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The interactions between *esp*, *fsr*, *gelE* genes and biofilm formation and pfge analysis of clinical *Enterococcus faecium* strains

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Enterococcus faecium has become an increasingly important nosocomial pathogen due to formation of biofilms on several surfaces. Sixty one (61) *E. faecium* strains isolated from blood, urine and fecal were assessed for biofilm production, the effect of different glucose concentration on biofilm production and also the presence of *esp*, *fsr* and *gelE* genes. Pulsed field gel electrophoresis (PFGE) method was performed to show chromosomal similarities and also to determine correlation between biofilm formation ability and genetic identity of *E. faecium* strains. It was observed that glucose concentration of the medium and incubation period can affect biofilm formation of the bacteria. When tested strains were incubated in a medium containing 1% glucose for 48 h, 66.66% of urine isolates, 60.71% fecal isolates and 25% of blood isolates produced strong biofilm structures. *esp*-positive strains (80% of the all isolates) were also identified as strong biofilm producers compared to *esp*-negative isolates. As a result of PFGE analyses, isolates numbered 14 (isolated from fecal sample) and 81 (isolated from blood sample) were classified in minor group B at a level of 48% similarity. Out of these two isolates, all the isolates were included in major group A with 43% similarity level and this group was subdivided into six subgroups.

Key words: *Enterococcus faecium,* biofilm, Pulsed Field Gel Electrophoresis (PFGE), *esp*, *fsr, gelE.*

INTRODUCTION

Biofilm formation is a dynamic process involving the attachment of bacteria to a biotic or abiotic surface and encased in a hydrated matrix of exopolymeric sub-stances, proteins, polysaccharides and nucleic acids (Tendolkar et al., 2006; Mohamed and Huang, 2007; Stepanovic et al., 2007). Biofilms are notoriously difficult to eradicate and are a source of many chronic infections. According to the National Institutes of Health, biofilms are medically important, accounting for over 80% of microbial infections in the body (Mohamed and Huang, 2007).

However, more than 30 species in the genus *Enterococcus* have been described to date; the two most studied enterococcal species are *Enterococcus. faecium* and *Enterococcus. faecalis* (van Schaik et al., 2010). Contrary to most of lactic acid bacteria, enterococci are not considered "generally recognized as safe (GRAS)" because of they are considered to be pathogens with low virulence (Hallgren et al., 2003; Ogier and Serror, 2008). Inhabitants of the human gastrointestinal and genitor-urinary tracts enterococci (Zhu et al., 2010) are also known to cause serious infections such as bacteraemia and endocarditis (Hallgren et al., 2003).

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Table 1. The strain numbers of *Enterococcus faecium* isolates.

Fecal isolates	Urine isolates	Blood isolates
1	3	2
7	5	4
13	8	6
14	24	11
15	25	12
17	26	21
18	29	28
19	30	46
36	32	69
38	33	71
39	35	77
41	37	81
42	43	
45	50	
55	52	
59	56	
63	60	
65	67	
68	72	
70	76	
84	94	
86		
87		
89		
90		
91		
93		
95		

Similarly, some researchers reported that enterococci have become increasingly important as nosocomial pathogens and have been found to form biofilms on several medical devices implanted in patients, such as central venous catheters, urinary catheters, intrauterine devices, and prosthetic heart valves (Cheng et al., 2002; Kristich et al., 2004; Laverde Gomez et al., 2011; Extremina et al., 2011). For biofilm formation of *Enterococcus* species, several genes have been found important such as *esp* (Shankar et al., 1999; van Schaik et al., 2010) and *fsr* via effect on gelatinase (Singh et al., 2007). Van Wamel et al. (2007) explained that Esp correlated with biofilm formation depending on growth conditions as well. The object of another study (Macovei et al., 2009) was to search relation between biofilm formation and gelatinase phenotype regulated by *fsr* operon in *E. faecalis* strains. As a result of that study, the researchers observed that *E. faecalis* with the complete *fsr* operon and the potential to form a biofilm were relatively common in the agricultural environment and might represent a source/reservoir of clinically relevant strains. Out of *esp* and *fsr* genes, several other factors have been

associated with biofilm development such as sugar-binding transcriptional regulator BopD, heterogeneity in surface charge, the *bee* locus and the secreted metalloprotease *gelE* (Van Wamel et al., 2007).

There are several factors used to identify differences between *Enterococcus* species, such as amplified rDNA restriction analysis (ARDRA), pulsed field gel electro-phoresis (PFGE) of DNA macro-restriction patterns, randomly amplified polymorphic DNA (RAPD-PCR), amplified fragment length polymorphisms (AFLP). Among these methods, PFGE has been successfully used to introduce the differences between clinical and food isolates, and between isolates from poultry and hospitalized patients (Ogier and Serror, 2008).

The aim of this study was to exhibit biofilm formation of *E. faecium* strains isolated from human urine, blood and fecal samples. To present the role of the genes, *esp*, *fsr*, *gelE*, correspond biofilm formation was also aimed in this study. PFGE method was used for illustrating chromosomal similarities between the isolates in order to determine correlation between biofilm formation ability and genetic identity of strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 61 previously isolated strains and identified as *E. faecium* strains (University of Ankara, Department of Biology, Prokaryotic Genetic Laboratory, Ankara-Turkey): 12 blood, 21 urine and 28 fecal originated human isolates (Table 1) were used in this research. *E. faecium* ATCC 6057 and *E. faecium* NCDO 942 were used as control strains. The stock cultures maintained in 40% glycerol solution at -80°C were activated in Tryptic Soy Broth (TSB, Merck®, Germany) for 24 h at 37°C prior to each trial.

Biofilm production and the effect of glucose concentration and incubation time on biofilm formation

A method described by Extremina et al. (2011) and Baldassari et al. (2001) was used to test the microorganisms for biofilm formation within the combination. Overnight cultures were diluted in fresh TSB until standardized the OD_{595} to 0.07 (CFU 10^7). Briefly, 200 µl of active cultures in TSB was inoculated into microtitre polystyrene plate wells. After 24 h growth at 37°C, the plates were gently washed three times with phosphate buffered saline (PBS, Sigma®, USA). The plates were allowed to dry for 1 h at 60°C and then fixed by using methanol (95%). For biofilm quantification, 200 µl of 1% crystal violet solution (Sigma®, USA) was added to each well, and the plates were allowed to stand for 20 min. The wells were subsequently washed thrice with sterile dH_2O to wash off the excess crystal violet. Crystal violet bound to the biofilm was extracted with 200 µl of ethanol-acetone (80/20%), and the absorbance of the extracted crystal violet was measured at 570 nm in ELISA Reader (Molecular Devices Spectra Max M2 Microplate Reader, USA). All biofilm assays were performed in triplicate.

The ability to form biofilm of the strains was scored as follows: OD < 0.120; non producers, 0.120 < OD < 0.240; weak producers, OD > 0.240; strong producers. Wells containing uninoculated served as negative controls (Tsikrikonis et al., 2012).

It is known that glucose concentration of the medium have effect on biofilm formation of the microorganisms (Pillai et al., 2004;

Table 2. The *esp*, *fsr*, and *gelE* primers and their properties.

Primer	Oligonucleotide sequence	Product size (bp)	Reference
esp	5' TTGCTAATGCTAGTCCACGACC 3' 3' GCGTCAACACTTGCATTGCCGAA 5'	955	Eaton and Gasson, 2001
fsr	5' AACCAGAATCGACCAATGAAT 3' 3' GCCCCTCATAACTCAATACC 5'	3268	Pillai et al., 2002
gelE	5' AGTTCATGTCTATTTTCTTCAC 3' 3' CTTCATTATTTACACGTTTG 5'	402	Mannu et al., 2003

Tendolkar et al., 2004). For this purpose, biofilm producing levels of standard strains (*E. faecium* ATCC 6057, *E. faecium* NCDO 942) and the strains determined as strong biofilm producers was assessed using TSB medium containing glucose at levels of 0.25, 0.50, 0.75, 1.00, 1.25%. After determining the optimum glucose concentration level, biofilm producing levels was identified at 24 and 48 h incubation periods as well. All experiments were performed in triplicate.

Isolation of *esp*, *fsr* and *gelE*

Enterococcal genomic DNA was used as the template for Polymerase Chain Reaction (PCR). Genomic DNAs of enterococcal strains were extracted by DNeasy Blood and Tissue Kit (Qiagen®, USA). Table 2 shows the specific primer pairs used for amplification of *esp*, *fsr*, *gelE* genes. PCR amplifications were performed in a ThermoCycler (Techne TC-512) in 0.2 ml reaction tubes each with 50 µl reaction mixtures composed of the 0.4 µM primer, 0.5 mM dNTP mix (Fermentas®, Finland), 1 X reaction buffer, 1.5 mM MgCl$_2$, 0.025u Taq polymerase (Promega®, USA) and 2.5 µl extracted enterococcal genomic DNA. PCR amplifications were performed for *esp*, *fsr* and *gelE* as previously described by Eaton and Gasson (2001), Pillai et al. (2002), and Mannu et al. (2003) respectively. According to this, after an initial denaturation procedure (94°C, 5 min) the reaction was subjected to 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final elongation procedure of 72°C for 10 min for the amplification of *gelE*. It was applied by adjusting the Tm temperature to 55°C for PCR reaction of *fsr*. Initial denaturation procedure was performed at 94°C, and 5 min for *esp* amplification. Then, reaction was subjected to 1 cycle of 94°C for 2 min, 56°C for 2 min and 72°C for 2 min, another 30 cycles of 92°C for 15 s, 56°C for 15s and 72° C for 15 s and a final elongation procedure of 72°C for 10 min. The PCR products were analysed on 1% agarose gel electrophoresis, stained in ethidium bromide solution and visualised under UV light.

PFGE analysis

Isolation of genomic DNAs

CHEF-DR III applications guide protocol (1992) (Bio-Rad®,USA) was modified for isolation of intact genomic DNAs of isolates with the purpose of PFGE analysis. *E. faecium* cultures were passed twice into the TSB medium at 37°C prior to use. An overnight culture was diluted in fresh broth until standardization at OD$_{600}$ between 0.5 and 1.0. The culture was centrifuged (10,000 x rpm, 5 min, 4°C) and then washed twice with cell suspension buffer [10 mMTris (pH 7.0), 20 mM NaCl, 50 mM EDTA (pH:8.0)] and resuspended in 100 µl of cell suspension buffer. Equal volumes of cells and 2% low melting grade agarose (BioShop®, Canada) were mixed in a microcentrifuge tube. Then approximately 100 µl of this mixture was pipetted into the disposable plug molds (10 mm x 5 mm x 1.5 mm, Bio-Rad Laboratories) before solidifying. In agarose, embedded cells were lysed *in situ* with lysis solution [30 mMTris (pH 8.0), 5 mM EDTA (pH 8.0), 50 mM NaCl, 10 mg/ml lysozyme] for 4 h at 37°C. Following this treatment agarose plugs were washed with 1x TE buffer [50 mM EDTA (pH 8.0), 20 mM Tris (pH 8.0)] so as to elimination lysis solution. Agarose plugs were added with proteinase K solution [100 mM EDTA (pH 8.0), 0.2% sodium deoxicholate, 1% sodium N-lauroylsarcosinate, 1 mg/ml proteinase K] and treated overnight without stirring at 50°C. Agarose plugs containing intact genomic DNA were washed ten times for half an hour at 50°C; four times with 1x TE supplemented by 1 mM NaCl, twice with 1x TE supplemented by 1 mM PMSF, twice with 1x TE and finally twice with 0.1x TE, respectively and then stored at 4°C in 0.1x TE.

Restriction enzyme digestion and electrophoresis

Each DNA embedded agarose plug was cut into about four slices. Slices of the plugs were digested for 16 h with 30 U of *SmaI* at 30°C in 80 µl of the 1x SE-Buffer Y (SibEnzyme®, Russia). Restriction enzyme mixture was removed on the slices of agarose plugs by washing with 0.5 x TBE prior to electrophoresis. After-wards, DNA fragments were resolved in 1% (w/v) pulsed-field certified agarose (BioShop®, Canada) in 0.5 x TBE buffer by pulsed field gel electrophoresis (PFGE) using CHEF-DR III System (Bio-Rad®, USA). Lambda ladder PFG Marker (New England Biolabs®, UK) was used as a molecular size standard. Electrophoresis was performed for 14 h at 120°C included angle. Pulse times for a total running time of 17h ranged from 0.1 to 5 s for 5 h, from 5 to 35 s for 6 h and from 40 to 125 s for 6 h at a constant voltage of 6 V/cm. The agarose gels were stained with ethidium bromide (10 µg/ml) and visualized under UV light. A digital image was obtained with Gel Logic 200 Imaging System (Kodak Company). NTSYS-pc version 2.2 (Rohlf, 1993) computer software was used for the cluster analysis of the enterococcal isolates. The Dice coefficient of similarity was calculated and comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Biofilm formation of the enterococcal strains and the effect of glucose concentration and incubation time

Biofilm assay was performed according to the method described by Extremina et al. (2011) and Baldassari et al. (2001). In this method, strains were grown in TSB medium without any supplementation and were incubated at
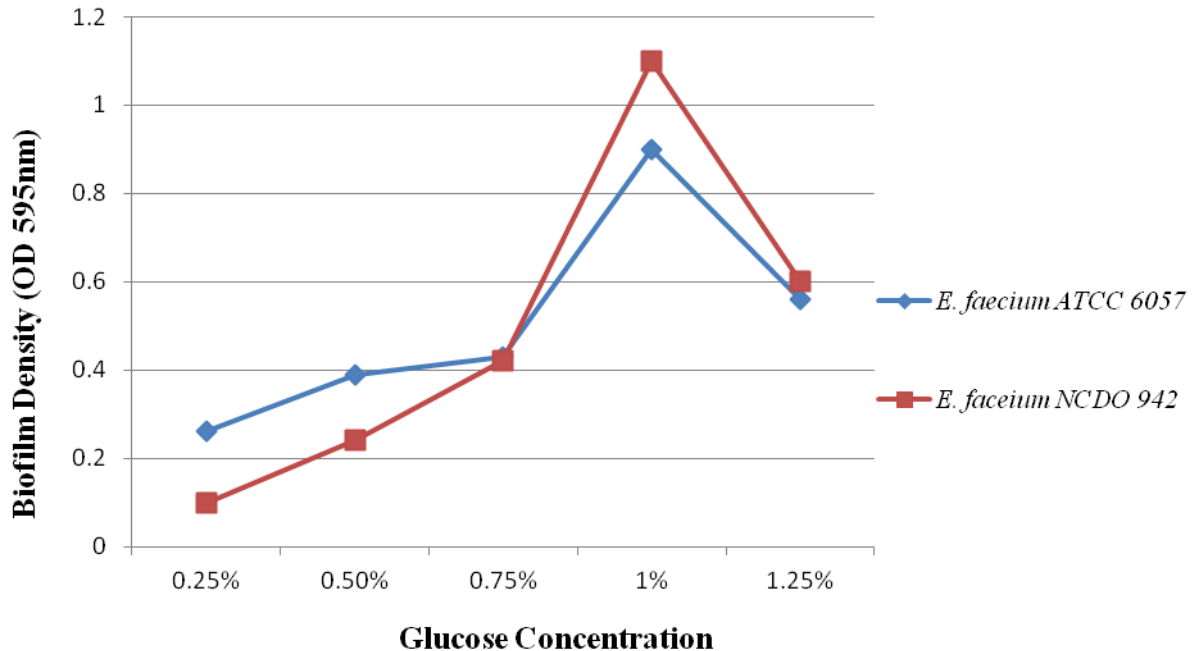
Figure 1. Effect of glucose concentration on biofilm formation of standard strains *E. faecium* ATCC 6057, *E. faecium* NCDO 942.

37°C for 24 h. According to results obtained in this experiment, 4 of 21 urine isolates, 9 of 12 blood isolates and 16 of 28 fecal isolates were able to produce biofilm. This means 48% of *E. faecium* strains could be defined as biofilm producers with the method comprising above. After this pre-trial, further experiments were performed.

Some researches indicated that factors such as nutrient concentration of the media can effect biofilm formation of the bacteria (Yoshida and Kuramitsu, 2002; Loo et al., 2000; Klausen et al., 2003). In addition to this knowledge, the enterococcal cell surface associated protein, Esp, may enhance biofilm formation by *E. faecalis* in a glucose-dependent manner (Tendolkar et al., 2005; van Wamel et al., 2007; Macovei et al., 2009). To demonstrate nutrient concentration effect on biofilm formation by *E. faecium*, we performed the microtiter plate assay to grow biofilms at the presence of different glucose concentration. Among the tested glucose concentrations (0.25, 0.50, 0.75, 1.00, 1.25%), 1.00% glucose concentration was used to detect an increased biofilm formation for standard and strong biofilm producer strains. Figure 1 shows the results for the standard strains. When the glucose concentration reached 1.25%, biofilm formation started to decline. Following this, all isolates were subjected to produce biofilm in TSB medium containing the mentioned glucose concentration. Out of our study, many researchers also reported on the effect of glucose concentration on biofilm formation. Whereas Pillai et al. (2004) detected 20-40% increased biofilm formation when they used 1.00% glucose added to TSB and Baldassarri et al. (2001) showed glucose supplemen-

tation may enhance biofilm formation. Kristich et al. (2004) demonstrated glucose-mediated inhibition of biofilm production among *Enterococcus* strains. In a study performed by Marinho et al. (2013), a synergistic effect on biofilm at 10, 28, 37 and 45°C and glucose was observed for *E. faecalis* and *E. faecium* as well. This thread can be taken in another angle; the glucose effect on biofilm formation can be considered important due to its ability to detect enterococcal biofilms in early stages or to select the biofilm producer bacteria in a more efficient way so that early detection of biofilm producing enterococci can be one of the essential steps towards prevention and management of nosocomial infections, as most of the hospital-acquired infections are biofilm related. Making changes in growth medium is one of the way to modify biofilm formation values output (Extremina et al., 2011).

In addition to glucose concentration assay, the effect of incubation time (24 and 48 h) on biofilm formation of *E. faecium* strains were investigated. As it is seen in Table 3, incubation for 48 h allowed strains to show more biofilm formation than 24 h incubation. Furthermore, the strains not producing biofilm after 24 h-incubation were able to produce biofilm by elongating incubation period to 48 h. Among all strains, 85.7% of urine and fecal isolates, 58.33% of blood isolates were determined as biofilm producers (Table 4). When comparing the isolates depending on origins, it can be reported that 66.66% of urine isolates, 60.71% fecal isolates and 25% of blood isolates were included in a group of strong biofilm producers (Table 4).

Table 3. Effect of incubation time and 1.00% glucose concentration on biofilm formation.

Parameter	Incubation time of the strains (h)	Non n (%)	Weak n (%)	Strong n (%)
Blood (12)	24	9 (75%)	2 (16.66%)	1 (8.33%)
	48	4 (33.33%)	5 (41.66%)	3 (25%)
Urine (21)	24	4 (19.04%)	6 (28.57%)	11 (52.38%)
	48	3 (14.28%)	4 (19.04%)	14 (66.66%)
Fecal (28)	24	16 (57.14%)	3 (10.71%)	9 (32.14%)
	48	4 (14.28%)	7 (25%)	17 (60.71%)

Table 4. *esp, fsr,* and *gelE* genes carriage, and biofilm formation of *Enterococcus faecium* isolates.

Origin of isolates (n)	*esp* gene n (%)	*fsr* gene n (%)	*gelE* gene n (%)	Biofilm formation (at 1.00 % glucose concentration and 48 h incubation)
Blood (12)	6 (46.15%)	0 (0%)	0 (0%)	7 (58.33%)
Urine (21)	8 (38.09%)	0 (0%)	0 (0%)	18 (85.7%)
Fecal (28)	6 (22.22%)	0 (0%)	0 (0%)	24 (85.7%)

Occurrence of *esp, fsr* and *gelE*

The presence of genes encoding the enterococcal surface protein (Esp), gelatinase enzymes (GelE), quorum-sensing locus *fsr*, were studied at 61 *E. faecium* isolates. The *esp* gene was detected for 46.15, 38.09 and 22.22% of blood, urine, and rectal isolates, respectively. *esp*-positive strains (80%) were significantly stronger biofilm producers than the *esp*-negative isolates (data not shown); this could indicate a role for *esp* in the formation of biofilm in clinical enterococci. The significantly higher incidence of *esp* gene in clinical isolates may reflect a role that Esp protein has in infection. Additionally to its role in adhesion, Esp is also thought to play a role in evasion of the host immune response, which is an important factor in the disease development (Shankar et al., 1999). There are conflicting results on the role of *esp* gene in biofilm formation but the opinions in general are in the line with positive effect of *esp* on biofilm production. Toledo-Arana et al. (2001) determined that *esp*-positive *E. faecalis* strains (93.5% in total) produce high level on abiotic surfaces whereas no biofilm formation was observed by *esp*-negative *E. faecalis* strains on the same surface. Another study performed by Tendolkar et al. (2005) aimed to localize the specific domain(s) of Esp that plays a role in Esp-mediated biofilm enhancement. It was reported by the researchers that an *E. faecalis* strain expressing only the N-terminal domain of Esp fused to a heterologous protein anchor formed biofilms that were quantitatively similar to those formed by a strain expressing full-length Esp. It is understood from this result that the minimal region contributing to Esp-mediated biofilm enhancement in *E. faecalis* was confined to the nonrepeat N-terminal domain. These results suggest that Esp may require interaction with an additional *E. faecalis*-specific factor(s) to result in biofilm enhancement. In contrast to our findings, there are also some studies that report that there is no relationship between *esp* gene and biofilm formation (Dworniczek et al., 2005; Ramadhan and Hegedus, 2005). Dupre et al. (2003) could not detect any relationship between the *esp* gene and biofilm formation of 15 clinical *E. faecalis* and 32 *E. faecium* strains. In addition, any bond between the presence of *esp* gene and biofilm formation of 70 *E. faecalis* and 38 *E. faecium* strains isolated from the circulatory system has not been established (Sandoe et al., 2003).

The relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *E. faecalis* and *E. faecium* was searched by Di Rosa et al. (2006). In the study, neither *esp* nor gelatinase seemed to be required for biofilm formation: both *E. faecalis* and *E. faecium* but in *E. faecium* while *esp* was found in isolates the presence of both *esp* and biofilm together was only found in strains from clinical settings, suggesting that there exists a synergy between these factors which serves as an advantage for the process of infection.

In a study (Top et al., 2013) very recently published, it was reported that the *E. faecium* enterococcal biofilm regulator, EbrB, regulates the *esp* operon and is implicated in biofilm formation and intestinal colonization. The study also determined that *esp* is part of an operon of at least three genes putatively involved in biofilm formation. In a mouse intestinal colonization model, the *ebrB* mutant was less able to colonize the gut compared to wild-type strain, especially in the small intestine. These data indicate that EbrB positively regulates the *esp* operon and is implicated in biofilm formation and intestinal colonization.

gelE, the other gene thought to have an effect on biofilm formation, is an extracellular zinc metalloprotease which can hydrolyze gelatin, collagen and casein (Qin et al., 2001). Some researchers showed that *gelE* has a critical role on development of the biofilms (Hancock and Perego, 2004; Kristich et al., 2004; Mohamed et al., 2004). Except *gelE*, it was demonstrated that *fsr* gene has a significant effect on enterococcal biofilms (Hancock and Perego, 2004; Mohamed et al., 2004; Pillai et al., 2004). Unlike all these literature knowledge, in our study, it was determined that *fsr* locus and *gelE* gene were not presence in any of tested *E. faecium* isolates. Further, any relationship between the genes (*fsr, gelE*) and biofilm producing capacity of the strains could not be detected. It suggests that the physiological factors may trigger the production of biofilm, instead of *fsr* locus and *gelE* gene.

In clinical enterococcal strains, gelatinase activity is generally associated with virulence factors (Pillai et al., 2002; Roberts et al., 2004). Because of no detection of gelatinase activity or presence of *fsr* and *gelE* genes in the tested clinical *E. faecium* isolates, no significant correlation was found between gelatinase production and *fsr*, *gelE* genes and biofilm formation. *fsr* locus (*fsr*ABDC) of *E. faecalis* is determined as a global regulator and it acts as a signal transmission system which controls biofilm formation of *E. faecalis* for only different environmental conditions. Besides of regulation function of gelatinase and serin protease expression, many genes like *bopD* can be one of the parts of *fsr* regulon (Paganelli et al., 2012). Different from our findings, Qin et al. (2000) found that 69% of clinical isolates were positive for *fsr B* gene. In another study performed by Pillai et al. (2002), all the isolates obtained from patients with endocarditis and 53% of fecal isolates were declared as positive for *fsr B* gene. Based on this data, the researchers offered to identify the enterococcal strains which have *fsr* locus among clinical isolate.

Further analysis of the subgroup of *esp* negative human and animal isolates showed that the ability to produce gelatinase was positively associated with biofilm formation only in animal originated *E. faecalis* isolates. This could indicate that production of this protease may be a selection mechanism for animal *E. faecalis*, as it may enable the *esp*-lacking animal isolates to produce biofilm. However genetic manipulation studies have offered that gelatinase is essential for biofilm formation, epidemiological studies have not supported the link between gelatinase and biofilm production among clinical *E. faecalis* isolates (Tsikrikonis et al., 2012).

PFGE assay results

PFGE has emerged as one of the most widely applicable, reproducible, and stable methods to examine strain identity in enterococci (Patterson and Kelly, 1998). In order to investigate relationship between genetic similari-

ties and biofilm formation abilities of clinical *E. faecium* isolates, the chromosomal DNA of the strains were cut with *Sma*I restriction endonuclease. Afterwards, macrorestriction fragment numbers and also sizes of all blood, urine and fecal strains were compared with PFGE. When the obtained data were analyzed, it was observed that there were high levels of heterogeneity between clinical isolates despite they all belong to same species. Cluster analysis of macrorestriction patterns generated by *Sma*I digestion of chromosomal DNAs revealed one major and a minor cluster for 61 *E. faecium* strains as shown in Figure 2. The fecal isolate numbered as 14 and the blood isolate numbered as 81 were classified in minor group B with around 48% similarity rate. Out of these two isolates, all the other isolates were included in a major cluster A at 43% similarity level and subdivided into six subgroups (Figure 2). Our PFGE results are similar from the point of low level homology among clinical *E. faecium* isolates with the study conducted by Weng et al. (2013). In another study performed previously by Bedendo and Pignatari (2000) were investigated genetic diversity among 20 clinical *E. faecium* isolates by using REP-PCR and PFGE, so PFGE had revealed easier interpreted band patterns in comparison with REP-PCR.

PFGE analysis of enterococci isolates from recreational and drinking water in Greece was searched by Grammenou et al. (2006). A collection of enterococci recovered from recreational and drinking water were applied to biotyping and DNA fingerprinting by PFGE, in order to identify possible genetic relationships. Even though genetic diversity was observed among the studied strains, common clonal types were also identified in different sources, suggesting a possible common origin of the enterococci. As a conclusion of that study, cluster analysis revealed a genetic relationship between certain environmental *E. faecium* and clinical strains.

The isolates numbered as 65, 68, 17, 18 and 41 constituted a subgroup A6 with their common traits, which are fecal isolates and also *esp* negative, although there was no found correlation in terms of biofilm formation abilities of them. On the contrary, isolates numbered as 42 and 45, which were previously isolated from the same medium (University of Ankara Department of Biology, Prokaryotic Genetic Laboratory, Ankara-Turkey) and exhibited the same *Sma*I band profile, were found also as the same with regard to all investigated properties in this study. Surprisingly, strain numbered 94, a urine isolate, showed strong biofilm formation and 70% chromosomal similarity with ATCC 6057 standard strain. However, we could not determine for all strains a correlation between all the tested strains biofilm formation and genetic similarity detected by PFGE. Increase in the ratio of differences between the strains indicates genetic distinctions, as well. It is possible to explain genetic differentiations among enterococcal strains with gaining linear plasmids, moderate prophages and transposones which can be integrated into chromosome via horizontal
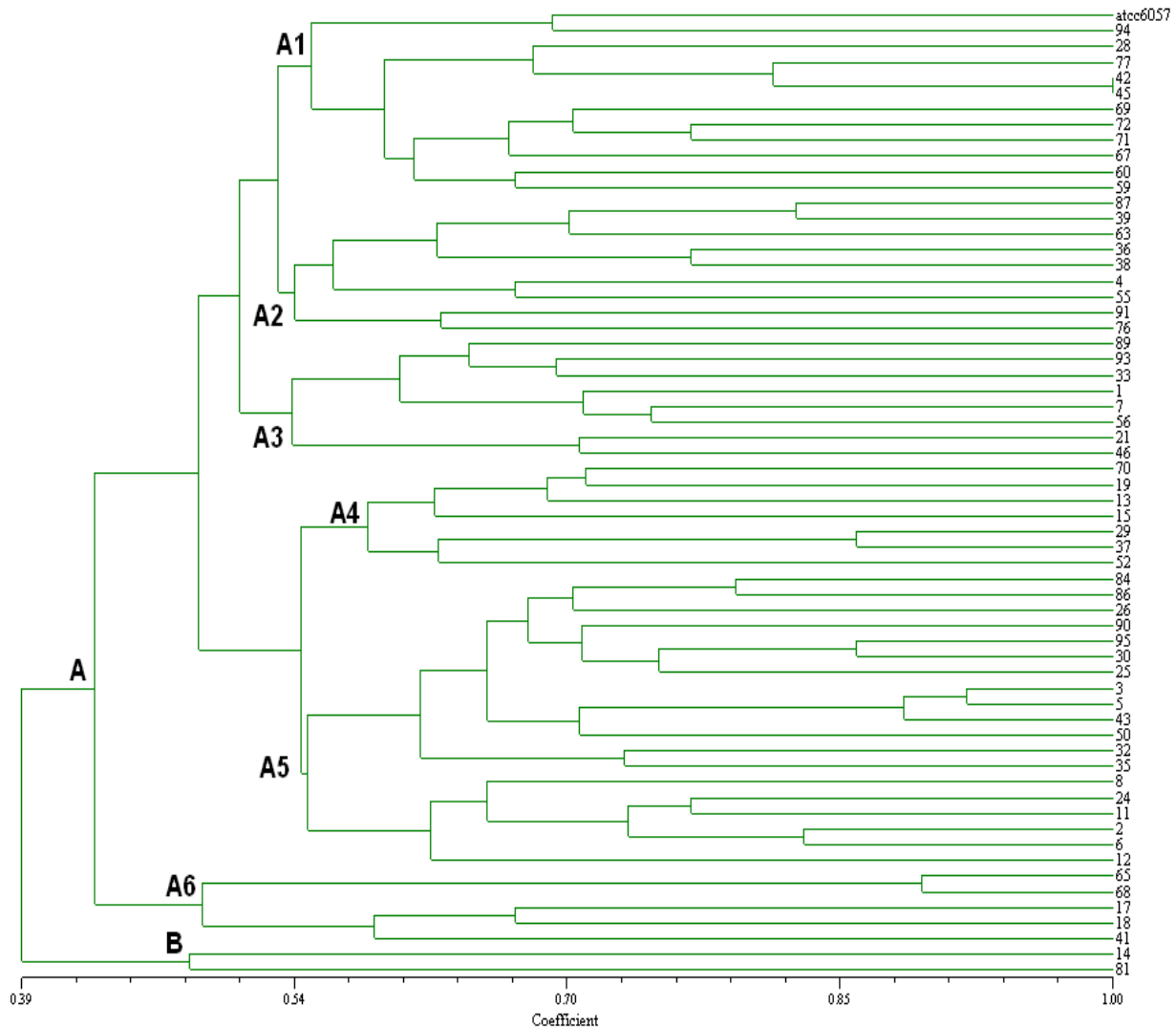
Figure 2. PFGE Dendrogram of *E. faecium* strains obtained by unweighted pair group method using average linkage of dice correlation coefficients

gene transfer between the isolates surviving in the same environment (Hacker et al., 2003; Paulsen et al., 2003). It is known that, this situation mentioned above can cause huge chromosomal differences between the strains belonging to *E. faecium* species and give a chance to them to show different phenotypic properties in evolutionary process, such as antibiotic resistance, virulence traits and also biofilm formation (Rice et al., 2005; Hegstad et al., 2010; Palmer et al., 2010).

As a result of PFGE assay, we can conclude that the chromosomal similarity detected around 60-70% between the clinical enterococcal isolates emerged due to gain or lose genes during evolutionary process. It was also found that biofilm production capacities of closely related isolates may vary. This assignation is another consequence of this study.

Conclusions

Enterococci are important pathogens and are one of the major causes of infection within hospitals. This study focused on the determination of phenotypic and molecular basis of biofilm formation traits in clinical *E. faecium* strains isolated from human origin blood, urine and rectal samples in Turkey. As a result of the present study, we can emphasize that glucose concentration has a significant effect on biofilm formation. The changes in incubation time may also introduce different biofilm formation cases. However, we focused on three genes, *esp*, *fsr*, *gelE*, thought as having relationship between biofilm formation of *E. faecium* strains; we could only detect a relation between *esp* gene and biofilm formation which was 80% of *esp*-positive strains and were strong biofilm

producers as well. A major and a minor group were determined by using PFGE analysis for *E. faecium* isolates. Surprisingly, it was observed that biofilm formation varied even among closely related species in PFGE assay.

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