at the hospital's pediatric units (intensive care, hemato-oncology, hematology, nephrology, gastroenterology, and emergency) and fifty-seven adult patients staying at the hospital's various units (intensive care, bone marrow transplant, hematology, neurosurgery, gastroenterology, internal medicine, and emergency) were found to be colonized or infected with VRE. Forty-two of the patients with VRE were female and fifty-eight were male. Ages of the children ranged from 1 month to 13 years and ages of the adults ranged from

18 to 83 years. The VRE-infected or colonized patients had a history of risk factors such as severe underlying disease or immuno-suppression, intra-abdominal or cardiothoracic surgical procedures, an indwelling urinary catheter or central venous catheter, vancomycin and/or multi-antimicrobial therapy, and extended length of hospital stay.

All of the VRE strains were identified as *E. faecium*. By the disk diffusion method, the resistance rates were found as 100% for vancomycin, teicoplanin, ampicillin, erythromycin, and ciprofloxacin, as 94% for nitrofurantoin, and as 83% for rifampicin. In addition, HLGR was found in 93% (MIC value > 1024 µg/mL) of strains. The rate of resistance to tetracycline (34%), chloramphenicol (36%), and linezolid (40%) was relatively low. Quinopristin-dalfopristin resistance rate (11%) was the lowest.

MIC of vancomycin ranged from 128 to greater than 256 µg/mL and MIC of teicoplanin ranged from 64 to greater than 256 µg/mL. Beta-lactamase activity in VRE strains was not detected. All the investigated VRE strains were *vanA* gene positive. The virulence factor *esp* gene was found positive in 87 of 100 isolates and four *E. faecium* strains were positive for the *cylB* gene (Figure 1 and 2). All the tested VRE strains were negative for *vanB*, *vanC1*, *vanC2/C3*, *vanD*, *vanE*, *vanG*, *cylA*, and *hyl* genes. The comparison of DNA sequences revealed a common origin of the investigated *E. faecium* strains. The representative results are shown in Figure 3.

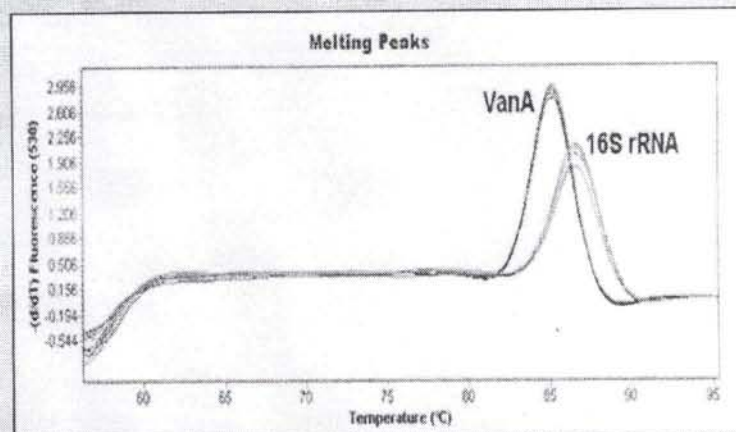


Figure 2. Representative real time PCR assay results showing VanA genotyping.



Figure 3. Representative CLUSTALW analysis result showing the alignment of 16S rRNA sequences of six different samples.

DISCUSSION

Enterococci are the second most common cause of urinary tract infection and third most common cause of bacteremia. Clonal spread is the dominant factor in the dissemination of multidrug-resistant enterococci [17].

The rapid progress in health care and the increase of quality and quantity of service delivery has also led to the proliferation of risk factors for colonization and infection [18,19]. VRE can spread from patient to patient via transient carriage on the hands of medical staff, contaminated environmental surface, or patient care equip-

ment [5,20]. In our study, the detection rate of VRE was found to be 10.3% in rectal swab samples of patients who were hospitalized in the intensive care unit and other critical clinics of our hospital, and almost all patients with VRE had risk factors (malignancy, renal failure, chronic diseases, diabetes, immune suppression, operations, invasive procedures, steroid therapy, extended length of hospital stay). In recent years, *E. faecium* has come to the fore between species of VRE. More than 70% of *E. faecium* isolates recovered from US hospitals are VRE [21]. In a multicenter study conducted in Italy, vancomycin-resistant *E. faecium* strains have been found to have increased from 0.9% to 32% [22].

In the present study, all isolated VRE strains were identified as *E. faecium*. It was understood that these strains are *vanA* genotype and originate from a single clone. The vast majority of VRE strains reported in our country show the *vanA* phenotype. This phenotype is more common in Europe and in the United States [5,9,23-25]. Enterococci are intrinsically resistant to betalactams, cephalosporins, and aminoglycosides (low-level). Additionally, enterococci can acquire resistance to other antimicrobial agents, including tetracyclines, macrolides, quinolones, streptogramins, and glycopeptides [26]. In our study, all strains showed high-level vancomycin resistance (MIC value 128 - greater than 256 µg/mL) and teicoplanin resistance (MIC value 64 - greater than 256 µg/mL). Also, the resistance to other antibiotics (ampicillin, erythromycin, ciprofloxacin, nitrofurantoin, and rifampicin) showed very high rates.

Severe enterococcal infections are usually treated with synergistic and bactericidal therapy with a combination of an aminoglycoside (gentamicin) and a beta-lactam (or other cell wall agents, such as vancomycin).

In our study, HLGR (MIC value > 1024 µg/mL) was determined in 93% of the strains. Recently, the increasing prevalence of high-level aminoglycoside resistance (HLAR) leads to failure of the synergistic effect of the combination of beta-lactam and aminoglycoside in the treatment resulting in failure of treatment. In a multicenter study in Europe, HLAR VRE strains have been reported as 1.5 - 48.9%. Turkey (48.1%) has attracted attention as a country with the second highest HLAR in 2000 [3,27]. Over the last decade, our data shows that the 45% increase occurred in HLAR rate in our country. Recently, two new antibiotics (linezolid and quinupristin/dalfopristin) started to be used in the treatment of multidrug-resistant VRE and have also emerged with a resistance problem [28,29]. In our study, linezolid resistance was 40% in isolated VRE. The least resistance was found for quinupristin-dalfopristin (11%). Quinupristin-dalfopristin is an antimicrobial agent that is not currently on the market in our country. In Europe, quinupristin-dalfopristin resistance in strains of vancomycin-resistant *E. faecium* is observed 10% and 0.6% in North America. Quinupristin-dalfopristin resistance is assumed to be linked with the use of more antibiotics associated with streptogramin in clinical practice and in animal feeds in Europe [25].

To understand persistent VRE colonization and disease progression, it is important to consider virulence factors. In our study, the *esp* gene was positive in 87 strains. Four strains were positive for *cytB* gene.

In many studies, molecular analyses showed the intra-hospital spread of *esp*-positive VRE clones. [7,30].

In conclusion, we determined that molecular typing suggests a clonal spread of multi-resistant *vanA E. faecium* strains and the isolates carry a high rate of *esp* genes. Therefore, identification of VRE strains to the species level and presence of virulence genes will assist in infection control practices in order to prevent VRE spread.

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Declaration of Interest:

The authors declare that they have no conflict of interest. We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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