Molecular characterization of vancomycin-resistant *Enterococcus faecium* strains isolated from carriage and clinical samples in a tertiary hospital, Turkey

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This study aimed to determine the presence of vancomycin resistance (*vanA* and *vanB*) and virulence genes (*esp*, *asa1*, *gelE*, *ace*, *hyl*, *cylA*, *cpd* and *ebpA*) in vancomycin-resistant *Enterococcus faecium* (VRE*fm*) strains and to analyse the clonal relationships among the strains. *E. faecium* strains were identified from rectal and clinical specimens by biochemical tests and the API-20 Strep kit. Susceptibility testing was performed using disc-diffusion and broth-dilution methods. PFGE was used for molecular typing of the VRE*fm* strains. The vancomycin resistance and virulence genes were amplified by two-step multiplex PCR. All 55 VRE*fm* isolates were resistant to penicillin G, ampicillin and high-level gentamicin but were susceptible to quinupristin/dalfopristin and linezolid. Multiplex PCR analysis indicated that all isolates harboured *vanA* and that 41 (75 %) were positive for virulence genes. The *esp* gene was the most common virulence factor and was detected in nine (41 %) invasive and 32 (96.7 %) non-invasive isolates. Multiple virulence genes were observed only in two non-invasive isolates; one harboured *esp* and *ebpA* and the other harboured *esp*, *ebpA*, *asa1*, *gelE* and *cpd*. PFGE typing yielded 16 different types, seven of which were clusters with two to 14 strains each. The clustering rates of the rectal swab, blood and urine isolates were 72.7 %, 61.5 % and 87.5 %, respectively. The genetic similarity observed among the VRE*fm* isolates indicated cross-transmission in the hospital. Further studies on the virulence factors present in the strains might provide insight into the acquisition of these traits and their contribution to increased prevalence of VRE*fm*.

INTRODUCTION

Enterococci are facultatively anaerobic, Gram-positive cocci, which are found in the human and animal gastrointestinal tract and frequently cause opportunistic infections in the hospital settings. *Enterococcus faecalis* and *Enterococcus faecium* are reported to be the third to fourth most prevalent nosocomial pathogens worldwide and this emergence has paralleled their development of antibiotic resistance (Werner *et al.*, 2008; Cattoir & Leclercq, 2013). Since

1980, these micro-organisms, especially *E. faecium*, have been a cause for concern due to limiting antimicrobial therapeutic options (Bourdon *et al.*, 2011; Cattoir & Leclercq, 2013); they have become increasingly resistant to many antimicrobial agents, including penicillin, aminoglycosides (high-level resistance) and glycopeptides (vancomycin-resistant enterococci; VRE). Vancomycin resistance is mainly due to the acquisition of *vanA* and to a lesser extent the *vanB* gene, which have been primarily detected in *E. faecium* (Werner *et al.*, 2008).

Gastrointestinal carriage is the major reservoir of VRE in hospitals, and once it colonizes, the organism might remain for

Abbreviations: CSF, cerebrospinal fluid; VRE, vancomycin-resistant enterococci; VRE*fm*, vancomycin-resistant *Enterococcus faecium*

weeks or even months after the patients have been discharged from the facilities (Orsi & Ciorba, 2013). VRE can be transmitted directly by patient-to-patient contact or indirectly via the hands of healthcare workers, contaminated medical equipment and environmental surfaces (Bourdon *et al.*, 2011; Orsi & Ciorba, 2013). Therefore, infection monitoring is the most important strategy for preventing colonization and limiting hospital spread of VRE (Orsi & Ciorba, 2013; De Angelis *et al.*, 2014). Molecular typing methods based on analysis of chromosomal DNA provide very useful information about the epidemiology of VRE infections and also for the development of effective strategies to limit the spread of VRE (Chuang *et al.*, 2010). Typing methods such as random-amplified PCR, DNA restriction fragment analysis, ribotyping, PFGE and DNA sequencing that vary in their reproducibility and discriminatory abilities are frequently used. PFGE has been reported to be superior to the other methods and is thus considered to be the gold standard for typing of a variety of bacterial species including enterococci (Bourdon *et al.*, 2011; Ranjbar *et al.*, 2014).

Several genes encoding virulence factors including *esp* (enterococcal surface protein), *asa1* (aggregation substance), *gelE* (gelatinase), *ace* (collagen-binding protein), *hyl* (hyaluronidase), *cylA* (cytolysin), *cpd* (sex pheromone determinant) and *ebpA* (endocarditis and biofilm-associated pilus subunit A) contribute to colonization and infection by enterococci (Shankar *et al.*, 1999; Vankerckhoven *et al.*, 2004). Many of these virulence factors have also been suggested to play a role in the virulence of *E. faecium* (Mannu *et al.*, 2003; Top *et al.*, 2008; Hällgren *et al.*, 2009; Kafil *et al.*, 2013).

The objectives of our study were (i) to investigate vancomycin resistance and associated virulence genes and (ii) to determine the clonal relationships among a total of 55 vancomycin-resistant *Enterococcus faecium* (VREfm) isolates (rectal swab and clinical isolates) collected from patients in a tertiary hospital.

METHODS

Study group. Clinical and rectal swab samples were taken from patients hospitalized between February 2012 and April 2013 at Ankara Atatürk Education and Research Hospital; this hospital serves both outpatients and inpatients with a total capacity of 522 beds. Rectal swab specimens were collected from all inpatients at the time of admission and repeated monthly. In case of VRE positivity, if the patient had no clinical findings, it was defined as rectal VRE colonization and the case was followed up weekly according to the institutional VRE surveillance program.

Enterococcal isolates. For VRE screening, rectal samples were cultured on Enterococcosel agar plates containing 6 μg vancomycin ml^{-1} (Becton Dickinson). After incubation at 35 °C for 24 h, colonies suspected of being of VRE were subcultured onto 5% sheep blood agar plates. Clinical samples such as blood, cerebrospinal fluid (CSF), pleural fluid, peritoneal fluid and urine were inoculated into convenient culture media including blood culture bottles or 5% sheep blood agar, and onto chocolate or eosin-methylene-blue agar plates; these were examined after 24–48 h of incubation at 35 °C. The colonies were identified as *Enterococcus* spp. based on their colony morphology, Gram-positive staining of cocci, catalase positivity, L-pyrrolidonyl-β-naphthylamidase activity, aesculin hydrolysis in the presence of 40% bile salts and growth in 6.5% sodium chloride (Teixeira *et al.*, 2011). Further identification was performed by using an API-20 Strep kit test (bioMérieux).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar (Oxoid) according to current Clinical and Laboratory Standards Institute (CLSI) guidelines. Penicillin G, ampicillin, high-level gentamicin and quinupristin/dalfopristin were tested. For urine isolates, nitrofurantoin, levofloxasin and tetracycline were also tested. The MICs of vancomycin were determined by the broth dilution method as given in the CLSI guidelines. Vancomycin MIC values ≤4 μg ml^{-1} were classified as susceptible, values ranging between 8 and 16 μg ml^{-1} were classified as moderately susceptible, and MIC values ≥32 μg ml^{-1} were classified as resistant (CLSI, 2015). The study protocol was approved by the Ethics Committee of the institution (number: 26379996/52).

PCR detection of vancomycin resistance and virulence genes. Two vancomycin resistance-associated genes, *vanA* and *vanB*, and eight virulence-associated genes, *esp*, *asa1*, *gelE*, *ace*, *hyl*, *cylA*, *cpd* and *ebpA*, were detected by two-step multiplex PCR using primers (Table 1) that were used in previous studies (Dutka-Malen *et al.*, 1995; Elsner *et al.*, 2000; Creti *et al.*, 2004; Depardieu *et al.*, 2004; Vankerckhoven *et al.*, 2004; Bourgogne *et al.*, 2007). Well-characterized VRE strains, and clinical and faecal isolates grown on 5% sheep blood agar were suspended in 1.0 ml of saline solution (0.85% NaCl) and the turbidity was adjusted to McFarland standard 1. Then, 100 μl of cell suspensions was pelleted, resuspended in 100 μl TE (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) buffer, and boiled for 5 min to prepare the template DNAs. Firstly, 5 μl cell lysates of reference strains harbouring *vanA*, *vanB*, *ace*, *asa1*, *cyl*, *cpd*, *ebpA*, *esp*, *gelE* and *hyl* genes were individually amplified with the corresponding primer pairs (5 pmol each) using HotStar *Taq* master mix (Qiagen) in a final volume of 25 μl. Monoplex PCR cycles were performed in a Corbett model thermocycler (Corbett Life Science) with an initial denaturation step at 95 °C for 15 min followed by 30 cycles at 95 °C for 50 s, an annealing step at 54 °C for 50 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Secondly, two multiplex PCR mixtures were prepared using HotStar *Taq* master mix, and cycling conditions were optimized to amplify the resistance and virulence genes at two steps. Multiplex PCR (mPCR) mix-I contained *vanA* (10 pmol) and *vanB* primers (3 pmol), whereas mPCR mix-II contained *ace*, *asa1*, *cyl*, *cpd*, *ebpA*, *esp*, *gelE* and *hyl* primers (in the range of 1.5 to 10 pmol). After optimization, 5 μl of cell lysate of each clinical and rectal VRE isolate was mixed with 20 μl of mPCR mix-I and mPCR mix-II separately and multiplex PCR cycling was performed under the following conditions: 95 °C for 15 min followed by 30 amplification cycles of 95 °C for 50 s, 56 °C for 50 s, 65 °C for 2.5 min, and 65 °C for 10 min. Amplicons were then analysed on 3% Nusieve agarose gel (Gamma micropor) containing ethidium bromide. Following gel electrophoresis at 150 V for 3 to 5 h, the images were recorded.

PFGE typing. PFGE was performed as described by Morrison *et al.* (1999). Briefly, bacterial cells embedded in 1.6% low-melting-point agarose (Gibco-BRL) plugs were lysed with lysozyme and proteinase K, and then chromosomal DNA was digested with 40 U *Sma*I (Fermentas). Fragmented DNA samples were electrophoresed in 1% pulsed-field certified agarose (Bio-Rad) using a CHEF-DR II system (Bio-Rad) with 5–30 s pulse times, for 20 h at 14 °C at 6 V cm^{-2} (Turabelidze *et al.*, 2000). The gel was stained with ethidium bromide (5 μg ml^{-1}), visualized under UV light, and photographed using a gel logic 2200 imaging system (resolution: 1708 × 1280 pixel; Kodak). The DNA band profiles were analysed with GelCompar software (version 3.0; Applied Maths). Band tolerances of 1.5% and 1% normalization were used for comparison of DNA profiles.

Table 1. Primers used for PCR detection of genes encoding vancomycin resistance and other virulence factors

Gene	Sequence (5′→3′)	Amplicon size (bp)	Reference
vanA	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Dutka-Malen *et al.* (1995)
vanB	ACGGAATGGGAAGCCGA TGCACCCGATTTCGTTC	647	Depardieu *et al.* (2004)
cpd	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	782	Elsner *et al.* (2000)
cyl	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	Vankerckhoven *et al.* (2004)
ace	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616	Creti *et al.* (2004)
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	Vankerckhoven *et al.* (2004)
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	Vankerckhoven *et al.* (2004)
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276	Vankerckhoven *et al.* (2004)
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	Vankerckhoven *et al.* (2004)
ebpA	AAAAATGATTCGGCTCCAGAA TGCCAGATTCGCTCTCAAAG	101	Bourgogne *et al.* (2007)

Since *Apa*I was reported as a useful restriction enzyme for PFGE typing of VRE strains (Turabelidze *et al.*, 2000), we performed *Apa*I digestion in order to confirm the high clustering rate obtained by *Sma*I digestion. For this purpose, the DNA plugs of 20 isolates clustered in two large PFGE types (II and IV) were digested with 40 U *Apa*I (Fermantas) using the same plug preparation, dialysis and electrophoresis conditions applied in *Sma*I digestion.

RESULTS

VRE isolates and antimicrobial susceptibility testing

A total of 55 non-duplicate VRE*fm* isolates were included in this study. Twenty-two isolates (13 blood, 8 urine and 1 CFS) obtained from inpatients (14 females and 8 males) with symptomatic infections were accepted as invasive. Thirty-three isolates collected from rectal swab screening cultures of inpatients (20 females and 13 males) were accepted as non-invasive rectal colonization. While 73% of isolates were from intensive care units, the remaining 27% were from other units including haematology, oncology and gastroenterology.

The results from disc diffusion analysis showed that all isolates were resistant to penicillin G, ampicillin and high-level gentamicin, whereas all were susceptible to quinupristin/dalfopristin and linezolid. Urine isolates were also found to be resistant to nitrofurantoin, levofloxasin and tetracycline. All 55 isolates were resistant to vancomycin; 48 isolates had MIC values ≥ 32 μg ml^{-1} and the remaining seven had MIC values ≥ 256 μg ml^{-1}.

Multiplex PCR investigation for vancomycin resistance and virulence genes

The results indicated that all VRE*fm* isolates harboured the *vanA* gene. Of the 55 (22 clinical and 33 faecal) isolates, 41 (75%) were positive and 14 (25%) were negative for the virulence genes tested (Table 2). The *cyl*, *hyl* and *ace* virulence genes were not detected in any of the isolates. The *esp* gene was present in 75% (41/55) of all isolates. In particular, 41% (9/22) of clinical isolates and 96.7% (32/33) of faecal isolates were positive for *esp*. None of the clinical isolates carried multiple virulence factors, whereas faecal isolates possessed more than one virulence

Table 2. Incidence (*n*, %) of genes encoding virulence factors among *E. faecium* strains isolated from different clinical specimens

Virulence factor	Blood (*n*=13)	Urine (*n*=8)	CSF (*n*=1)	Faecal (*n*=33)	Total (*n*=55)
Negative	12 (92.3)	–	1 (100)	1 (3)	14 (25)
esp	1 (7.7)	8 (100)	–	30 (91)	39 (71)
esp+ebpA	–	–	–	1 (3)	1 (1.8)
esp+ebpA+ asa1+gelE+cpd	–	–	–	1 (3)	1 (1.8)

factor. One isolate had the *esp* and *ebpA* genes and the other possessed *esp*, *ebpA*, *asa*, *gelE* and *cpd* genes.

PFGE typing with *Sma*I and *Apa*I

PFGE typing of 55 VRE*fm* isolates using *Sma*I demonstrated that the clustering rate was 83.6% (46/55 isolates), and three PFGE groups (A, B and C) were recognized based on the application of a similarity coefficient higher than 85%. As shown in Fig. 1, group A contained the largest number of isolates, whereas only one isolate was recognized in each of groups B and C. Moreover, 16 distinct PFGE types were distinguished; seven of them (I, II, III, IV, VI, VII and XI) were clusters with a size ranging from two to 14 isolates, while the remaining nine types (V, VIII, IX, X, XII, XIII, XIV, XV and XVI) were unique as they were represented by only one isolate. Except for four faecal isolates grouped in type I and eight clinical isolates grouped in type VI, the faecal and clinical isolates did not show a particular allocation. As for invasive isolates, 13 blood isolates were distributed into eight different types: II, III, IV, VI, VII and XI were clusters comprising 11 blood isolates, while XIII and XVI were unique, comprising two

Isolate	PFGE type	PFGE group	Sampling date	Sample	Service	Virulence factor
25	I	A	23.07.2012	RS	Oncology	None
26	I	A	29.01.2013	RS	Brain Surgery ICU-1	esp
27	I	A	13.04.2012	RS	Reanimation ICU-2	esp
28	I	A	08.03.2013	RS	Transplantation ICU	esp
6a	II	A	17.01.2013	Blood	Transplantation ICU	None
18b	II	A	27.10.2013	RS	Reanimation ICU-1	esp
30b	II	A	15.02.2013	RS	Reanimation ICU-2	esp
30a	II	A	12.02.2013	Blood	Reanimation ICU-2	None
31	II	A	10.12.2012	Blood	Haematology	None
32b	II	A	10.08.2012	RS	Reanimation ICU-2	esp
33	II	A	11.07.2012	RS	Reanimation ICU-2	esp
34	II	A	20.07.2012	RS	Reanimation ICU-1	esp
35	II	A	13.08.2012	RS	Reanimation ICU-1	esp
36	II	A	06.02.2013	Blood	Reanimation ICU-2	None
37	II	A	12.10.2012	RS	Reanimation ICU-1	esp
38	II	A	27.10.2012	RS	Reanimation ICU-2	esp+ebpA
39	II	A	10.01.2013	RS	Reanimation ICU-2	esp
40	II	A	06.02.2013	RS	Brain Surgery ICU-1	esp
32a	III	A	09.07.2012	Blood	Reanimation ICU-2	None
41	III	A	13.07.2012	Blood	Reanimation ICU-2	None
1	IV	A	24.09.2012	RS	Infection	esp
2	IV	A	17.02.2012	RS	Reanimation ICU-1	esp
3	IV	A	10.12.2012	RS	Reanimation ICU-1	esp
4	IV	A	15.02.2013	RS	Reanimation ICU-1	esp
5	IV	A	08.12.2012	Blood	Coronary ICU	None
6b	IV	A	21.01.2013	RS	Transplantation ICU	esp
7a	IV	A	21.01.2013	Urine	Reanimation ICU-1	esp
7b	IV	A	21.01.2013	RS	Reanimation ICU-1	esp
8b	IV	A	01.02.2013	RS	Brain Surgery ICU-2	esp
9	IV	A	06.02.2013	RS	Reanimation ICU-1	esp
10a	IV	A	17.07.2012	Blood	Reanimation ICU-1	None
11	IV	A	10.08.2012	RS	Reanimation ICU-1	esp
29	V	A	15.03.2013	RS	Neurology ICU	esp+ebpA+asa1+gelE+cpd
16a	VI	A	11.11.2012	Urine	Neurology ICU	esp
18a	VI	A	17.11.2012	Urine	Reanimation ICU-1	esp
19	VI	A	05.08.2012	Urine	Infection	esp
20	VI	A	24.01.2013	Blood	Gastroenterology	esp
21	VI	A	05.03.2013	Urine	Infection	esp
22	VI	A	07.03.2013	Urine	Neurology	esp
23	VI	A	31.10.2012	Urine	Neurology ICU	esp
24	VI	A	08.11.2012	Urine	Internal Medicine	esp
16b	VII	A	05.11.2012	RS	Neurology ICU	esp
17	VII	A	25.07.2012	Blood	Reanimation ICU-2	None
13	VII	A	19.05.2012	RS	Reanimation ICU-1	esp
43	IX	A	05.04.2012	RS	Internal Medicine-Oncology	esp
42	X	A	17.02.2012	RS	Reanimation ICU-1	esp
8a	XI	A	09.02.2013	RS	Brain Surgery ICU-1	None
10b	XI	A	16.02.2013	RS	Reanimation ICU-1	esp
14b	XI	A	08.02.2013	RS	Reanimation ICU-2	esp
15	XI	A	21.09.2012	Blood	Transplantation ICU	None
46	XII	A	06.07.2012	RS	Oncology	esp
14a	XIII	A	23.01.2013	Blood	Reanimation ICU-2	None
45	XIV	A	21.06.2012	RS	Reanimation ICU-1	esp
44	XV	B	11.07.2012	RS	Reanimation ICU-2	esp
47	XVI	C	02.10.2012	Blood	Reanimation ICU-2	None

Fig. 1. Dendrogram of *Sma*I PFGE typing of 55 VRE*fm* isolates. PFGE types are indicated as Roman numerals. PFGE groups A, B and C comprised the isolates having a similarity coefficient ⩾85 %. Invasive and non-invasive isolates of the same patient are indicated as a and b, respectively. Numbers given on branches indicate cophenetic correlation coefficient values. ICU, intensive care unit; RS, rectal swab. The scale bar given on the top indicates similarity percentages detected for pulsotypes.

blood isolates. From a total of eight urine isolates, seven were clustered in type VI. The clustering rates of the blood and urine isolates were 61.5% and 87.5%, respectively. The single CSF invasive isolate clustered with blood and rectal swab isolates in type XI. As for the 33 non-invasive (rectal swab) isolates, 12 different PFGE types were distinguished. Twenty-six faecal isolates clustered in five types (I, II, IV, V, VII and XI) and the clustering rate was 72.7%. The rest of them were distributed as single isolates in seven types (V, VIII, IX, X, XII, XIV and XV). The 20 isolates that clustered in type II and IV with *Sma*I were retyped by *Apa*I and all of them clustered in the same type (Fig. 2).

PFGE typing and virulence gene types of isolates

As shown in Fig. 1, the results did not reveal a strong association between clustering profile and virulence gene content of isolates, especially at the group level. Among 55 VRE*fm* isolates, *esp* was the most common virulence gene and it was detected in 75% (41/55) of isolates regardless of their invasive or non-invasive status. However, both 41 *esp*-positive and 14 *esp*-negative isolates clustered in group A. It was noteworthy that at the type level, eight clinical isolates clustered in type VI and they were all positive for *esp*.
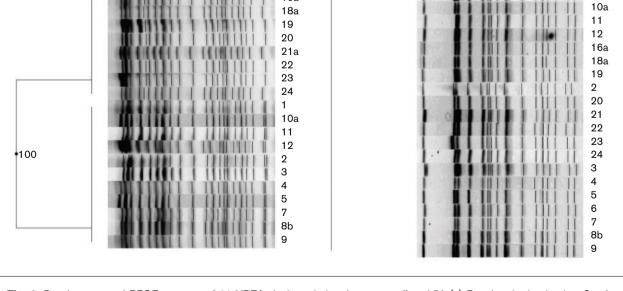
DISCUSSION

VRE were first identified as hospital-associated pathogens in Europe in the late 1980s (Leclercq *et al.*, 1988; Uttley *et al.*,

1988) and thereafter were found to be disseminated worldwide (Bourdon *et al.*, 2011; De Angelis *et al.*, 2014). The resistance rates reported to the National Healthcare Safety Network during 2009–2010 were between 62.3% and 82.6% for *E. faecium*, and between 6.2% and 9.8% for *E. faecalis* depending on the site of infection (Sievert *et al.*, 2013). In contrast to the USA data, during 2011 and 2012, VRE resistance remained low in Europe as the reported rates were 19.0% to 5.5% for *E. faecium* and *E. faecalis* in hospital settings, respectively (European Centre for Disease Prevention and Control Annual Epidemiological Report 2013, reporting on 2011 surveillance data and 2012 epidemic intelligence data; http://www.ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf). In Turkey, Basustaoglu *et al.* (2001) reported the first VRE*fm* in 2001. Subsequently, the first outbreak of VRE*fm* in a tertiary hospital was notified by Colak *et al.* (2002). Later on, other reports were published on VRE*fm* phenotype (Kilic *et al.*, 2005; Baylan *et al.*, 2011) and also on the low incidence of the VRE*fm vanB* genotype (Coşkun *et al.*, 2012; Altun *et al.*, 2014; Terkuran *et al.*, 2014). In our country, the VRE isolation percentage from intensive care units was notified as 21.2% in 2013 (http://www.shgm.saglik.gov.tr/dosya/1-88693/h/uhesa-analiz-2013.pdf).

PFGE typing is a cost-effective method, which provides information about the relatedness of bacterial isolates and has been extensively used for the surveillance of hospital-associated infections (Teixeira *et al.*, 2011). In our study, according to a similarity coefficient higher than 85%, 55 VRE*fm* isolates were distributed into three groups (A,

Fig. 2. Dendrogram and PFGE patterns of 20 VRE*fm* isolates belonging to type II and IV. (a) Results obtained using *Sma*I-digested DNAs. (b) Results obtained using *Apa*I-digested DNAs.

B and C) and 53 (96.4%) isolates belonging to group A were considered as closely related. The high rate (83.6%) of clustering and close relatedness of isolates indicated cross-transmission among different clinics including ICU-1 and ICU-2 in our hospital during February 2012 and February 2013. Although this high clonal relatedness was also confirmed by a second restriction enzyme, *Apa* I, the available epidemiological data did not show a link for some of the patients clustered in the same type. For instance, 14 VRE*fm* isolates clustered in the largest type (II) were isolated from five different units in a six-month period. The second largest type (IV) comprised 12 isolates collected from two blood, one urine and nine rectal swab samples from nine patients in five units during a period of 12 months. The type IV included eight invasive isolates from patients hospitalized in five different units, of which seven were urine isolates and one was a blood sample. All four isolates that clustered in type I were rectal swab isolates of patients from four different units. As reported previously by Yetkin *et al.* (2006), the observation of clonally related isolates among different clinics may suggest cross-transmission by the hands of health care staff and/or medical devices. Nevertheless, we do not have any data to substantiate this claim as the mode of VRE*fm* transmission was outside the scope of this study. As indicated in the literature, enterococcal infection might originate from both endogenous and exogenous sources (Teixeira *et al.*, 2011). In agreement with these data, we found that some of the VRE*fm* isolates from the stool of patients had the same DNA profiles as those of blood isolates. On the other hand, most of the PFGE profiles of the invasive isolates were not identical to those of the non-invasive isolates obtained from the same patients, suggesting exogenous sources.

The VRE*fm* isolates pose major therapeutic problems because the majority of these strains are also resistant to multiple antimicrobials (Cattoir & Leclercq, 2013). Bourdon *et al.* (2011) reported that around 95% of VRE*fm* isolates were resistant to ampicillin and erythromycin, and only around 22% were resistant to high levels of gentamicin. According to our results, 100% of our isolates carried the *vanA* gene and they were resistant to multiple antimicrobials except quinupristin/dalfopristin and linezolid, as determined by the disc diffusion method.

In the present study, 75% (41/55) of VRE*fm* isolates were positive for virulence genes and only 25% were clear of all the virulence genes tested. The *cylA*, *hyl* and *ace* genes were not detected and this is consistent with the findings of other studies (Duprè *et al.*, 2003; Vankerckhoven *et al.*, 2004; Billström *et al.*, 2008; Worth *et al.*, 2008; Hällgren *et al.*, 2009; Sharifi *et al.*, 2012). Nevertheless, high rates of *cylA*- or *hyl*-positive *E. faecium* isolates in humans have also been documented (Rice *et al.*, 2003; Cha *et al.*, 2012; Kwon *et al.*, 2012). In Turkey, studies on vancomycin resistance and virulence factors in VRE*fm* isolates of clinical origin have been reported previously (Baylan *et al.*, 2011; Terkuran *et al.*, 2014) and the findings

regarding these virulence factors differed from our observations.

Esp is a cell-wall-associated protein that promotes adhesion, colonization and evasion of the immune system and contributes to biofilm formation (Shankar *et al.*, 1999; Waar *et al.*, 2002). High prevalence of the *esp* gene in both invasive (53% to 92%) and non-invasive (61% to 81.5%) VRE*fm* isolates has been reported previously (Vankerckhoven *et al.*, 2004; Raad *et al.*, 2005; Worth *et al.*, 2008; Cha *et al.*, 2012). Similarly, we observed that *esp* was the most common virulence gene with a total prevalence of 75% (41/55) in VRE*fm* isolates (100% in urine and 96.7% in faecal isolates). In a recent study from Turkey, Terkuran *et al.* (2014) observed a high frequency of *esp* in urinary tract VRE*fm* isolates, whereas the intestinal isolates carried *esp* at very low rates. Nevertheless, a low incidence (6.5%) of the *esp* gene in urinary *E. faecium* isolates from Turkey has also been reported (Baylan *et al.*, 2011).

In our study, none of the clinical isolates harboured the *asa1* gene and this is consistent with other studies (Eaton & Gasson, 2001; Duprè *et al.*, 2003; Vankerckhoven *et al.*, 2004; Abriouel *et al.*, 2008; Hällgren *et al.*, 2009). In contrast to these data, 31.4% of *E. faecium* isolates collected from human samples were found to be *asa1*-positive in a study from Turkey (Terkuran *et al.*, 2014). We found that *asa1* was coexisting with *esp*, *ebpA*, *gelE* and *cpd* genes in only one faecal VRE*fm* isolate.

The incidence of the *gelE* gene in clinical isolates varied between 7.9% and 71% in previous studies (Sabia *et al.*, 2008; Terkuran *et al.*, 2014; Sharifi *et al.*, 2012). However, Vankerckhoven *et al.* (2008) did not find the *gelE* gene in any of their *E. faecium* isolates. Only one faecal VRE*fm* isolate harbouring *gelE* along with *esp*, *ebpA*, *asa1* and *cpd* genes was detected in our study.

The *ebp* (encoding endocarditis and biofilm-associated pili) operon consisting of *ebpA*, *ebpB*, *ebpC* and *srtC* genes has been shown to be important for biofilm formation and pathogenesis of enterococci (Budzik & Schneewind, 2006; Nallapareddy *et al.*, 2006; Singh *et al.*, 2007). The presence of *ebp* was observed in almost all clinical *E. faecalis* and *E. faecium* isolates (Kafil *et al.*, 2013). In our study, the clinical isolates were negative for *ebpA*. However, we detected the *ebpA* gene coexisting with one or more virulence genes in two faecal isolates.

As reported in several previous studies, the *E. faecium* isolates tested were devoid of multiple virulence factors (Sharifi *et al.*, 2012; Hällgren *et al.*, 2009; Terkuran *et al.*, 2014). On the other hand, clinical VRE*fm* isolates possessing multiple virulence factors have recently been reported in Turkey (Baylan *et al.*, 2011; Terkuran *et al.*, 2014). In our study, the presence of multiple virulence genes was also detected in two out of 33 faecal isolates; one isolate had the *esp* and *ebpA* genes and the other had *esp*, *ebpA*, *asa1*, *gelE* and *cpd* genes. Although the numbers are small, our

results suggest that the number of virulence genes among colonizing strains is increasing.

In conclusion, PFGE typing of the VRE*fm* isolates showed that 97.6% of isolates were clustered in group A, suggesting a cross-transmission between invasive and non-invasive isolates among different clinics during the study period. In order to reduce this high cross-transmission rate, we started to follow the infection control rules more strictly including contact tracing, taking barrier precautions and educating the health-care staff. According to our data, there was no direct relationship between the types of virulence genes and PFGE profiles of VRE*fm* isolates. The *esp* gene was the most dominant virulence gene detected in both clinical and commensal VRE*fm* strains. These data obtained from inpatient isolates may indicate the association of this gene with a high prevalence of VRE*fm* in hospital settings. Further investigations on virulence factors using larger isolate collections might provide insight into the acquisition of these traits and their contribution to increased prevalence of VRE*fm*.

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