Turk J Intensive Care 2019;17:204-8
DOI: 10.4274/tybd.galenos.2019.20438

ORIGINAL RESEARCH / *ÖZGÜN ARAŞTIRMA*

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

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

Received/Geliş Tarihi : 08.10.2018
Accepted/Kabul Tarihi : 06.02.2019

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ABSTRACT *Objective:* In this study, it was aimed to determine vancomycin resistance mechanisms, virulence genes and clonal relationships of 23 vancomycin-resistant *Enterococcus faecium* (VRE*fae*) strains isolated from patients admitted to intensive care unit of Hatay State Hospital.

Materials and Methods: Minimal inhibitor concentration (MIC) values of vancomycin were determined by E-test and antimicrobial susceptibility of the strains was determined by disc diffusion method. The clonal relationship among VRE*fae* isolates was determined by pulsed-field gel electrophoresis (PFGE). Additionally, vancomycin resistance (*van*A, *van*B, *van*C, *van*D, *van*E and *van*G) and virulence genes (*asa*1, *esp, gel*E, *hyl* and *cyl*A) were investigated by polymerase chain reaction (PCR) method.

Results: All isolates harbored *van*A gene and 17 isolates (73.9%) were positive for at least one virulence gene. 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 the other virulence genes (*asa*1, *gel*E and *cyl*A) were not observed in any isolates tested. All isolates showed multidrug resistance phenotype. Vancomycin MIC values of the isolates were found to be ≥256 µg/mL by E-test. PFGE analysis revealed 14 different pulsotypes in five clusters (A, B, C, D and E). The presence of identical PFGE patterns indicated that there was a cross-transmission at hospital settings.

Conclusion: Continuous surveillance of resistance patterns and molecular characteristics of VRE are needed to reduce the prevalence of infections caused by VRE and to take effective control measures in hospitals.

Keywords: Vancomycin-resistant *Enterococcus faecium*, pulsed-field gel electrophoresis, virulence genes

ÖZ *Amaç:* Bu çalışmada, Hatay Devlet Hastanesi yoğun bakım ünitesine başvuran hastalardan izole edilen 23 vankomisine dirençli *Enterococcus faecium* (VRE*fae*) suşunun vankomisine olan direnç mekanizmalarının, virülans genlerinin ve klonal ilişkilerinin belirlenmesi amaçlandı.

Gereç ve Yöntem: İzolatların vankomisin minimal inhibitör konsantrasyon (MİK) değerleri E-testi ile antimikrobiyallere olan duyarlılıkları ise disk difüzyon yöntemi ile saptandı. VRE*fae* izolatları arasında klonal ilişki pulsed-field jel elektroforezi (PFGE) ile belirlendi. Vankomisin direnç (*van*A, *van*B, *van*C, *van*D, v*an*E ve v*an*G) ve virülans genleri (*asa*1, *esp, gel*E, *hyl* ve *cyl*A) polimeraz zincir reaksiyonu (PZR) yöntemi ile araştırıldı.

Bulgular: Bütün izolatlar *van*A geni taşıdığı tespit edildi 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 (*asa*1, *gel*E ve *cyl*A) ise hiçbir izolatta tespit edilmedi. İzolatların tamamı çoğul direnç fenotipi 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 profillerinin görülmesi hastane ortamları ile hastalar arasında karşılıklı bulaşmanın olduğunu gösterdi.
Sonuç: VRE kaynaklı enfeksiyonların prevalansını azaltmak ve hastane ortamlarında etkili kontrol önlemleri almak için VRE'nin direnç patternlerini ve moleküler özelliklerinin sürekli olarak izlenmesi gerekmektedir.
Anahtar Kelimeler: Vankomisine dirençli *Enterococcus faecium*, pulsed-field jel elektroforezi, virülens 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, pulsed field gel electrophoresis (PFGE), multi locus sequence typing, 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 Hatay State 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.

As VRE strains,which were isolated before, are used in the study (due to being a retrospective study), ethics committee decision is not taken.

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 by 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 minimal inhibitory concentration (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 VRE*fae* 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

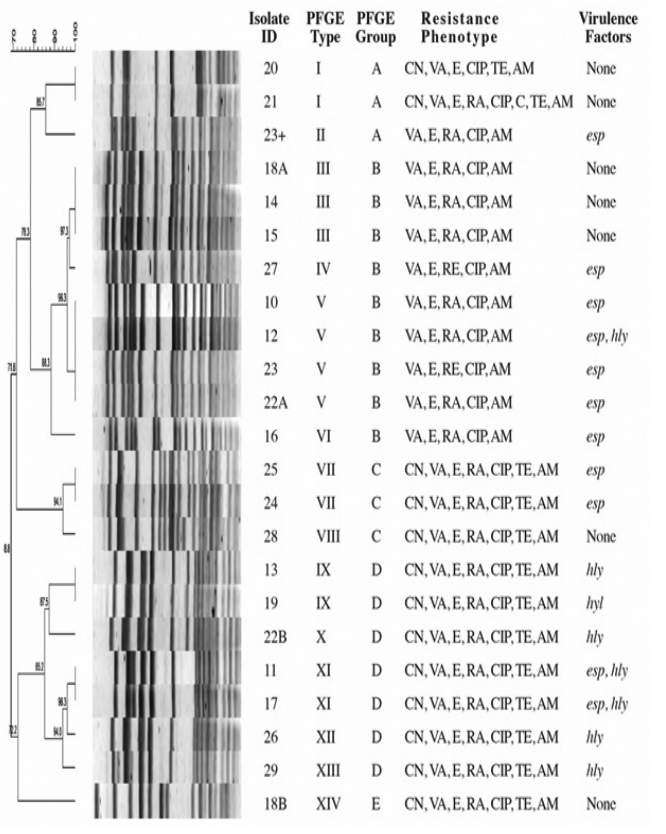
All VRE*fae* isolates carried the *van*A gene. Out of 23 VRE*fae* isolates, 17 (73.9%) were positive for virulence genes examined. Of the virulence genes examined, only *esp* and *hly* genes were observed among the isolates. The most common virulence gene was *esp*, which was detected in nine isolates 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 VRE*fae* 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



Isolate ID	PFGE Type	PFGE Group	Resistance Phenotype	Virulence Factors
20	I	A	CN, VA, E, CIP, TE, AM	None
21	I	A	CN, VA, E, RA, CIP, C, TE, AM	None
23+	II	A	VA, E, RA, CIP, AM	*esp*
18A	III	B	VA, E, RA, CIP, AM	None
14	III	B	VA, E, RA, CIP, AM	None
15	III	B	VA, E, RA, CIP, AM	None
27	IV	B	VA, E, RE, CIP, AM	*esp*
10	V	B	VA, E, RA, CIP, AM	*esp*
12	V	B	VA, E, RA, CIP, AM	*esp, hly*
23	V	B	VA, E, RE, CIP, AM	*esp*
22A	V	B	VA, E, RA, CIP, AM	*esp*
16	VI	B	VA, E, RA, CIP, AM	*esp*
25	VII	C	CN, VA, E, RA, CIP, TE, AM	*esp*
24	VII	C	CN, VA, E, RA, CIP, TE, AM	*esp*
28	VIII	C	CN, VA, E, RA, CIP, TE, AM	None
13	IX	D	CN, VA, E, RA, CIP, TE, AM	*hly*
19	IX	D	CN, VA, E, RA, CIP, TE, AM	*hyl*
22B	X	D	CN, VA, E, RA, CIP, TE, AM	*hly*
11	XI	D	CN, VA, E, RA, CIP, TE, AM	*esp, hly*
17	XI	D	CN, VA, E, RA, CIP, TE, AM	*esp, hly*
26	XII	D	CN, VA, E, RA, CIP, TE, AM	*hly*
29	XIII	D	CN, VA, E, RA, CIP, TE, AM	*hly*
18B	XIV	E	CN, VA, E, RA, CIP, TE, AM	None

Figure 1. Dendogram showing the results of *sma*I PFGE 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

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 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 to 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 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 VRE*fae* 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*.

Conclusion

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 VRE*fae* 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.

Ethics

Ethics Committee Approval: As VRE strains, which were isolated before, are used in the study (due to being a retrospective study), ethics committee decision is not taken.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: F.S., Ö.A., Design: F.S., Ö.A., Data Collection or Processing: Ö.A., F.B., Analysis or Interpretation: Ö.A., Literature Search: Ö.A., Writing: Ö.A., F.S.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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