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Epidemiological linkage of vancomycin-resistant Enterococcus faecium from different sources in Ahwaz, Iran

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One sentence summary: Epidemiological linkage of VREfm

ABSTRACT

This study was set to determine the genetic linkage and the clonal relationship between vancomycin-resistant Enterococcus faecium (VREfm) isolates in three hospitals of Ahwaz city. In this study, 1050 samples were collected from various rectal swabs, hands of health care workers, environmental surfaces, medical equipment and 146 enterococci isolates from clinical sources of three hospitals from March to September 2015. Antimicrobial resistance patterns in VREfm were detected by disk diffusion method. Genetic linkages of VREfm strains were investigated by pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) methods. Out of 366 enterococcal isolates, 163 Enterococcus faecium isolates were found to be resistant to vancomycin. PFGE and MLST analysis showed the presence of 79 pulsotypes and 11 sequence types (ST), respectively. In total, 90% of the isolates belonged to clonal complex 17 (CC17). Three new STs were reported for the first time in this study and ST80 was the predominant ST. We found a high prevalence of diverse VREfm with threatening antibiotic resistance patterns in all the studied sources with the dominance of CC17 VREfm strains in Ahwaz hospitals. Also, the results of typing method showed inter- and intra-hospital circulation of VREfm and similar pulsotypes and STs among different sources.

Keywords: vancomycin-resistant Enterococcus faecium; PFGE; MLST; different sources

INTRODUCTION

Enterococci are opportunistic pathogens living in human and animals’ gastrointestinal tract. Although most enterococcal infections are caused by Enterococcus faecalis, vancomycin resistance is more common in Enterococcus faecium strains (Higuita and Huycke 2014). The epidemiological importance of E. faecium
is that it is not only intrinsically insensitive to some antimicrobials, but also able to obtain antimicrobial-resistant genes such as fluoroquinolones, aminoglycosides (high level), ampicillin and vancomycin (Leclercq et al. 2013). In recent years, vancomycin-resistant Enterococcus (VRE) has emerged as a major public health concern in many parts of the world (Emaneini et al. 2016).

The acquisition of resistance genes by *E. faecium* presents a serious challenge to clinicians in treating enterococcal infections. Furthermore, resistant clones can spread within hospitals as well as between cities or countries (Humphreys 2014). It is known that the major vancomycin-resistant Enterococcus faecium (VREfm) isolates causing hospital infection in the world belong to a particular clonal complex designated as ‘clonal complex 17’ (CC17). Clones belonging to the CC17 are mostly resistant to ampicillin and ciprofloxacin and possess a pathogenicity island carrying virulence genes such as esp and hyl which altogether increase their fitness with the hospital environment (Cetinkaya, Falk and Mayhall 2000; Lee et al. 2013; Hammerum et al. 2017).

Different studies have shown the important role of medical equipment, the environment surrounding the patient and intestinally colonized patients in the spread of VREfm in hospitals. These results have been obtained by using accurate and fast typing methods such as pulse field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Talebi et al. 2008; Lee et al. 2013; Shokohizadeh et al. 2017).

Studies in Iran show an increasing incidence of VREfm infections in Iranian hospitals (Shokohizadeh et al. 2013; Talebi, Sadeghi and Poursaheb 2014). However, there is little information available about the major sequence types (STs) of VREfm and the clone types from different sources have not been compared much (Shokohizadeh et al. 2017).

This study was set to determine: 1. the frequency of Enterococcus in clinical and environmental specimens as well as those from rectal swabs and hands of health care workers, 2. Antimicrobial resistance patterns and 3. The clonal relatedness between VREfm isolates from different sources in three major teaching hospitals in Ahvaz, southwest of Iran, using PFGE and MLST. Furthermore, the dominant STs of VREfm isolates were determined.

**MATERIAL AND METHODS**

**Enterococcus faecium isolates**

A total of 800 environmental samples (from beds, bed sheets, tables, stations, cell phones, ventilators, suction and nebulizers), 200 rectal swab samples of patients and 50 swabs from the hands of health care workers were collected from different wards of three major hospitals in Ahvaz from March to September 2015. Furthermore, a total of 146 enterococcal isolates from clinical sources (urine, blood, endotracheal tube aspiration, wound and abscess) were collected from patients with nosocomial infections according to the standard set by the Center for Disease Control (Atlanta, USA) definitions (Garner et al. 1988). Rectal swabs were collected from hospitalized patients in the studied wards.

All samples were cultured on bile-esculin agar (Merck, Germany) and blood agar (Merck, Germany) at 37°C for 24–48 h. All enterococci isolates were identified as previously described by Collin and Facklam (Facklam and Collins 1989). Enterococcus faecium and E. faecalis isolates were confirmed by polymerase chain reaction using ddl genes (Facklam and Collins 1989).

**Vancomycin-resistant isolates**

VREfm strains were detected by disk diffusion method according to CLSI guidelines (2016) and growth in the bile-esculin agar containing 6 mg/ml vancomycin (Sigma Aldrich, Germany). The minimum inhibitory concentrations of vancomycin and teicoplanin were determined by E-test (Liofilchem, Italy). The genes encoding resistance to vancomycin (vanA and vanB) were also identified by a duplex polymerase chain reaction (Kariyama et al. 2000).

**Antimicrobial susceptibility testing**

The antimicrobial susceptibility of the VREfm isolates to 10 antibiotics including vancomycin (30 µg), teicoplanin (30 µg), gentamicin (120 µg), ampicillin (10 µg), erythromycin (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg) and linezolid (30 µg) was determined by disk diffusion method in our previous study (Arshadi et al. 2018).

**Pulsed-field gel electrophoresis**

PFGE technique was used to analyze the genetic linkage among VREfm isolates from different sources. Agarose plugs containing the genomic DNAs of the isolates were prepared in low-melting agarose. Small slices of the agarose plugs were used to be digested by Smal restriction enzyme (Thermo Scientific, Lithuania). PFGE was performed in CHEF-DRIII (Bio-Rad, US) apparatus as described previously (Talebi et al. 2008). The band patterns of PFGE were analyzed by a trial version of Gelcompar II software (version 6.6) in which band patterns are compared by Dice method and clustered by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and interpreted using the guidelines proposed by Tenover et al. (Tenover et al. 1995).

**Multilocus sequence typing**

The STs of VREfm isolates in Ahvaz hospitals were determined by MLST. A total of 21 VREfm isolates were designated for analysis by MLST based on PFGE patterns (one isolate per common type was selected for MLST analysis). MLST for *E. faecium* was performed using the scheme suggested by Homan et al. (Homan et al. 2002). The internal fragments of seven housekeeping genes (*atpA, ddl, gdh, purK, glyd, pstS* and *adk*) of *E. faecium* were amplified by polymerase chain reaction and were sequenced. Allele numbers and STs were detected according to the instructions in the online MLST database of *E. faecium*. Phylogenetic analysis of the isolates was performed by eBURST (http://eburst.mlst.net/v3/instructions/).

**Ethics approval and consent to participate**

The present study was ethically approved by the Institutional Review Board of Tehran University of Medical Sciences (Code No: IR.TUMS.REC.1394.1671). Permission was granted from the Tehran University of Medical Sciences and the hospitals to access the samples.

**RESULTS**

A total of 383 enterococci isolates were collected from clinical and environmental samples as well as from rectal swabs and...
hands of health care workers. Among 383 isolates, 61% (n = 233), 35% (n = 133) and 4% (n = 17) isolates were identified as E. faecium, E. faecalis and other species of enterococci. In the present study, E. faecium (64%) showed a higher rate than E. faecalis (35%). The frequency of E. faecium, E. faecalis and other enterococci in different sources from the studied hospitals has been compared in Table 1.

According to antimicrobial susceptibility testing results, 45.6% (167) of the isolates showed resistance to vancomycin. Among 167 VRE isolates, 163 belonged to E. faecium and 4 belonged to E. faecalis. The frequency of VREfm isolates has been shown in Fig. 1 according to the sources of samples and the studied hospitals.

Minimum inhibitory concentration values for vancomycin and teicoplanin ranged from 64 to 256 µg/ml and from 16 to 256 µg/ml, respectively, in all sources. All vancomycin-resistant isolates harbored the vanA gene and vanB was not detected in any VRE isolate. More than 90% of the VRE isolates in different sources showed resistance to ampicillin, ciprofloxacin and erythromycin. Simultaneous resistance to teicoplanin, ampicillin, ciprofloxacin, erythromycin, gentamicin, tetracycline and chloramphenicol was the most frequent (25%) antibiotic resistance pattern among VRE isolates.

The analysis of 104 VREfm isolates with a similarity cut-off of 80% determined by PFGE revealed 79 pulatypes which comprised 21 common types or CT and 58 single types. The results of this study showed inter- and intra-hospital dissemination of some clones of VREfm from different sources with similar pulotypes among clinical, environmental, hands of health care workers and rectal swab VREfm isolates (Fig. 2).

Clonal diversity among VREfm isolates was also confirmed by MLST, as 11 different STs were identified. Most isolates (90.4%, n = 19) belonged to CC17. MLST data analysis by eBURST showed that these isolates were placed in three large lineages: ST17, ST18 and ST117. ST80 and ST117 were detected in 24 and 14.3% of the isolates. ST121, ST169 and ST1338 were singletons. A total of three new STs (ST13338, ST13339 and ST1340) were also detected and submitted to MLST database of E. faecium. Isolates with the same ST (e.g. ST80 and ST18) showed different PFGE patterns and sources. However, ST80 from similar hospitals showed the same virulence genes patterns (Table 2).

DISCUSSION

In the 1980s and early 1990s, more than 90% of enterococcal infections were caused by E. faecalis and only 10% were caused by E. faecium (Treichman et al. 2005). In contrast, nowadays, 38–75% of Enterococcus infections are associated with E. faecium (Chiang et al. 2007; Hidron et al. 2008). This shift is due to the fact that resistance to ampicillin and vancomycin is more common in E. faecium isolates than in E. faecalis isolates (Sharifi et al. 2012). So far, several studies have been conducted on the prevalence of enterococci in Iran, most of which have been conducted on clinical sources. The prevalence of E. faecium in Iranian hospitals has increased in recent years. In a study conducted in Iran, the ratio of E. faecalis to E. faecium showed a decrease, reaching 1.2–1 (53 versus 44%), which was higher than the results of other reports (Shokohizadeh et al. 2013). In our study, the ratio of E. faecium to E. faecalis was 1.8–1. The increase in the ratio of E. faecium to E. faecalis was observed in isolates from all sources.

Many studies have been conducted around the world on the prevalence of vancomycin-resistant enterococci. According to a report from US hospitals, there is a high prevalence of VREfm isolates, with 80% of E. faecium clinical isolates resistant to vancomycin and 90% resistant to ampicillin. The level of resistance to vancomycin in Asia is not as high as in the United States, probably due to its recent occurrence in this continent. The VREfm isolates have increased in recent years in Iran (Shokohizadeh et al. 2013). The results of our study indicate an increase in VREfm isolates compared to some studies conducted in Iran (Emaneini et al. 2016). In this study, 45% of the isolates were identified as VRE including resistant isolates from all sources. Since enterococci is one of the most important factors in the incidence of nosocomial infection, the samples were taken from patients hospitalized for more than 3 days in the hospital and mainly in high-risk wards such as ICU and nephrology.

High levels of resistance to ampicillin and ciprofloxacin in VREfm isolates collected from all sources are important because this phenotype is one of the major indexes of the presence of VREfm in hospitals.

In early studies using the PFGE technique, especially in hospitals in the United States, the prevalence and outbreak of VRE isolates was clonal; one or two of the enterococci clones were the cause of infections in the hospitals. However, in recent years, polyclonal distribution has been observed in most hospitals in the United States, Europe, Asia and the Middle East (Kayser 2003; Willems and Van Schaik 2009). According to reports published in Iran, the distribution of polyclonal isolates, especially VRE isolates, has been observed in Iranian hospitals (Talebi et al. 2008; Saif et al. 2009; Shokohizadeh et al. 2013; Arshadi et al. 2017).

The presence of different clones of VRE isolates and their frequent emergence in hospitals and the high rate of transmission of antibiotic-resistant genes to E. faecium clones in hospitals can play a role in the genetic variation of these isolates. As indicated in the results section, PFGE results indicate the inter- and intra-hospital distribution of VREfm clones. Also, among the common clones, some isolates were obtained at different times which could be due to the fact that the isolates were stable and could have been circulating for a long time in the hospital contaminating different parts of the hospital. Also, given that 71% of the common types were derived from isolates of different origins, the transmission of resistant isolates was likely to occur over time, interacting with contaminated surfaces or the hands of the health care workers. Our hospitals are among the general hospitals in the province which admit patients from Ahvaz and also from neighboring provinces.

In the present study, 57% of the common types were detected in rectal swab specimens which can indicate the important role of gastrointestinal colonization of people with VRE in the spread of infection, especially in high risk populations. Since in Ahvaz hospitals, like other hospitals in Iran, patients are not screened for VRE before and during hospitalization, implementing national and regional guidelines is crucial for controlling the distribution of VRE from gastrointestinal colonization in hospitals.

Based on the results of MLST, CC17 E. faecium has been reported as the dominant clone in hospitals worldwide (Donskey et al. 2000; Titze-De-Almeida et al. 2006).

In this study, according to the results of MLST and their analysis by eBURST algorithm, 90% of the isolates of E. faecium belong to the CC17 which is highly consistent with hospital-adapted clones (Willems et al. 2005). In addition, all isolates belonging to the CC17 were resistant to ciprofloxacin, ampicillin and gentamicin.

In the present study, PFGE and MLST methods showed a high genetic diversity among VREfm isolates. The isolates with different pulotypes were examined by the MLST method and in some
Table 1. The frequency (N) of Enterococcal isolates in different sources from the studied hospitals.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sample size</th>
<th>E. faecium</th>
<th>E. faecalis</th>
<th>Sample size</th>
<th>E. faecium</th>
<th>E. faecalis</th>
<th>Sample size</th>
<th>E. faecium</th>
<th>E. faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical</td>
<td>150</td>
<td>35</td>
<td>27</td>
<td>136</td>
<td>30</td>
<td>15</td>
<td>127</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>75</td>
<td>25</td>
<td>23</td>
<td>60</td>
<td>20</td>
<td>11</td>
<td>65</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Environmental</td>
<td>250</td>
<td>18</td>
<td>8</td>
<td>300</td>
<td>26</td>
<td>10</td>
<td>250</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>Health care workers</td>
<td>20</td>
<td>6</td>
<td>0</td>
<td>15</td>
<td>5</td>
<td>1</td>
<td>15</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 1. The frequency (N) of VREfm from different sources in studied hospitals.

Table 2. Genotypic characteristics of VREfm isolates from three university hospitals based on PFGE and MLST.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Ward</th>
<th>GM</th>
<th>AMP</th>
<th>CIP</th>
<th>ST</th>
<th>PT</th>
<th>Source</th>
<th>Hospital</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>Neuro ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>80</td>
<td>CT14</td>
<td>Rectal</td>
<td>Imam</td>
</tr>
<tr>
<td>7</td>
<td>ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>121</td>
<td>CT3</td>
<td>Urine</td>
<td>Golestan</td>
</tr>
<tr>
<td>5</td>
<td>ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1338</td>
<td>CT20</td>
<td>Catheter</td>
<td>Abouzar</td>
</tr>
<tr>
<td>79</td>
<td>ICU2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>80</td>
<td>CT16</td>
<td>Rectal</td>
<td>Golestan</td>
</tr>
<tr>
<td>65</td>
<td>Internal ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1340</td>
<td>CT4</td>
<td>Urine</td>
<td>Imam</td>
</tr>
<tr>
<td>74</td>
<td>Internal ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1339</td>
<td>CT2</td>
<td>Nurse station</td>
<td>Imam</td>
</tr>
<tr>
<td>60</td>
<td>ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1301</td>
<td>CT1</td>
<td>Urine</td>
<td>Abouzar</td>
</tr>
<tr>
<td>50</td>
<td>Nephrology</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>761</td>
<td>CT11</td>
<td>Rectal</td>
<td>Abouzar</td>
</tr>
<tr>
<td>24</td>
<td>ICU1</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1340</td>
<td>CT17</td>
<td>Vent</td>
<td>Golestan</td>
</tr>
<tr>
<td>23</td>
<td>ICU2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>80</td>
<td>CT13</td>
<td>Health care workers</td>
<td>Golestan</td>
</tr>
<tr>
<td>19</td>
<td>Internal ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>80</td>
<td>CT15</td>
<td>Rectal</td>
<td>Imam</td>
</tr>
<tr>
<td>16</td>
<td>ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>761</td>
<td>CT8</td>
<td>Blood</td>
<td>Abouzar</td>
</tr>
<tr>
<td>8</td>
<td>Nephrology</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1301</td>
<td>CT10</td>
<td>Health care workers</td>
<td>Abouzar</td>
</tr>
<tr>
<td>3</td>
<td>ICU Surgery</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>169</td>
<td>CT9</td>
<td>Rectal</td>
<td>Imam</td>
</tr>
<tr>
<td>132</td>
<td>Nephrology</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>80</td>
<td>CT21</td>
<td>Urine</td>
<td>Imam</td>
</tr>
<tr>
<td>42</td>
<td>Nephrology</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>117</td>
<td>CT5</td>
<td>Vent</td>
<td>Abouzar</td>
</tr>
<tr>
<td>44</td>
<td>ICU1</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>117</td>
<td>CT6</td>
<td>Health care workers</td>
<td>Golestan</td>
</tr>
<tr>
<td>68</td>
<td>Nephrology</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>18</td>
<td>CT7</td>
<td>Urine</td>
<td>Golestan</td>
</tr>
<tr>
<td>80</td>
<td>ICU2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>132</td>
<td>CT12</td>
<td>Rectal</td>
<td>Golestan</td>
</tr>
<tr>
<td>85</td>
<td>Nephrology</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>117</td>
<td>CT18</td>
<td>Rectal</td>
<td>Abouzar</td>
</tr>
<tr>
<td>97</td>
<td>Neuro ICU</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>18</td>
<td>CT19</td>
<td>Urine</td>
<td>Golestan</td>
</tr>
</tbody>
</table>

Neuro ICU: Neurosurgery ICU; GM: Gentamicin; AMP: Ampicillin; CIP: Ciprofloxacin; ST: Sequence type; PT: Pulsotype; Vent: Ventilator

In our study, ST80 was known as the dominant ST. This ST is included in CC17 and has been detected in samples of patients admitted to European and Asian countries such as the UK, Denmark, Sweden and South Korea (Billström et al. 2010; Cha et al. 2013; Pinholt et al. 2015; Brodrick et al. 2016; Lee et al.).
ST117 was the second most common ST recovered in this research. Based on the epidemiological data at PubMLST, ST117 was detected in the clinical samples of hospitalized patients in Germany in 1996. In 2003–2009 and 2014, ST117 was reported from clinical isolates of hospitalized patients in Turkey and Netherlands, respectively (Arslan et al. 2013; Zhou et al. 2018). According to the results of a research in Iran, MLST analysis revealed the presence of 15 different subtypes of VREfm isolated from Tehran hospitals, 86% of which belonged to CC17 and 50% were associated with ST203 (Shokoohizadeh et al. 2017). In conclusion, the results of this study showed a higher incidence of E. faecium than E. faecalis in all the studied sources as well as a high prevalence of VREfm isolates in samples collected from all sources including clinical and environmental samples (surfaces and equipment) as well as rectal colonization and health care workers’ hands. The results of PFGE and MLST in this study indicate the presence of hospital-adapted E. faecium clones in clinical, gastrointestinal colonized and environmental isolates of E. faecium, as well as inter- and intra-hospital distribution of vancomycin-resistant strains with high risk resistance patterns and the majority of CC17. In order to reduce the risk of contamination in hospitals with high risk clones of E. faecium, it is suggested that 1) a regional protocol and then a national protocol be implemented to identify gastrointestinal colonization in patients with VREfm at admission and hospitalization, 2) coherent monitoring plans track the status of resistant isolates in high-risk hospital wards such as ICUs and nephrology, 3) appropriate typing methods be applied and launched for epidemiological studies on hospital-resistant strains by academic research centers or provincial reference laboratories, 4) the surfaces and equipment in different parts of the hospital be disinfected and properly cleaned and 5) more effective communication exist between the clinic and the laboratory in order to prescribe the antimicrobials logically according to the results of standard antimicrobial sensitivity tests.

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Conflict of interest. None declared.

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