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DOES SUBINHIBITORY CONCENTRATIONS OF CLINICALLY IMPORTANT ANTIBIOTIC INDUCE BIOFILM PRODUCTION OF *ENTEROCOCCUS FAECIUM* STRAINS?

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Biofilm structures are the most resistant form of active microorganisms against sanitation, disinfection, and sterilization processes. One of the specific properties of biofilm is the development of antibiotic resistance that can be up to 1,000-fold greater than planktonic cells. *Enterococcus faecium* is a human pathogen that causes nosocomial bacteremia and at the present time, it is well known that most of the chronic infections are biofilm-based. Recent evidence suggested that subinhibitory concentrations (sub-MICs) of antibiotics have an important role in the evolution of antibiotic resistance and induction on biofilm formation. Based on this information, we aimed to determine the effect of subinhibitory antibiotic concentrations on biofilm formation and the role of the antibiotic concentrations on the enterococcal surface protein gene (*esp*). To determine the impact of clinically important antibiotics on biofilm production, crystal violet assay was used. Then, the effect of sub-MICs of antibiotics on the expression of the *esp* gene was investigated by quantitative real-time PCR. Biofilm production assays show that MIC/2 of erythromycin (ERT; 512 μg/ml), MIC/32 of vancomycin (VAN; 16 μg/ml), MIC/64 of streptomycin (STR; 32 μg/ml), and MIC/128 of kanamycin (KAN; 4 μg/ml) values induce maximum biofilm production compared with the control. According to q-PCR results, sub-MIC values of ERT, VAN, and STR antibiotics were found to enhance *esp* gene expression. In addition, despite the increasing biofilm production after KAN treatment, the antibiotic was not effective on the *esp* expression.

Keywords: *Enterococcus faecium*, sub-MIC, biofilm formation, *esp* gene, quantitative real-time PCR

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Introduction

Antibiotics target essential bacterial structures, such as cell wall and cellular pathways including DNA, RNA, and protein synthesis mechanism. They have been used to treat several infectious diseases. The long-term use of antibiotics in recent years has resulted in appearance of multidrug-resistant (MDR) bacterial pathogens, such as Enterococci [1, 2].

Enterococci, known as opportunistic pathogens, are naturally found in intestinal microflora and oral cavity of humans and animals. Two most common *Enterococcus* species (*Enterococcus faecalis* and *Enterococcus faecium*) are capable of producing biofilms, which are bacterial communities attached to a biotic or an abiotic substrate encased in a matrix. *E. faecium* is an important global cause of biofilm-related infections. Biofilms are dependent on multiple genetic factors, such as *esp, gelE*, and *fsr* locus [3, 4]. Cell wall-associated protein implicated in biofilm formation is an enterococcal surface protein (Esp) coded by *esp* gene. It was first identified in *E. faecalis* as a large surface-anchored protein from infection-derived isolates [5]. An *esp* homologue has been identified in *E. faecium* and this gene is localized on pathogenicity islands in both species [6–8]. Studies suggested that there was a strong correlation between *esp* and the forming of biofilms. Toledo-Arana et al. [9] reported that 93.5% of *esp*-positive isolates could form biofilms on polystyrene, whereas none of the *esp*-negative isolates could produce biofilms. The investigators suggested that the N-terminal domain of Esp is sufficient for biofilm production, mutation on the N-terminal domain region of Esp in *E. faecalis* strain causes less biofilm production [10]. The *esp*-positive strains were also identified as strong biofilm producers compared with *esp*-negative isolates [11, 12]. In addition to that, researchers presented that presence of a higher glucose concentration in the growth medium-regulated biofilm production [12–14]. In spite of that, other studies suggested that there was no association between the presence of *esp* and biofilm-forming ability and the *esp* gene was not necessary for the production of biofilm in *E. faecalis* and *E. faecium* [15–18]. While some studies showed that *esp* is a certain factor for biofilm formation, others presented that biofilm production needs other necessary factors with *esp*.

Recent studies suggested that sub-MICs of antibiotics acted as signaling molecules mediating variety of cell processes, such as gene transcription and expression, quorum sensing, inter- or intra-species communication, and biofilm formation [19–23]. In addition, low concentrations of antibiotics may stimulate different stress responses that might enable horizontal transfer of antibiotic resistance genes among bacterial communities, which are found on biofilm [23–26].

Studies showed that some using antibiotic concentrations below the MIC can significantly induce biofilm formation in a variety of Gram-positive and Gram-negative bacterial species. Subinhibitory concentration of an aminoglycoside antibiotic tobramycin induced biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* [27]. Similarly, gentamicin (GEN) (>64 mg/ml, 2 × MIC) and tetracycline (>128 mg/ml, 4 × MIC) were determined as the most effective antibiotics against *Salmonella* Infantis biofilm formation; however, biofilm structure was induced with sub-MICs of nalidixic acid, spectinomycin, tetracycline, and neomycin antibiotics treatment [28]. Balaji et al. [29] proved that 1/16 MIC value of fluoroquinolones increased biofilm formation, whereas 1/2 MIC value of them occurred inhibition effect on clinical isolates of *Streptococcus pyogenes* biofilm. The proof presented that inhibition and biofilm formation were dose-dependent [29]. Previous studies showed that sub-MIC amoxicillin antibiotic levels induce methicillin-resistant *Staphylococcus aureus* biofilm and this biofilm was thicker, contained more pillar and channel structures compared with the control [30]. Kafil et al. [31] investigated the effects of ampicillin (AMP), vancomycin (VAN), GEN, and ceftizoxime antibiotics on biofilm formation and gene expression of colonization factors, such as *E. faecalis* antigen A gene (*efaA*), aggregation substance gene (*asa1*), endocarditis and biofilm-associated pilli gene (*ebpA*), *esp*, and collagen adhesin gene (*ace*), in *E. faecalis*. They found that AMP, VAN, and ceftizoxime did not have any significant effect on biofilm formation while GEN induced biofilm formation. And also for 12 strains, GEN, VAN, and AMP increased expression of *esp* in the ratio of 50.9%, 89.1%, 131%, respectively, by contrast, ceftizoxime reduced expression of *esp* (35%) [31]. One of the most important goals in clinical microbiology is to prevent biofilm-associated infections. However, strategies for the treatment of biofilm-related infections should not be according to antibiotic concentrations that are effective only against to planktonic cells. Currently, very little is known about the mechanism of antibiotic induced biofilm formation in genus *Enterococcus.* This study aims that quantifying the use of low concentrations of antibiotics induces enterococcal biofilm formation and biofilm-related gene expression.

Material and Methods

Bacterial isolates and culture conditions

Two biofilm producer *E. faecium* strains, isolated from rectal sample, were selected for this research. *E. faecalis* OG1RF was used as biofilm producer control

strain. All strains were obtained from Prokaryote Genetic Laboratory Culture Collection of Ankara University (Ankara, Turkey). Glycerol stock cultures were activated in Tryptic Soy Broth (TSB, Merck, Germany) for overnight (18 h) at 37 °C.

Determination of minimum inhibitory concentration (MIC)

The MIC values of antibiotics were determined by the Clinical and Laboratory Standards Institute broth microdilution method using Mueller–Hinton Broth (Oxoid, UK). The strains were treated with clinically important antibiotics, which are commonly used for the treatment of *Enterococcus* infections (Table I). *E. faecalis* ATCC 29212 was used as the control strain and each experiment was performed in duplicate.

Effect of subinhibitory concentrations (sub-MICs) of antibiotics on enterococcal biofilm formation

To determine induction of antibiotics on biofilm production level of *E. faecium* isolate, chloramphenicol (CHL), kanamycin (KAN), erythromycin (ERT), AMP, VAN, streptomycin (STR), and GEN antibiotics were selected. A method described by Extremina et al. [32] and Baldassarri et al. [33] was used to test the microorganisms for biofilm formation with minor modifications. The serial twofold dilution of the antibiotics (from MIC/2 to MIC/128), 10^7 CFU/ml ($OD_{595} = 0.07$) overnight in *E. faecium* culture, and TSB supplemented with 1% glucose were added to 96 well plates and incubated at 37 °C for 48 h. Negative control was only test broth, and positive control was only bacteria. After 48 h growth at 37 °C, the plates were gently washed thrice with phosphate buffered saline. The plates were allowed to dry

Table I. Antibiotic resistance phenotypes and MIC values of the strains

Strain	VAN (μg/ml)	AMP (μg/ml)	ERT (μg/ml)	KAN (μg/ml)	CHL (μg/ml)	GEN (μg/ml)	STR (μg/ml)
E. faecium 84	R, 512	R, 4096	R, 512	R, 4096	R, 256	S	R, 2048
E. faecium 95	R, 512	R, 4096	R, 1024	R, 512	R, 512	R, 2048	R, 2048
E. faecalis OG1RF	S	R, 2048	I, 4	R, 256	R, 64	R, 128	R, 512
E. faecalis ATCC29212	S	S	S	R, 32	S	S	S

Note: AMP: ampicillin; GEN: gentamicin; KAN: kanamycin; STR: streptomycin; CHL: chloramphenicol; ERT: erythromycin; VAN: vancomycin; R: resistance; I: intermediate-level resistance; S: susceptible; MIC: minimum inhibitory concentration.

for 1 h at 60 °C and then fixed using methanol (95%). For biofilm quantification, 200 µl of 1% crystal violet (CV) solution was added to each well and the plates were allowed to stand for 30 min. The wells were subsequently washed thrice with steriled H_2O to wash off the excess CV. CV bounded to the biofilm was extracted with 200 µl of ethanol–acetone (80/20% v/v) and the absorbance of the extracted CV was measured at 595 nm on ELISA Reader (ThermoScientific, Multiskan Go, USA).

Determination of esp gene expression by quantitative real-time PCR assay

Statistically significant sub-MIC antibiotic values leading to biofilm formation in *E. faecium* 95 were selected for *esp* gene expression experiments. Antibiotic-induced total RNA of clinical *E. faecium* 95 strain was extracted using Promega RNA Isolation kit (Promega, USA). RNA concentrations were measured by NanoDrop 2000 (ThermoScientific, USA). cDNA synthesis was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). Quantitative real-time PCR was done by *esp* primers; *esp*-F 5′TGGTGATGGAAACCCTGACGA-3′, and *esp*-R 5′-TTGCGCTTTGTGACCTGTTCC-3′ [34]. The q-PCR assay was performed in Roche Light Cycler® 480 II (Roche, Switzerland). The q-PCR amplifications were performed in 10 µl reactions containing 1× Hot FirePol® EvaGreen® qPCR master mix (Solis BioDyne, Esthonia), which includes Hot FirePol® DNA Polymerase, EvaGreen® qPCR Buffer, 2.5 mM $MgCl_2$, ultrapure dNTPs, EvaGreen® dye and RNase-free H_2O, 0.5 pmol each primer, and 1 µl of the respective template cDNA dilution. The q-PCR assay was optimized to the initial activation step of 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 20 s for all the studied genes, extension at 72 °C for 20 s. All experiments were performed in triplicate. The threshold cycle (Ct) of each well and data acquisition were carried out using a software program from Real-Time Analysis Software Programme (Light Cycler® 480 SW 1.5.0 SP4). The delta Ct (ΔCt) method was used for PCR single gene data analysis. The normalized (ΔCt) for *esp* gene was calculated by subtracting the mean Ct of the 16S rDNA housekeeping gene from the Ct of *esp*.

Statistical analysis

All biofilm assays were performed in triplicate. In CV quantitative analysis, the results were calculated by subtracting the median OD_{595} of the triplicates of the control (test broth and *E. faecium* isolate, without antibiotic) from the median OD_{595} of the triplicates of the sample. Statistical analysis was carried out by SPSS (version 18, USA). One-way ANOVA test was preferred for microtiter plate assay data. A p value less than 0.05 was considered statistically significant.

Results and Discussion

In this study, two *E. faecium* strains isolated from clinical samples were subjected. Isolates were investigated for resistance against seven antibiotics that are important to clinical treatment of *Enterococcus* infections (Table I). Both strains were resistant to AMP, KAN, STR, CHL, ERT, and VAN at high levels. In addition, *E. faecium* 95 strain showed resistance to GEN (MIC; 2,048 μg/ml), whereas *E. faecium* 84 strain was sensitive. According to this result, both strains were detected to exhibit multidrug resistance to clinically important antibiotics.

Biofilm producing *E. faecalis* OG1RF as a control strain was found sensitive to VAN only, whereas non-biofilm producer *E. faecalis* ATCC29212 was sensitive to all treated antibiotics except KAN (Table I). Considering this assay results, it can be indicated that the strains capable of producing biofilms are more resistant to antibiotics. MDR strains may have acquired antibiotic-resistant genes between bacteria that are present inside the biofilm structure causing to be in-hospital adapted clones. In addition, the uncontrolled use of the most common antibiotics may have influenced the rise in prevalence of enterococcal infections in humans. Recent reports showed that *E. faecium* strains isolated from clinical samples had high degree of resistance to antibiotics [35–38]. In comparing the biofilm-forming strains (BIO+) with the non-biofilm-forming strains (BIO−), BIO+ strains were high frequency resistant than BIO− strains in clinical *E. faecium* isolates [39]. This observation was also declared in some other studies conducted by Sindhanai et al. [40] and Bhardwaj et al. [41].These results support that the biofilm forming promotes the virulence profile to microorganisms [41].

The adherence ability of biofilm was determined by estimation of obtained OD values of *E. faecium* clinical isolates according to growth conditions including planktonic culture and different sub-MIC antibiotic dilutions-broth media supplemented with 1% glucose. After 48 h exposure to sub-MIC antibiotics, the biofilm exhibited for these antibiotics on the plates the highest adherence ratio, which was statistically significant difference ($p < 0.05$). The activities of antimicrobial agents were tested at sub-MICs against *E. faecium* 95, 84, and *E. faecalis* OG1RF biofilms are shown in Figures 1–3, respectively. According to biofilm induction with sub-MIC of antibiotics results; CHL, AMP, and GEN antibiotic values did not induce biofilm formation of *E. faecium* 95 strain. On the other hand, sub-MIC antibiotic values of inducing maximum biofilm formation in *E. faecium* 95 were MIC/128 of KAN (4 μg/ml), MIC/2 of ERT (512 μg/ml), MIC/32 of VAN (16 μg/ml), and MIC/64 of STR (32 μg/ml) compared with control. The results proved that the different concentrations of each antibiotic promoted the maximum biofilm production. In addition, concentration of antibiotic decreased except ERT,

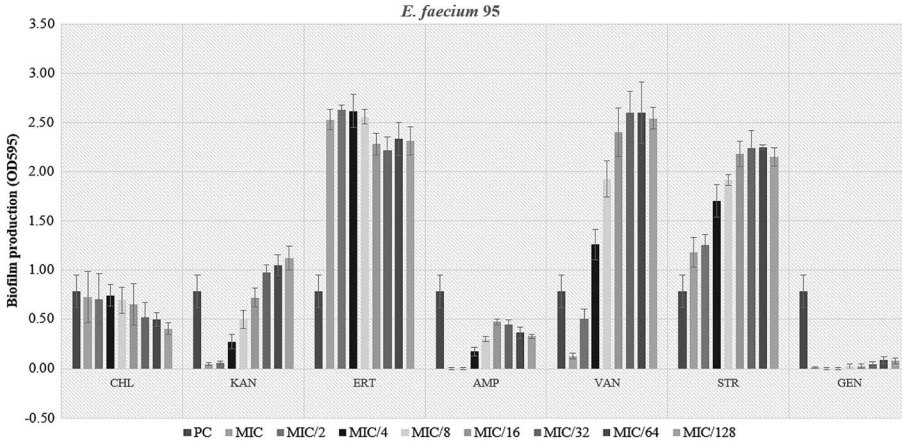

Figure 1. Biofilm production levels of *E. faecium* 95 strain following incubation with sub-MIC antibiotic levels. PC: positive control; CHL: chloramphenicol; KAN: kanamycin; ERT: erythromycin; AMP: ampicillin; VAN: vancomycin; STR: streptomycin; GEN: gentamicin

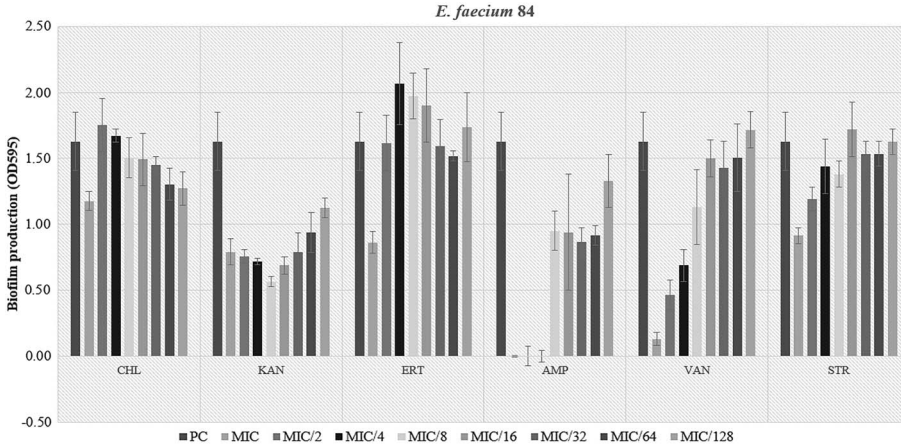

Figure 2. Biofilm production levels of *E. faecium* 84 strain following incubation with sub-MIC antibiotic levels. PC: positive control; CHL: chloramphenicol; KAN: kanamycin; ERT: erythromycin; AMP: ampicillin; VAN: vancomycin; STR: streptomycin

and the biofilm formation was also induced. Only the ERT was the cause of the increased biofilm formation on *E. faecium* 84 strain whose value was found to be MIC/4 (256 μg/ml) whereas tested sub-MIC antibiotic values had any significant difference on biofilm formation of *E. faecalis* OG1RF. This study presented that

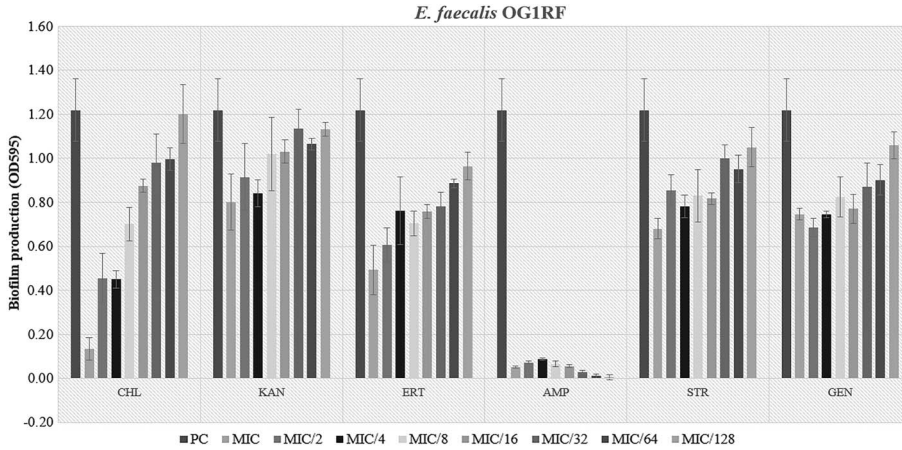

Figure 3. Biofilm production levels of *E. faecalis* OG1RF strain following incubation with sub-MIC antibiotic levels. PC: positive control; CHL: chloramphenicol; KAN: kanamycin; ERT: erythromycin; AMP: ampicillin; STR: streptomycin; GEN: gentamicin

strains formed different amounts of biofilm productivity. *E. faecium* 84 and *E. faecalis* OG1RF were more powerful biofilm producers compared with *E. faecium* 95. Strains producing high levels of biofilm in the absence of antibiotics may not form biofilm induction in the presence of sub-MIC antibiotic values [42], for instance, sub-MIC levels of azithromycin showed inhibition effect on *P. aeruginosa* biofilm formation [43]. The demonstration of differences in the formation of antibiotic-induced biofilms according to strains shows that a single model system cannot be established for treatment. In this case, it is necessary to conduct strain-based control.

Quantitive real-time PCR assay was used to evaluate the effect of sub-MIC of KAN, ERT, VAN, and STR on *esp* gene as one of the responsible genes on colonization of enterococci in selected strain *E. faecium* 95. Transcriptions of *esp* gene were strongly increased by MIC/2 of ERT (512 µg/ml), MIC/32 of VAN (16 µg/ml), and MIC/64 of STR (32 µg/ml). Although the MIC/128 of KAN (4 µg/ml) induced biofilm formation, this concentration has no effect on the *esp* expression (Figure 4). As the similar results, Kafil et al. [31] showed, although expression of *esp* gene increased with GEN and VAN, it was reduced with ceftizoxime and AMP antibiotics. There are several virulence factors relating to biofilm, such as *ace, esp, efaA, ebpA*, and *asa1* in Enterococci [32, 44]. As based to this study, KAN-induced biofilm formation may not only depend on the induction of *esp* but also other virulence genes. Other components, such as extracellular DNA, may also be effective on the biofilm.

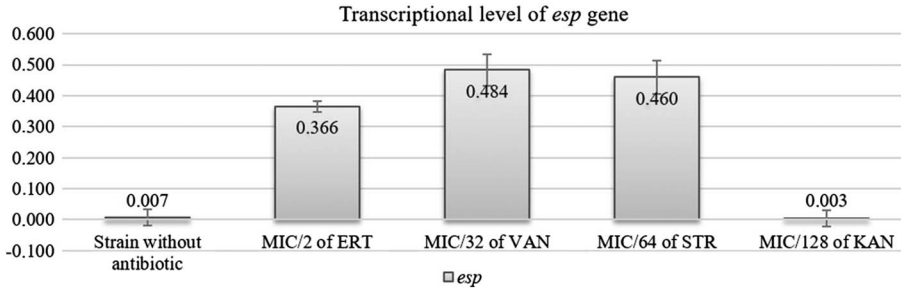

Figure 4. *esp* gene expression rate after antibiotic exposure in *E. faecium* 95

We showed that virulence gene expression patterns can be changed by exposure to antibiotics below MIC and there is no single mechanism of antibiotic-induced biofilm formation. There are limited studies about the role of antibiotics on enterococcal biofilm formation. More studies are needed to determine whether there is a relationship between biofilm inducibility and response to therapy. Genes responsible for biofilm formation should be investigated by knockout studies.

Conclusions

Antibiotic dosages in clinical treatment are generally chosen according to MICs of antibiotics against planktonic cells. Although MIC has been used as a gold standard to determine antimicrobial sensitivities of bacteria, the MIC value is not predictive of a particular antibiotic choosing in clinical efficacy because of the biofilm forming ability in bacteria. In addition, cells buried in deep within a biofilm matrix may be exposed to sub-MIC concentrations of antibiotics because of diffusion gradients. The data from our experiments showed that certain concentrations of chosen antibiotics stimulate biofilm production of clinically isolated *E. faecium* strain. Commonly using antibiotics as VAN in treatment of clinical enterococcal infections provokes the biofilm formation at low concentrations. We concluded that antibiotic concentrations for struggle with pathogens need to be designated carefully. In addition, induction of *esp* expression via sub-MICs of antibiotics may cause difficulties at accomplished by antibiotic therapy for eradicating persistent enterococcal infections associated with biofilms. Future studies as generation of mutant libraries will probably elucidate the mechanisms of biofilm induction by sub-MIC antibiotic levels.

Conflict of Interest

The authors declare no conflict of interest.

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