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ORIGINAL RESEARCH / *ÖZGÜN ARAŞTIRMA*

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Hatay Devlet Hastanesi'nde Yoğun Bakım Ünitesine Başvuran Hastalardan İzole Edilen Vankomisine Dirençli *Enterococcus faecium* İzolatlarının Moleküler Karakterizasyonu

Molecular Characterization of Vancomycin Resistant *Enterococcus faecium* Isolates from Patients Admitted to Intensive Care Unit in Hatay State Hospital

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ABSTRACT *Objective:* In this study, it was aimed to determine the mechanisms of resistance to vancomycin , virulence genes and the clonal relationships of 23 vancomycin resistant *Enterococcus faecium* (VREfae) strains isolated from patients admitted to intensive care unit in XXXXXXXX
Materials and Methods: Vancomycin minimal inhibitor concentration (MIC) values of isolates were determined by E-test and sensitivity of isolates to antimicrobial agents was measured by disc diffusion method. Clonal relationship among the VREfae isolates was determined by pulsed-field gel electrophoresis (PFGE). Additionally, vancomycin resistance (*vanA, vanB, vanC, vanD, vanE* and *vanG*) and virulence genes (asa1, esp, gelE, hyl and cylA) were investigated by polymerase chain reaction (PCR) method.
Results: All isolates harbored vanA gene, and17 (73.9%) isolates were positive for at least one virulence genes. The most common virulence gene was esp, which was detected in nine isolates alone and in two isolates together with hly gene. The *hly* gene was detected in five isolates alone and other virulence genes (*asa*1, *gelE* and *cylA*) were not observed in any isolates tested. All isolates showed multiresistant resistance phenotype. Vancomycin MIC values of the isolates were measured ≥256 µg/ml by E test. PFGE analysis revealed 14 different pulsotypes in five clusters (A, B, C, D and E). Presence of identical PFGE patterns in isolates indicated that cross-transmission existed between hospital settings and patients.
Conclusion: To reduce the prevalence of VRE-induced infections and to take effective control measures in hospital settings; VRE resistance patterns and molecular characteristics should be monitored continuously.
Keywords: Vancomycin resistant *Enterococcus faecium*, Pulsed-field gel electrophoresis (PFGE), Virulence genes

ÖZ *Giriş:* Bu çalışmada, Hatay Devlet Hastanesi Yoğun Bakım Ünitesi'ne başvuran hastalardan izole edilen 23 vankomisine dirençli E*nterococcus faecium* (VREfae) suşunun vankomisine olan direnç mekanizmalarının, virülans genlerinin ve klonal ilişkilerinin belirlenmesi amaçlandı.
Gereç ve Yöntemler: İzolatların vankomisin minimal inhibitör konsantrasyon (MIC) değerleri E-testi ile antimikrobiyallere olan duyarlılıkları ise disk difüzyon yöntemi ile saptandı. VREfae izolatları arasında klonal ilişki pulsed-field jel elektroforezi (PFGE) ile belirlendi. Vankomisin direnç (*vanA, vanB, vanC, vanD, vanE* ve *vanG*) ve virülans genleri (*asa1, esp, gelE, hyl* ve *cylA*) polimeraz zincir reaksiyonu (PZR) yöntemi ile araştırıldı.
Bulgular: Bütün izolatlar *van*A geni taşıdığı bulundu ve izolatların 17'si (%73.9) en az bir virülans geni yönünden pozitif bulundu. En yaygın virülans geni olan esp, dokuz izolatta tek başına ve iki izolatta ise *hly* geni ile birlikte saptandı. *hly* geni tek başına beş izolatta tespit edilirken ve incelenen diğer virülans genleri (*asa1*, *gelE* ve *cylA*) ise hiçbir izolatta tespit edilmedi. İzolatların tamamı çoğul

direnç fenotipi (MDR) gösterdi. İzolatların vankomisin MİK değerleri E test ile ≥256 µg/ml olarak bulundu. PFGE analizi izolatların beş farklı klustera (A, B, C, D ve E) ve 14 farklı pulsotipe ayrıldığını gösterdi. İzolatlar arasında aynı PFGE band profili göstermeleri hastane ortamları ile hastalar arasında karşılıklı bulaşmanın olduğunu gösterdi.

Sonuç: almak için VRE'nin direnç patternlerini ve moleküler özelliklerinin sürekli olarak izlenmesi gerekmektedir.

Anahtar Kelimeler: Vancomycin dirençli *Enterococcus faecium*, Pulsed-field gel electrophoresis (PFGE), virulens genleri

Introduction

Following the first report of vancomycin resistant enterococci (VRE) in England 1988 (1) and shortly afterwards in France (2), presence and distribution of these agents were increasingly reported in both USA and European countries (3). In Turkey, for the first time, vancomycin resistant *Enterococcus faecium* (VRE*fae*) was isolated from pleural fluid taken from 11-month-old boy with bronchopulmonary infection, empirically treated with vancomycin and amikacin in 1998 by Vural et al. (4). In following years, incidence of these microorganisms, especially VRE*fae*, were increasingly reported from different settings and clinical cases in Turkey (5-7). Although enterococci are known to have intrinsic resistance to certain antimicrobials such as β-lactams, aminoglycosides; recently, vancomycin resistance together with high level of ampicillin and aminoglycoside resistance have frequently been reported, and today is a major cause of concern due to the limited antimicrobial treatment options (8, 9).

In previous studies carried out in Turkey, rectal colonization rate of VRE was reported between 4.9 and 15% (10, 11). Effective and continuous surveillance of cultures in patients hospitalized intensive care units has great advantages such as: (i) detection of asymptomatic carriers with multiple resistant microorganisms, (ii) implementation of effective isolation measures for patients and settings, (iii) obtaining successful results for the eradication of these microorganisms (12). Therefore, it is recommended to investigate the patients hospitalized in intensive care units for these microorganisms (13).

Enterococci have the ability to produce several virulence factors contributing their pathogenesis such as *esp* (enterococcal surface protein), *asa*1 (aggregation substance), *gel*E (gelatinase), *cyl*A (cytolysin) and *hyl* (hyalurinidase) (14). These virulence factors have been reported in VRE*fae* isolates with varying rates (7, 15).

It is of great importance to determine the clonal relationships among VRE isolates in order to take effective control measures and limiting spread of these microorganisms. For this purpose, numerous molecular typing methods such as randomly amplified polymorphic DNA (RAPD), Pulsed field gel electrophoresis (PFGE), Multi locus sequence typing (MLST), have been developed (16-18). However, each of these methods has differences in the reproducibility and discriminatory abilities. Of these methods, PFGE is accepted as gold standard for typing various bacterial species and is widely used (19, 20).

The aims of the study were (i) to investigate vancomycin resistance mechanism and virulence genes and (ii) to determine clonal relationship and antimicrobial susceptibility of 23 VRE***fae*** strains isolated from patients hospitalized in intensive care unit.

Materials and Methods

Study Group

Rectal swab samples were taken from patients hospitalized in intensive care unit of XXXXXXXX Hospital, Turkey between January and June 2017. Rectal swab samples were collected from all in patients at the time of admission and repeated monthly. In case of VRE positivity, if the patients had no clinical signs, it was defined as rectal VRE colonization and the case was followed up weekly according to institutional VRE surveillance program.

Isolation of VRE Isolates

For VRE screening, rectal swab samples were directly inoculated onto chromID VRE agar plates (**bioMérieux**, France) and incubated at 35 °C for 48 h. Then, the colonies (blue-green or violet color) of being suspected of VRE were passaged on blood agar plates supplemented with 5% defibrinated sheep blood in order to obtain pure cultures. The colonies were identified as *Enterococcus* spp. based on biochemical tests (colony morphology, Gram staining, catalase test, growth in 6.5% sodium chloride, L-pyrrolidonyl-ß-naphthlamidase activity and, aesculin hydrolysis in the presence of 40% bile salts) (21). The isolates were identified by VITEK and confirmed polymerase chain reaction (PCR) (22). In addition, all isolates were characterized by PFGE, which was performed in Public Health Institution of Turkey (Ankara) as described previously by Morrison et al. (23).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates was determined using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar (Merck, Germany) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (24). Following disks were used: high level gentamicin (120 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampicin, ampicillin (10 µg), vancomycin (30 µg), erythromycin (15 µg) and ciprofloxacin (5 µg). The MIC values of vancomycin were determined by E-test (Liofilchem, Italy). ***Enterococcus faecalis*** ATCC29212 was used as control strain. The isolates resistant to at least three different antimicrobial classes were deemed as multidrug resistant (MDR).

PCR Detection of Vancomycin Resistance Genes

Vancomycin resistance genes (*van*A, *van*B, *van*C, *van*D, *van*E and *van*G) were detected as previously reported by Depardieu et al. (25).

PCR Detection of Virulence Genes

Multiplex PCR was performed to screen specific virulence genes (***esp***, ***hyl***, ***asa***1, ***cyl***A and ***gel***E) as previously reported by Vankerckhoven et al. (14).

Results

Antimicrobial Susceptibility Testing

All 23 VREfae isolates were resistant to vancomycin, ampicillin, ciprofloxacin and erythromycin. Resistance rates for rifampicin, tetracycline, gentamicin and chloramphenicol were 95.7% (22), 56.5% (13), 56.5% (13) and 4.3% (1), respectively (Figure 1). All isolates showed MDR phenotype. Vancomycin MIC values of the isolates were found to be ≥256 µg/ml by E test.

mPCR Investigation for Vancomycin Resistance and Virulence Genes

All VREfae isolates carried the vanA gene. Out of 23 VREfae isolates, 17 (73.9%) were positive for virulence genes examined. Of the virulence genes examined, only esp and hly genes were observed among the isolates. The most common virulence gene was esp, which was detected in nine isolates alone and in two isolates together with hly gene. The hly gene was detected in five isolates alone (Figure 1).

PFGE Analysis

A shown in Figure 1, PFGE typing of 23 VREfae isolates showed 14 distinct PGFE types, clustering in five (A, B, C, D and E) PFGE groups based on a similarity coefficient of ≥85.

Discussion

Multiple resistant microorganisms including VRE have increasingly become important pathogens in relation to both colonization and hospital infections and have caused considerable concern throughout world (3). As in all over the world, an increasing rate of prevalence of VRE agents isolated from nosocomial infections were observed in Turkey. Namely, prevalence rates of VRE based on data from the National Nosocomial Infections Surveillance System was reported as 5.4%, 6.1%, 11.2%, 17.7% and 21.2% for 2008, 2009, 2010, 2012 and 2013, respectively (26, 27).

The vancomycin resistance in enterococci is mediated through the ***van*** gene operons, and so far eight operons (*van*A, *van*B, *van*C, *van*D, ***van***E, *van*G, *van*L and *van*M), each with a different resistance phenotype have been described. Of these, the isolates only carrying *van*A, *van*B and *van*M gene have a high level of vancomycin resistance (≥128 µg/ml) (28). In previous studies conducted in Turkey (5, 7, 29), ***van***A is frequently determined in VRE isolates. Similarly, in the current study, all isolates were also found to carry *van*A gene.

VRE isolates are often resistant to other classes of antimicrobial agents and show MDR phenotype, making treatment options very limited (9). In this study, all isolates

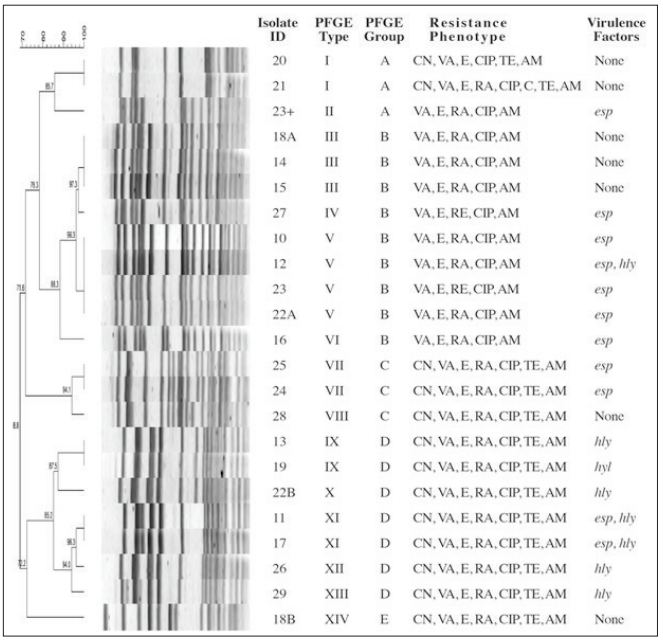

Figure 1. Dendogram showing the results of *SmaI* PGFE 23 VRE*fae* isolates. PFGE types are indicated as Roman numerals. PFGE groups A, B, C, D and E consisted of the isolates having a similarity coefficient ≥85%. The scale bar given on the top indicates similarity percentages detected for pulsotypes

showed MDR phenotype. Similarly, Gozalan et al. (7) found that all isolates were MDR phenotype except quinopristin/dalfopristin and linezolid.

Determination of clonal relationships between VRE strains isolated from hospital settings and clinical cases are of great value in detecting outbreak strains and infection sources. For this purpose, PFGE technique is one of the most preferred molecular techniques used for typing bacterial isolates due to high reproducibility and discrimination power (23). According to PFGE results, 23 VRE***fae*** isolates were distributed in five groups (A, B, C, D and E). As seen Figure 1, most of the isolates were found to be belonged in B and D group, accounting for 69.6% of the isolates, indicating occurrence of clonal spread in intensive care unit. Santajit and Indrawattana (30) have reported that nosocomial infections may originate from exogenous or endogenous sources and may be transferred by either direct or indirect contact between patients, health care workers, contaminated objects and, even medical devices. Although the mode of transmission is not the scope of this study, it might be suggested that VRE*fae* isolates might originated from exogenous or endogenous sources. Another study carried out by Gozalan et al. (7) reported identical PFGE profiles between non-invasive and invasive VRE*fae* isolates vice versa, indicating exogenous or endogenous sources.

Presence of virulence genes in enterococci plays an important role for colonization and pathogenesis of infection (14). In this study, 73.9% of the isolates were positive for the presence of virulence genes and *esp* and *hly* genes were only detected alone or together among virulence genes tested. Similar observation was reported by Saba Çopur et al. (31), who detected only ***esp*** (80.6%, n=75), *hly* (15.1%, n=14) and *gel*E (3.2%, n=3) genes among 93 VRE*fae* isolates and stressed that vancomycin sensitive enterococci (VRS) isolates had more virulence genes than VRE isolates. Another study carried out by Gozalan et al. (7), virulence genes were detected in 75% (n=41) of 55 VR*Efae* isolates regardless of their invasive or non-invasive status and found ***esp*** gene as the most common virulence gene, in one isolate together with ***ebp***A and in one isolate together with *ebp*A, *asa*1, *gel*E and *cpc*.

PFGE results indicated cross-transmission in intensive care unit due to identical PFGE profiles shared by isolates. Thus, infection control measures should strictly be applied in hospital settings. All VRE*fae* isolates had MDR phenotype including ampicillin and high level gentamicin resistance. For the treatment of infections caused by VR*Efae* strains, antibiotic susceptibilities of these agents should be taken into consideration. The ***esp*** gene was detected as the most common virulence gene considering the studies conducted in Turkey, it might be suggested that *esp* gene is highly prevalent in both invasive and non-invasive VRE isolates. Comprehensive molecular epidemiological studies should be conducted to determine the possible roles of non-invasive isolates in nosocomial infections and to take the effective measures to reduce the spread of these bacteria and the infections that may arise from these bacteria.

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