ORIGINAL ARTICLE

Investigation of Gelatinase Gene Expression and Growth of *Enterococcus faecalis* Clinical Isolates in Biofilm Models

Enterococcus faecalis Klinik İzolatlarının Üreme ve Gelatinaz Gene Ekspresyonlarının Biyofilm Modellerinde Araştırılması

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ABSTRACT

Objectives: *Enterococcus faecalis* is the major reason for biofilm-related infections and it also interacts with *Staphylococcus aureus* in biofilms. Gelatinase (gelE) enzyme is an important virulence factor of *E. faecalis* for biofilm formation. This study aimed to compare the biofilm producing *E. faecalis* isolates from urine and urinary catheters. The influence of *S. aureus* on the growth of *E. faecalis* biofilm cells was also investigated in a dual biofilm model *in vitro*. Another aim was to evaluate *E. faecalis* gelE gene expression during biofilm formation.

Materials and Methods: Firstly, crystal violet staining was used to measure the total biofilm biomass of the isolates. Secondly, plate counting was performed to determine the biofilm formation ability of *E. faecalis* isolates and the effect of *S. aureus* on *E. faecalis* biofilm formation. Finally, the gelE expression profile of the isolates was assessed by quantitative real time-polymerase chain reaction.

Results: According to crystal violet staining and plate counting, all *E. faecalis* isolates were biofilm producers and the number of *E. faecalis* sessile cells increased in the presence of *S. aureus*. Among the 21 *E. faecalis* isolates, ten expressed high levels of the gelE gene, while eight of them had low expression profiles (p<0.05).

Conclusion: When they grow together, *S. aureus* may give some advantages to *E. faecalis* such as increasing sessile cell growth. The expression of the gelE gene was not affected by *E. faecalis* biofilm formation of the isolates collected from the patients with urinary tract infections.

Key words: Dual biofilm, *E. faecalis*, *S. aureus*, gelatinase, quantitative reverse transcription polymerase chain reaction

ÖZ

Amaç: *Enterococcus faecalis* biyofilm ilişkili enfeksiyonların ana sebebidir ve biyofilmlerde *Staphylococcus aureus* ile de etkileşimde bulunurlar. Jelatinaz (gelE) enzimi biyofilm oluşumunda *E. faecalis* için önemli bir virulans faktörüdür. Bu çalışma idrar ve üriner kateterlerden izole edilmiş biyofilm oluşturan *E. faecalis* izolatlarını karşılaştırmayı amaçlamaktadır. Aynı zamanda *S. aureus*'un *E. faecalis* biyofilm hücrelerinin üremesi üzerindeki etkisi de *in vitro* iki türlü biyofilm modelinde incelenmiştir. Bir diğer amacımız biyofilm oluşumu sırasında *E. faecalis* gelE gen ekspresyonunu değerlendirmektir.

Gereç ve Yöntemler: İzolatların total biyofilm biyokütlesinin ölçümünde ilk olarak kristal viyole boyama yöntemi kullanılmıştır. İkinci olarak, *E. faecalis* izolatlarının biyofilm oluşturma kapasitesini ve *S. aureus*'un *E. faecalis* biyofilmleri üzerinde etkisini değerlendirmek için plak sayım yöntemi uygulanmıştır. Son olarak, izolatların gelE ekspresyon profilleri kantitatif gerçek zamanlı-polimeraz zincir reaksiyonu ile belirlenmiştir.

Bulgular: Kristal viyole ve plak sayım yöntemine göre, tüm *E. faecalis* izolatlarının biyofilm oluşturdukları ve *E. faecalis* sesil hücre sayılarının *S. aureus* varlığında arttığı belirlenmiştir. Yirmi bir *E. faecalis* izolatı arasında, 10'u gelE gen ekspresyonunu yüksek oranda arttırmış, ancak 8'i azaltmıştır (p<0.05).

Sonuç: Birlikte üredikleri zaman; *S. aureus*, *E. faecalis*'e sesil hücre üremesini arttırmak gibi bazı avantajlar sağlayabilmektedir. GelE gen ekspresyonu idrar yolu enfeksiyonlu hastalardan izole edilmiş *E. faecalis* izolatlarının biyofilm oluşumundan etkilenmemiştir.

Anahtar kelimeler: İkili biyofilm, *E. faecalis*, *S. aureus*, jelatinaz, kantitatif gerçek zamanlı-polimeraz zincir reaksiyonu

INTRODUCTION

Biofilms are defined as biotic or abiotic surface-attached microbial consortia and have multiple stages such as initial reversible attachment; production of an extracellular polymeric matrix (EPM) including proteins, polysaccharides, and nucleic acids; irreversible attachment; etc.[1,2] Biofilm formation is an important problem causing failure in antimicrobial treatment because sessile cells in the biofilm are highly resistant to antimicrobial agents. It has been highlighted that 65%-80% of all infections are biofilm-related. Biofilm cells are phenotypically, physiologically, and genotypically different from nonattached (planktonic) cells. Moreover, high concentrations of antimicrobial agents are necessary to kill sessile cells in a mature biofilm vs planktonic cells.[3]

It has been recently shown that most diseases are caused by polymicrobial communities.[4-8] Although some infections are considered predominantly monomicrobial, they may be influenced by other microorganismal associations during active infection.[4] The physiology of microbial cells in the biofilm has been frequently changed by these interactions and leads to various advantages being obtained, such as resistance to antimicrobials or the human immune system, metabolic cooperation, quorum sensing systems, and more productive gene sharing.[9-12]

Enterococcus species have been recognized as opportunistic pathogens for many nosocomial infections and are natural inhabitants of the human intestinal and oral flora. *Enterococcus faecalis* is the most common species leading to many infections among the other enterococcus species.[13,14] They can readily form biofilms and keep growing on various medical devices' surfaces such as urinary catheters despite a serious inflammatory response.[15] *Staphylococcus aureus* has become an important cause of hospital-acquired infection associated with indwelling medical devices and surgical wounds. It may cause chronic infections that cannot be treated with antibiotics because of the ineffective host immune response. Moreover, staphylococci have nonspecific resistance mechanisms such as biofilm formation.[16-18]

Changing expression levels of virulence factors of *E. faecalis* have been shown whether they formed a biofilm or not. Among the virulence factors, the gelatinase (gelE) enzyme is an important factor that hydrolyzes gelatin, casein, and collagen.[19] Although there have been many studies on biofilm formation and gelE expression by *E. faecalis*, it is still not clear how gelE expression levels change in mono- or polymicrobial biofilms.[20-22]

In the present study, we evaluated the biofilm ability of *E. faecalis* isolates by quantification assays and then we set up an *in vitro* dual biofilm model in a repeatable style and determined the influence of the presence of *S. aureus* on the growth of *E. faecalis* by plating assay. Finally, the *gelE* gene expression levels of *E. faecalis* were measured by quantitative real time-polymerase chain reactions (qRT-PCRs).

MATERIALS AND METHODS

Strains used in the study

A total of 20 *E. faecalis* clinical isolates and a strain as a positive control (*E. faecalis* ATCC 29212) were used in this study. These isolates were taken from urinary catheter (n=10) and urine samples (n=10) from hospitalized intensive care unit patients admitted to a University Hospital from 2000 to 2011.

For dual biofilm formation, all the *E. faecalis* isolates and *E. faecalis* ATCC 29212 were cultured with *S. aureus* ATCC 29213.

Mono and dual biofilm formation in microtiter plates

Final inoculum suspensions of all clinical *E. faecalis* strains were adjusted to approximately 10^6 colony-forming units (CFU) mL^{-1}. Each experiment included the biofilm-forming *E. faecalis* ATCC 29212 strain as a positive control. For dual species biofilms, *E. faecalis* isolates were co-cultured with a laboratory strain of *S. aureus* (10^6 CFU/mL) and incubated at 37°C without shaking. Sterile tryptic soy broth (TSB) (Becton Dickinson GmbH, Heidelberg, Germany) with 0.25% glucose was used as a blank. For each test condition, 12 wells of a flat-bottomed polystyrene 96-well microtiter plate were inoculated with 100 µL of the final inoculum suspension. After 4 h of incubation at 37°C without shaking, nonadhered cells were removed and rinsed with 100 µL of 0.9% physiological saline (PS), then 100 µL of fresh TSB with 0.25% glucose was added, and the plates were incubated for an additional 20 h for biofilm maturation. After 24 h, the supernatants were removed and each well was rinsed with PS before the sessile cells were quantified.

Quantification of the biofilms

Crystal violet staining

The biomass quantification of *E. faecalis* biofilms was performed according to an optimized assay.[19] After washing with sterile PBS, the wells were stained with 100 µL of a solution of 0.2% crystal violet for 15 min. The stained biofilms were rinsed again three times with PBS to remove excess dye and dried for 15 min at room temperature. The bound dye was solubilized in 150 µL of acetone/ethanol solution. The optical densities (ODs) of the stained adherent cells were read at 570 nm using a micro-ELISA plate reader. We defined the cut-off OD (0.282) as three standard deviations above mean OD of the negative control. Each isolate was tested in 12 wells in each assay and each assay was carried out in duplicate (n=24).

Plate counting

Quantification of the number of cells in mature biofilms was done via plate counting using tryptic soy agar (TSA) medium.

Table 1. The primers for quantifying the genes of *E. faecalis* by RT-qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
16sRNA	CCGAGTGCTTGCACTCAATTGG	CTCTTATGCCATGCGGCATAAAC
gelE	TGGATTAGATGCACCCGAAAT	CGGAACATACTGCCGGTTTAGA

Biofilms were detached by vortexing (5 min) followed by sonication (5 min). The sonicated fluids were serially diluted and plated on TSA to determine the number of CFU per mL of the isolates. Bile esculin azide agar was used for plating of *E. faecalis* isolates in mature dual biofilms that were formed by *E. faecalis* and *S. aureus*.

Expression of the gelE gene in planktonic and biofilm cells of E. faecalis

Total RNA was extracted from the mono- and dual-species biofilm cells with the RNeasy® Mini Kit according to the manufacturer's recommendations (Qiagen GmbH, Germany). All RNA extracts were prepared as 100 ng µL^{-1} per sample and transcribed into cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). RT-PCR (Roche Light Cycler 2.0) was performed with LightCycler Faststart DNA Master SYBR Green1 (Roche Diagnostics GmbH) in a total volume of 20 µL. Primer sequences for the housekeeping gene 16sRNA and gelE were obtained from the literature and are listed in Table 1.[17] The 16sRNA gene was used to normalize the expression level of gelE. Melt curve analysis was carried out to assess the specificity of each primer pair. The comparative C_T method for relative quantification ($^{\Delta\Delta}C_T$ method) was performed to analyze the data.[23]

Statistical analysis

The independent samples t-test was used to compare biofilm cell CFU counts between the two groups (with/without *S. aureus* biofilms). One-way ANOVA was used to evaluate CFU differences within a group. The CFU counts were log-transformed before the statistical tests. A p value <0.05 was considered significant. For gene expression, the results were analyzed by t-tests and only differences of more than twofold up- or down- regulation and with a p-value <0.05 were considered significant.

RESULTS

Detection of biofilm production by E. faecalis isolates

In total 20 *E. faecalis* isolates were analyzed to determine the ability of biofilm formation. All of the isolates were found to be biofilm-positive by plate counting and crystal violet staining (Figures 1 and 2). In terms of biofilm-forming ability no statistically significant difference was determined between the isolates from catheters and not from catheters (Figure 2).

In the co-culture of *E. faecalis* with *S. aureus*, the cell counts of *E. faecalis* were significantly higher than those in their mono-species biofilm (Figure 2). Our results showed that *S. aureus* contributed to the growth of *E. faecalis* biofilm cells by an unknown mechanism.

GelE gene expression in planktonic and biofilm cells of E. faecalis clinical isolates

We used qRT-PCR to compare the expression levels of gelE in planktonic and biofilm cells of 21 *E. faecalis* isolates (including a positive control) from urine and urinary catheter samples from

hospitalized patients and *E. faecalis* ATCC 29212. According to the results obtained from the mRNA levels of gelE in planktonic and biofilm cells of *E. faecalis*, 12 of the 21 *E. faecalis* strains (including the positive control) exhibited increased gelE gene
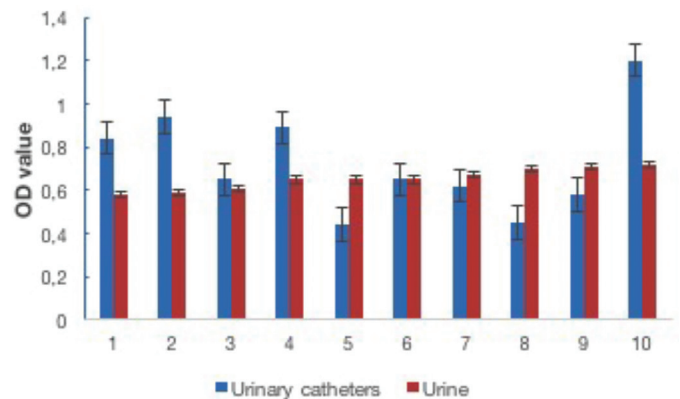

Figure 1. Biofilm forming ability of the *Enterococcus faecalis* isolates from the urine and urinary catheter samples of hospitalized patients by crystal violet staining assay

[a]Biofilm formation degrees of the isolates were determined by crystal violet staining assay. Results are means of at least three different experiments, OD: Optical density
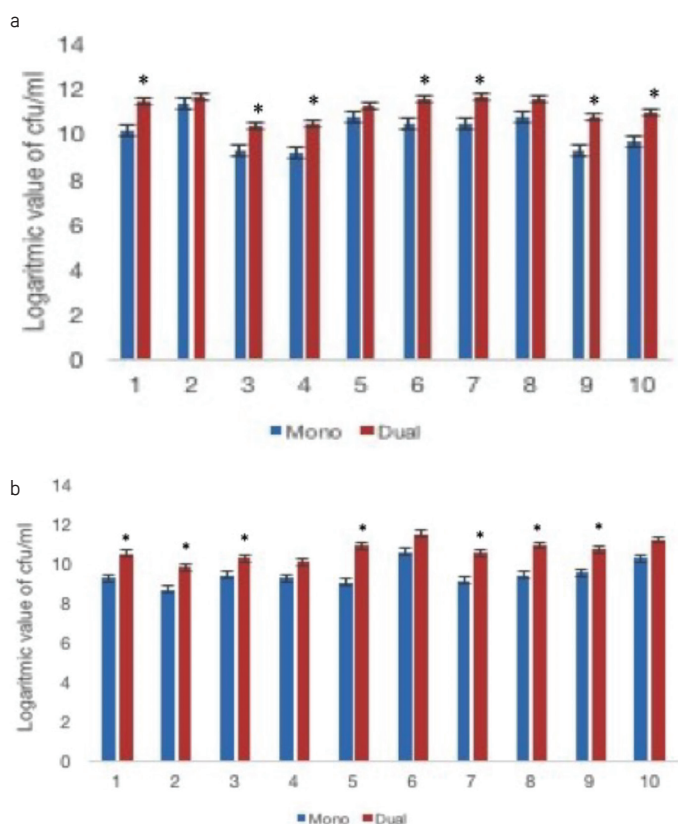

Figure 2. Biofilm forming ability of the *Enterococcus faecalis* isolates in mono and dual biofilms. Numbers of the sessile cells of the *E. faecalis* isolates in mono and dual species biofilms were determined by plate counting assay. (a) The results of isolates from the urinary catheter samples, (b) The results of isolates from the urine samples. Results are means of at least three different experiments. Mono: only *E. faecalis* biofilms, Dual: *E. faecalis* and *S. aureus* biofilms, CFU: Colony forming unit,

*: Statistically significant

expression; however, only ten of them (including the positive control) were statistically significant (p<0.05). Eight isolates showed significantly decreased expression levels (p<0.05) (Figure 3).
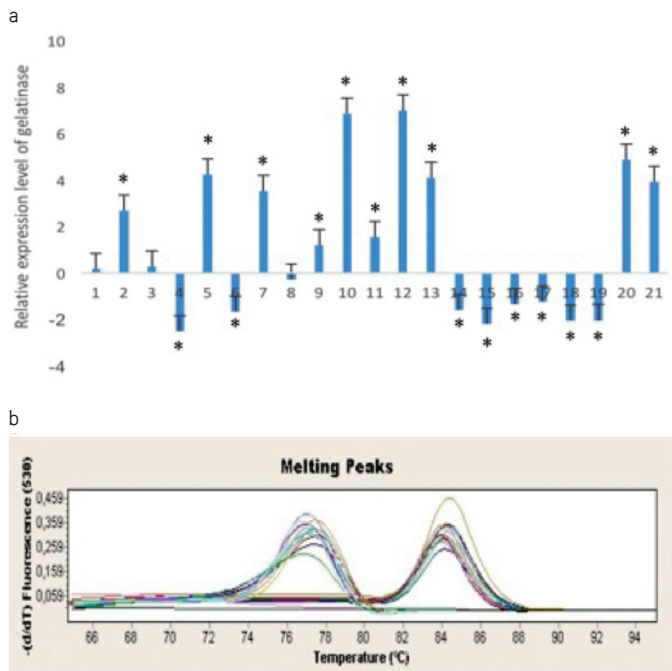
a

b

Figure 3. Gelatinase gene relative expression ratios of *Enterococcus faecalis* ATCC 29212 and clinical isolates in planktonic and biofilm cells.

*: Statistically significant (p value<0.05), (a, b) Melting curves of gelE and 16srRNA (housekeeping gene) of the bacteria, respectively

DISCUSSION

Biofilm-related urinary tract infections represent the main cause of nosocomial infections. Enterococci (especially *E. faecalis*) and *S. aureus* are a major challenging problem for treatment of urinary tract infections.[24] It is widely known that the presence of bacterial biofilms on the inner or outer surface of the catheter leads to catheter-associated urinary tract infections (CAUTIs).[15] The occurrence of CAUTIs, as the most common hospital-acquired infection, has an important economic and clinical impact and is directly related to the majority of uropathogens such as *E. faecalis* and *S. aureus* that may form biofilms. In the current study, we assessed the ability of biofilm formation of clinical *E. faecalis* isolates in alone and co-culture with *S. aureus in vitro*. Our results indicated that all isolates from inpatients with and without urinary catheters were biofilm positive with regard to the plate counting and crystal violet staining methods (Figures 1 and 2). The starting bacteria concentration was normalized as 6 $\log_{10}$ (10^6 CFU/mL). However, after the incubation period, the lowest bacteria number in the well plates was found to be 8.7 $\log_{10}$. This result showed that all the mono-species biofilm isolates of *E. faecalis* attached and grew on the walls of the wells in microtiter plates (Figure 2).

Interspecies interactions in polyspecies biofilm usually provide various advantages for the inhabitant species such

as increased tolerance against several antimicrobials and increased virulence in infections.[25] Pastar et al.[26] showed that the presence of *Pseudomonas* inhibited the growth of *S. aureus in vitro* and induced expression of *S. aureus* virulence factors in polymicrobial wound infection. In another study, the effect of *Streptococcus mutans* on *E. faecalis* biofilm formation was investigated and an increase in biofilm formation of *E. faecalis* by *S. mutans* was obtained.[27] It has been previously shown that the combined effect of *C. albicans* and *E. faecalis* in a mouse model resulted in increased growth of enterococci in the animals when *C. albicans* had been introduced.[28] In a *P. aeruginosa* and *C. albicans* dual biofilm model, it was observed that *P. aeruginosa* formed biofilms on the fungal filaments of *C. albicans* and this close contact caused the killing of the fungal filaments.[29] In our study, the number of sessile cells of *E. faecalis* in dual-species biofilms with *S. aureus* was significantly higher than in their mono-species biofilm. We concluded that the growth and biofilm formation of the *E. faecalis* isolates were increased by *S. aureus* sessile cells. According to the biofilm cell counts between the urine and urinary catheter samples, the counts of *E. faecalis* isolates from the urinary catheters were greater than those of the isolates from urine.

Many virulence factors have significant roles in the pathogenesis of enterococcal infections such as adhesion, colonization, and invasion. Although it has been indicated that some of the major virulence genes were related to biofilm formation on abiotic surfaces in hospital environments, research on the virulence mechanism and related genes in biofilm formation is still needed.[30-32] A high amount of gelE gene expression in *E. faecalis* biofilm cells was shown in some studies, whereas others were in contradiction with this finding.[33-35] Arciola et al.[15] showed the importance of gelE in biofilm formation in implant infections. In a recent study, the prevalence of the gelE gene was determined as 64.3% among 510 clinical *Enterococcus* spp. isolates from UTI and wound infections.[30] However, Kafil and Mobarez[24] did not find a significant effect of the presence or absence of gelE on biofilm production by *Enterococcus* species. We examined the gelE mRNA levels of both planktonic and sessile cells of *E. faecalis* ATCC 29212 and 20 *E. faecalis* isolates by RT-qPCR. Our results showed that the gelE expression levels of ten isolates were significantly enhanced, but eight of the isolates were significantly decreased in biofilms when compared to their planktonic forms (p≤0.05) (Figure 3). Based on this result, we concluded that gelE expression had no effect on biofilm formation of the isolates collected from urinary tract infections (p>0.05) (Figure 3). The comparison of gelE mRNA levels of the isolates from the two different samples showed no significant difference either.

CONCLUSIONS

There was no statistical significance between the isolates from catheters and not from catheters in terms of biofilm-forming capability. *E. faecalis* sessile cell counts were increased in the presence of *S. aureus*. Expression of the gelE gene was not affected by *E. faecalis* biofilm formation of the isolates collected from the patients with urinary tract infections.

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