Survey on Phenotypic Resistance in *Enterococcus Faecalis*: Comparison of Expression of Biofilm Related Genes in Persister and Non Persister Cells

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Abstract

Background

Currently, phenotypic resistance is a serious therapeutic challenge, and a definitive remedy has not been discovered yet. Biofilm formation and persister cells are two well-studied phenotypic resistance, leading to the recalcitrance and relapse of different chronic infections. It appears that the presence of persister cells in biofilm is the main factor in the relapse of infections and treatment failure. Thus, we aimed to evaluate the expression of biofilm-associated genes in persister and non-persister *E. faecalis* isolates.

Methods

Ninety-five clinical *E. faecalis* isolates were investigated using microtiter broth dilution (MBD) and microtiter plate (MTP) assay to determine the vancomycin-sensitive isolates and biofilm formation, respectively. To this end, 91 vancomycin-sensitive *E. faecalis* isolates (biofilm producers) were screened by PCR to determine the presence of biofilm-related genes (*gelE*, *esp*, and *agg*). The vancomycin-tolerant isolates were determined by MTP assay. Bacterial persister assay was performed using an enzymatic lysis assay. Finally, the expression of biofilm-related genes was evaluated in persisters and non-persister isolates of *E. faecalis* by real-time PCR assay.

Results

E. faecalis isolates indicated a high (95.8%) sensitivity to vancomycin. PCR assay identified *gelE*, *esp*, and *agg* genes in 91 (100%), 72(79.12), and 74(81.32) of isolates, respectively. All *E. faecalis* biofilm producers were tolerant to vancomycin, and minimum bactericidal concentration for biofilms (MBCB) was > 2500 µg/ml. Based on enzymatic lysis assay, among 91 isolates, only 3 persister were isolated. The increased expression level of biofilm-related genes was observed in persister than non-persister *E. faecalis* isolates.

Conclusion

The expression of biofilm-related genes is higher in persister than non-persister isolates of *E. faecalis*.

1. Introduction

Enterococcus faecalis emerges as the most common hospital-acquired pathogen(1). A major attribute of enterococcal infections is phenotypic and genotypic resistance (2, 3). The phenotypic resistance, recognized as a prominent achievement of bacteria, which dramatically enhances the tolerance against a variety of antibiotic classes (4). Currently, investigating the phenotypic resistance remains as a controversial concern that has received particular attention in microbiology.

Biofilm formation, the first well-studied phenotypic resistance, is associated with a broad range of infections ranging from exogenous device-related to chronic tissue infections (5–7). Now, the question arises that what factor(s) do (es) increase the biofilm tolerance. The existence of persister cells defined as dormant cells or sub-population with low-metabolic activity and also known as other phenotypic resistance in biofilms might represent the reason for the drug tolerance (8). Indeed, persisters are dormant (slow-growing) or growth-arrested phenotypic variants of normal cells in bacterial populations and are transiently antibiotic-tolerant cells. The presence of persister cells in biofilm is deemed as the main factor responsible for the relapse of infections and treatment failure (8–10). Given that persisters are dormant and inert cells, they can be prey for the immune system factors. Biofilm formation would be a survival strategy for defenseless

persister cells (11). Consequently, these observations raised the probability that persister isolates might show more proclivity to biofilm formation. The combination of these phenotypic resistances (biofilm/persister infections) are thought to underlie the dramatically enhances the antibiotic tolerance, treatment failure and relapse of chronic infections. Thus, research in this field is valuable for public health (12). Herein, we aimed to investigate the antibiotic-tolerant biofilm to identify the persisters and evaluate the expression of biofilm-associated genes in persisters and non-persisters of *E. faecalis* isolates.

2. Methods

2.1. Study Design and *E. faecalis* Isolates

This cross-sectional study was conducted on 95 clinical *E. faecalis* isolates from September 2019 to October 2020. The isolates were previously obtained from Medical Microbiology Department, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran and also from Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran. All the *E. faecalis* isolates were confirmed by polymerase chain reaction (PCR) with specific primers for the 16SrRNA gene (Table 1). The clinical isolates were stored in Tryptic soy broth (TSB; Merck, Germany) containing 20% glycerol at -70°C until further analysis. The study protocol was approved by the Ethics Committee of Ilam University of Medical Sciences (IR.MEDILAM.REC.1397.046).

Table 1
Specific primer pairs used for PCR and Real Time-PCR

Gene		Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Cycle number
16S rRNA	Temperature (°C)/Time	95 / 5 min	95 / 45 sec	62 / 40 sec	72 / 1 min	72 / 10min	37
<i>gelE</i>	Temperature (°C)/Time	95 / 5 min	95 / 50 sec	57 / 1 min	72 / 50 sec	72 / 10min	
<i>esp</i>	Temperature (°C)/Time	95 / 5 min	95 / 50 sec	50 / 1 min	72 / 50 sec	72 / 10 min	
<i>agg</i>	Temperature (°C)/Time	95 / 5 min	95 / 45 sec	58 / 1 min	72 / 50 sec	72 / 10min	

2.2. Minimum Inhibitory Concentration (MIC) of Vancomycin in *E. faecalis* Isolates

In order to determine vancomycin-sensitive strains, the MICs of vancomycin (Sigma Aldrich, USA) in *E. faecalis* isolates were determined by the microtiter broth dilution method in Mueller-Hinton Broth (MHB, Condalab, Spain) according to clinical and laboratory standard institute (CLSI) guidelines (13). The MIC ≥ 32 $\mu\text{g/mL}$ and ≤ 4 $\mu\text{g/mL}$ was proposed as the breakpoint of resistance and sensitive, respectively. *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used for the quality control.

2.3. Biofilm Assay

Biofilm formation was performed by the microtiter plate (MTP) and crystal violet assay, as described elsewhere (14). Briefly, *E. faecalis* overnight cultures were inoculated in TSB (Merck) containing 0.25% glucose. The culture was adjusted to obtain 0.5 McFarland standard, and bacterial suspension was diluted 1:100 in a fresh TSB. Thereafter, 200 μL of each bacterial suspension was distributed to three wells of the sterile 96-well polystyrene MTP, and the content was incubated at 37°C for 48 h. After three times washes with phosphate-buffered saline (PBS), unattached bacterial cells were removed and

then left to dry. Biofilm was stained with 200 µL of crystal violet 2% (w/v) for 15 min, and wells were gently rewashed with water. After the wells were dried, 200 µL of 33% (v/v) glacial acetic acid was used to re-solubilize the dye bound to the adherent cells. The optical density (OD) value was measured at 545 nm using an ELISA reader (Biotech, USA), and the mean OD of the three wells was then calculated. The adherence capabilities of the isolates were classified into four categories; three standard deviations (SDs) above the mean OD of the negative control (broth only) was considered as the cut-off optical density (OD_c). The isolates were categorized as follows: $OD \leq OD_c$, $OD_c < OD \leq 2 \times OD_c$, $2 \times OD_c < OD \leq 4 \times OD_c$ and $4 \times OD_c < OD$, implying that the bacteria were non-adherent, weakly adherent, moderately adherent, and strongly adherent, respectively.

2.4. Molecular Detection of Biofilm-Related Genes

Genomic DNA was extracted from the fresh overnight cultures of the isolates as explained before (15). PCR was carried out for the detection of biofilm-related genes (*esp*, *agg* and *gelE*) as previously described (16–18). The primers were synthesized by Macrogen (Macrogen, South Korea), and specific primer pairs for different genes are shown in Table 1. PCR was performed in a thermocycler (Bio-Rad, USA) with 25 µl of reaction mixture containing 2 µL of each forward and reverse primer, 12.5 µL of Master Mix 2× (Solis BioDyne, Estonia), 7 µL of nuclease-free water, and 1.5 µL of template DNA. The PCR program is represented in Table 2. PCR products were analyzed by electrophoresis (Bio-Rad, USA) on 1% (w/v) agarose gels (SinaClon, Iran), and the amplified products were subjected to DNA sequencing by Bioneer (South Korea).

Table 2
PCR program for molecular detection of genes

Gene	Sequence (5'-3')	Amplicon size (bp)	Reference
<i>agg</i>	F : GATACAAAGCCAATGTCGTTTCCT R: TAAAGAGTCGCCACGTTTCACA	101	(16)
<i>esp</i>	F: GCATCAGTATTAGTTGGT R: TTCCTTGTAACACATCAC	196	(17)
<i>gelE</i>	F: CGGAACATACTGCCGGTTTAGA R: TGGATTAGATGCACCCGAAAT	101	(22)
16S rRNA	F: CCGAGTGCTTGCACTCAATTGG R: CTCTTATGCCATGCGGCATAAAC	137	(39)

2.5. Minimal Bactericidal Concentration for Biofilm (MBCB) of E. faecalis Isolates

The MBCB of *E. faecalis* isolates was determined as per a method described formerly (19) with modifications in the isolation of persister cells. In brief, 200 µL of each diluted culture (as previously noted) was distributed to the individual wells of 96-well MTP and incubated at

37°C for 48 h. The following day, the suspension was discarded and washed with sterile PBS. Afterward, 200 µl of the desired antibiotic dilution was added (diluted in normal saline, ranging from 0 to 2,500 µg/ml in 250-µg/ml increments). After the incubation of the plates at 35°C for 24 h, the antibiotic was discarded, and then the biofilms were slowly sonicated. The content of each well was diluted with fresh media and cultured on blood agar (BA; Condalab, Spain) plates.

2.6. Persister Assay

The enzymatic lysis method was carried out as previously described (20). Briefly, for persister isolation, 1 mL of overnight culture and 200 µl of the lysis solution (SinaClon, Iran) were added to a 15 mL falcon. The mixture was homogenized by vortex for 10 seconds, and then incubated at room temperature. After 10 minutes, 200 µl of the enzymatic lysis solution (45 mg, 48539 units/mg, of lysozyme [Sigma Aldrich] in 1 ml TE buffer) was added to the falcon and slowly mixed by inverting. The mixture was incubated at 200 rpm at 37 °C for 15 min, serially diluted and eventually plated on blood agar (Condalab, Spain). The *E. coli* TOP10 was used for negative control.

2.7. Persister Killing Assay

Persister killing assay was accomplished in compliance with a former method (21). In summary, the stock solution was prepared by 0.5 mg of mitomycin C (MMC; Sigma Aldrich) in 1 ml of distilled water. The MIC of different concentration of MMC was measured by broth microdilution in TSB. Based on the lack of turbidity, the wells were considered as MIC. For planktonic cells, 1× MIC and 5× MIC were affected at 3 h-interval incubation, and then cell viability was determined by serially diluted and plated on blood agar. For biofilm, 1× MIC and 5× MIC were affected on biofilm formed for 16 h. The next day, the suspension was discarded and washed with sterile PBS. Finally, biofilms were gently sonicated, and the content of each well was serially diluted and plated on BA.

2.8. Expression of Biofilm-Related Genes

Quantitative real-time PCR (qPCR) assay was performed to determine the expression level of biofilm-related genes (*esp*, *agg*, and *gelE*) in persister and non-persister cells in triplicate. RNA was extracted with the RNA extraction kit (SinaClon, Iran). The quality and integrity of the total RNA assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were electrophoresed on 1% agarose gel. In following, cDNA was synthesized by oligo dT primers according to the manufacturer's instructions (cDNA synthesis kit; Takara, Tokyo, Japan). The specific primers are presented in Table 1. To carry out qPCR, the 16srRNA gene was used as the internal reference gene. The reaction mixture, in a total volume of 25 µL, contained 2 µL of each forward and reverse primer, 12.5 µL of 2× real-time PCR Master Mix (SYBR Green; Fermentas, Lithuania), 7 µL of nuclease-free water, and 1.5 µL of cDNA. Moreover, negative controls including all the elements of the reaction mixture except the template cDNA were performed in every analysis, and no amplified cDNA product was ever detected. Real-time PCR system (CFX96 real-time PCR detection system, Bio-Rad, California, USA) was carried out in triplicate according to the following conditions: an initial holding at 95°C for 5 minutes, followed by 38 cycles of denaturation at 95°C for 45 seconds, annealing at 58°C for 60 seconds, and extension at 68°C for 30 seconds. The relative expression fold changes of mRNAs were calculated using the $2^{-\Delta\Delta C_t}$ method.

3. Results

3.1. Biofilm Formation and detection of related genes

The vancomycin sensitivity rate was 95.8% (91/95) in all the clinical *E. faecalis* isolates. In this study, the vancomycin-sensitive isolates were selected for subsequent analysis. Our findings showed the high ability (98.9%) of biofilm formation in clinical *E. faecalis* isolates. Vancomycin-sensitive isolates (n = 91) were characterized into four groups: strong biofilm (24/91; 26.38%), moderate (44/91; 48.35%), weak (22/91; 24.17%), and non-biofilm producer (1/91; 1.1%).

Based on the results, *esp*, *agg*, and *gelE* genes were detected in 72 (79.12%), 74 (81.32%) and 91 (100%) isolates, respectively (Table 3). Fifty- six (61.53%) isolates had these genes simultaneously, and all the isolates included at least one biofilm-related gene. Coexistence genes, *esp*, *agg*, and *gelE*, were observed in 22 strong biofilm producer *E. faecalis* isolates. The relationship between the biofilm formation and related genes in *E. faecalis* isolates is presented in Table 4.

Table 3
Characteristics of *E. faecalis* isolates

Strain ID	Vancomycin MIC (µg/ml)	Biofilm formation	Biofilm Genes	Persister cell	Strain ID	Vancomycin MIC (µg/ml)	Biofilm formation	Biofilm Genes	Persister cell
Ef 1	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 47	0.5	Weak	<i>gelE</i> , <i>esp</i>	-
Ef 2	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	Persister	Ef 48	0.5	Moderate	<i>gelE</i> , <i>esp</i>	-
Ef 3	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 49	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 4	0.5	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 50	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	Persister
Ef 5	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 51	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 6	0.25	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 52	0.5	Weak	<i>gelE</i> , <i>agg</i>	-
Ef 7	0.5	Strong	<i>gelE</i> , <i>esp</i>	-	Ef 53	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 8	0.125	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 54	0.5	Moderate	<i>gelE</i> , <i>esp</i>	-
Ef 9	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 55	0.25	Weak	<i>gelE</i> , <i>esp</i>	-
Ef 10	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 56	0.25	Weak	<i>gelE</i> , <i>agg</i>	-
Ef 11	0.5	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 57	0.5	Strong	<i>gelE</i> , <i>esp</i>	-
Ef 12	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 58	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 13	0.125	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 59	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 14	0.25	Moderate	<i>gelE</i> , <i>agg</i>	-	Ef 60	0.125	Moderate	<i>gelE</i> , <i>agg</i>	-
Ef 15	0.5	Weak	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 61	0.5	Weak	<i>gelE</i> , <i>esp</i>	-
Ef 16	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 62	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-

Strain ID	<i>Vancomycin</i> MIC (µg/ml)	Biofilm formation	Biofilm Genes	Persister cell	Strain ID	<i>Vancomycin</i> MIC (µg/ml)	Biofilm formation	Biofilm Genes	Persister cell
Ef 17	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 63	0.25	Weak	<i>gelE</i> , <i>agg</i>	-
Ef 18	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	Persister	Ef 64	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 19	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 65	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 20	0.125	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 66	0.5	Moderate	<i>gelE</i> , <i>esp</i>	-
Ef 21	0.5	Moderate	<i>gelE</i> , <i>esp</i>	-	Ef 67	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 22	0.5	Moderate	<i>gelE</i> , <i>agg</i>	-	Ef 68	0.25	Weak	<i>gelE</i> , <i>agg</i>	-
Ef 23	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 69	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 24	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 70	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 25	0.25	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 71	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 26	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 72	0.5	Weak	<i>gelE</i> , <i>agg</i>	-
Ef 27	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 73	0.5	Weak	<i>gelE</i> , <i>esp</i>	-
Ef 28	0.25	Moderate	<i>gelE</i> , <i>esp</i>	-	Ef 74	0.25	Moderate	<i>gelE</i> , <i>esp</i>	-
Ef 29	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 75	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 30	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 76	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 31	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 77	0.5	Moderate	<i>gelE</i> , <i>esp</i>	-
Ef 32	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 78	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 33	0.5	Moderate	<i>gelE</i> , <i>esp</i>	-	Ef 79	0.5	Weak	<i>gelE</i> , <i>agg</i>	-

Strain ID	<i>Vancomycin</i> MIC (µg/ml)	Biofilm formation	Biofilm Genes	Persister cell	Strain ID	<i>Vancomycin</i> MIC (µg/ml)	Biofilm formation	Biofilm Genes	Persister cell
Ef 34	0.5	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 80	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 35	0.25	Weak	<i>gelE</i> , <i>esp</i>	-	Ef 81	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 36	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 82	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 37	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 83	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 38	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 84	0.5	Weak	<i>gelE</i> , <i>esp</i>	-
Ef 39	0.125	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 85	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 40	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 86	0.25	Non-Biofilm	<i>gelE</i>	-
Ef 41	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 87	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 42	0.5	Weak	<i>gelE</i> , <i>agg</i>	-	Ef 88	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 43	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 89	0.125	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 44	0.25	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 90	0.5	Moderate	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 45	0.125	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-	Ef 91	0.5	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-
Ef 46	0.25	Strong	<i>gelE</i> , <i>esp</i> , <i>agg</i>	-					

Table 4
The Relationship between biofilm formation and related genes in *E. faecalis* Strains

Biofilm Intensity (No.)	<i>gelE</i> No (%)		<i>esp</i> No (%)		<i>agg</i> No (%)	
	Positive	Negative	Positive	Negative	Positive	Negative
Strong (24)	24(100)	0	24(100)	0	22(92)	2(8)
Moderate (44)	44(100)	0	41(93)	3(7)	36(82)	8(18)
Weak (22)	22(100)	0	7(32)	15(68)	16(73)	6(27)
Non- Biofilm forming (1)	1(100)	0	0	1(100)	0	1(100)
Total (91)	91(100)	0	72(79.12)	19(20.88)	74(81.32)	17(18.68)

3.2. Minimum Bactericidal Concentration for Biofilms (MBCB)

About determination of the MBCB of *E. faecalis* isolates for identification of tolerant biofilm and persister cells, the cells resided in all of the biofilms indicated extremely high tolerance to vancomycin with MBCB > 2500 µg/ml.

3.3. Persister Assay

Although all biofilms had a high tolerance to vancomycin, but the enzymatic lysis assay displayed that 3 isolates (strong biofilm) were persister and other 88 isolates were non-persister cells. About persister killing assay, the results suggested that 5× MIC (5 µg/ml) of MMC could completely eradicate the planktonic persister cells at 3 h-interval incubation and led to the reduction of viable cells in the biofilm. However, it could not fully eliminate the biofilm at 24 h-interval incubation. We tested the 10 µg/ml but could not due to dispersal of biofilm. The MBC of MMC for *E. faecalis* persister cells in biofilm state was > 10 µg/ml.

3.4. Biofilm-Associated Gene Expression in Persisters and Non-Persisters

In following, we determined the expression of the *esp*, *agg*, and *gelE* genes in three persisters (Ef 2, Ef 18 and Ef 50) and seven non-persisters isolates (Ef 3, Ef 12, Ef 30, Ef 37, Ef 43, Ef 46 and Ef 58). Overexpression means that the isolates have fourfold increase in the expression level of genes compared with the reference strain *E. faecalis* ATCC 29212. The *esp*, *agg*, and *gelE* genes were overexpressed in three persisters isolates, but their expression did not change in seven non persisters isolates. In persister isolates, the *agg* gene had lower expression than the *esp* and *gelE* genes (Fig. 1).

4. Discussion

Biofilms are actually the architecture of an intelligent response made by certain bacteria as has long been considered as the main cause of their survival. In contrast to some hypotheses that antibiotics can readily traverse the biofilm layers and resulting in dispersion of them, it has been established in earlier studies, some biofilm-forming bacteria are able to temporarily tolerate extremely high concentration of antibiotics (22, 23). Indeed, biofilm barriers are a type of scape strategy to circumvent the immune system factors (8, 12, 24). Our results revealed that the expression of biofilm-related genes was higher in persister than non-persister isolates of *E. faecalis*. Moreover, most of *E. faecalis* isolates (95.8%) were vancomycin sensitive, which supports the finding of a meta-analysis study (25). The presence of *gelE* in all of our strains reflected the significant function of gelatinase in biofilm formation (26), which has previously been reported in the literature (27, 28). Additionally, our findings, in line with that of Tendulkar *et al.* (28), unmasked a direct link between the presence of *esp* gene with the biofilm intensity. Evidence has pointed out that persisters are the main factor underlying drug tolerance (8, 29, 30). Interestingly, in the current study, 3 isolates were persisters but, the cells embedded in all of the biofilms could dramatically tolerate vancomycin (MBCB > 2500 µg/ml), which is consistent with the finding of Butini *et al.* (31) who

indicated that the high concentration of vancomycin (i.e. >1024 µg/ml) is required for biofilm eradication. Of note, several investigations have evinced that Gram-positive cocci might have tolerance to vancomycin and other cell wall-effective antibiotics; besides, the molecular and phenotypic mechanisms of this bacterium are still unclear (32–34).

Abranches *et al.* (35) have reported that tolerance is related to the intracellular level of (p) ppGpp, and mutation in (p) ppGpp- regulator (*relQ*) leads to reduction of vancomycin tolerance. In another report, Abranches and coworkers (36) directed inherently tolerance to cell wall-effective antibiotic due to metabolic downshift by suppressing of genes that implicated in translation, transport, energy metabolism and binding. Furthermore, Brauner *et al.* (37) have highlighted discrepancy among resistance, persistence, and tolerance, which the last two have been classified as time-dependent and dose-dependent persistence as well as tolerance by slow growth and by lag, respectively. It would also be of interest to know that they have suggested that resistance and tolerance are the characteristics of whole bacterial populations, while persistence is a strategy to survive a subpopulation of a clonal bacterial population. Thus, given that only 3 persister were identified, increasing tolerance of biofilm-producing *E. faecalis* isolates against vancomycin would reflect the obscure mechanism of antibiotic tolerance of non-persister cells. The enzymatic lysis assay is the most appropriate method for persister isolation because it is not affected by environmental conditions and has a potential ability to discriminate between two types of persister cells. Using this method, we identified only 3 isolates as persister. Conventional antibiotic assay is dependent on environmental conditions, and more importantly, antibiotics can induce stress response and also leads to increasing persister cells (20).

Persister killing by the anti-cancer drug was subsequent focus of our study. MMC was effectively affected planktonic cells but could not thoroughly eradicate biofilm. Our result was in agreement with that of Kwan *et al.* (21) who denoted that MMC was unable to completely eliminate biofilm dispersal. Chowdhury *et al.* (38) have also determined that the anti-cancer drug such as cisplatin can fully eradicate biofilms which could be an effective treatment. Currently, numerous methods have been designed for the dispersal of biofilm dispersion but it should be noted that the dispersion of persister-containing biofilm might lead to persister dissemination and recurrent infection that anti-persister treatment needs to be considered.

In this study, we evaluated the expression of biofilm-related genes (*esp*, *agg*, *gelE*) in persister and non-persisters of biofilm-producing *E. faecalis* isolates. We determined that the expression of biofilm-related genes were higher in persister than non-persisters *E. faecalis* isolates. There was a difference between the expression of these genes in the persister and non-persister isolates, which can highlight this function in the persister cells. However, our result would confirm this hypothesis that biofilm is a strategy for survival of persisters, but further studies are necessitated to completely prove this hypothesis, because we only studied 3 persister isolates. If this hypothesis is completely proven, a hypothesis arises whether might be a common mechanism for the simultaneous regulation of these two types of phenotypic resistance. Several studies have been supported that toxin-antitoxin system play a key role in biofilm formation and persistence that could be an evidence to this hypothesis.

5. Conclusion

4. Conclusion

This study highlighted that the associated biofilm-related genes in the persister cells are expressed at a higher rate in comparative with non-persister cells.

Declarations

Author contribution

Dr Iraj Pakzad and Vahab Hassan Kaviar contributed to the conception, design of the work, acquisition, analysis and interpretation of data. Abbas Maleki and Sanaz Yarahmadi contributed in administrative, technical, and material support. Ebrahim Kouhsari, Saeed Khoshnood and Vahab Hassan Kaviar contributed in drafting of the manuscript, revising and final approval of the version to be published.

New sequences

Not detected in this study.

Conflict of interest

The authors report no conflicts of interest in this work.

Ethics approval and consent to participate and publish

This investigation was approved by the Medical Research and Ethics Committee of Ilam University of Medical Science (IR.MEDILAM.REC.1397.046) (Ethics approval number: 971013/38).

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Figures

Figure 1

Gene expression comparison of *esp*, *agg* and *gelE* in persister (Ef2, Ef18 and Ef 50) and non-persister isolates (Ef58, Ef46, Ef43, Ef37, Ef 30, Ef12, and Ef3) and *E. faecalis* ATCC 2921