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Virulence Gene's Relationship With Biofilm Formation and Detection of *aac (6')/aph (2")* in *Enterococcus faecalis* Isolated From Patients With Urinary Tract Infection

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Background: *Enterococcus faecalis* is part of the human gastrointestinal tract normal flora, which is considered as a major cause of nosocomial infections. One of the important virulence factors of enterococci is their ability to biofilms formation.

Objectives: We investigated the potential relationships between biofilm formation and prevalence of virulence genes (*asa1, esp, cylA*, and *gelE/sprE*), and antimicrobial resistance genes (*aac (6')/aph (2")*) in *Enterococcus faecalis* isolated from patients with urinary tract infection.

Patients and Methods: In this survey 95 *E. faecalis* isolates from patients with urinary tract infections staying at Shahid Beheshti hospital in Kashan, Iran, between 2007 and 2008 were studied. We analyzed the prevalence of genes encoding virulence factors (asa1, esp, cylA and *gelE/ sprE*), and antimicrobial resistance genes [*(aac (6')/aph (2")*] by PCR. In addition, the production of biofilm and extracellular enzymes, hemolysin (Hln) and Gelatinase were examined.

Results: The *asa1,(aac (6')/aph (2"), esp, cylA*, and *gelE/sprE* were detected in 94.7%, 68.4%, 61.1%, 50.5% and 21.1% of *E. faecalis* isolates, respectively. The hemolysin production and gelatinase activity were seen in 44.2% and 20% of isolates, respectively. 16.8% of *E. faecalis* isolates showed strong and 83.2% exhibited weak biofilm formation. The percentages of genes encoding virulence factors in E. faecalis which had the ability of strong biofilm formation were as follows: *gelE/sprE* 25%, esp22.4%, (*aac (6')/aph (2")* 18.5%, asa1 16.7% and cylA 14.6%. The presence of both aac *(6')/aph (2")* and esp positive act as a risk factor for biofilm formation (P value < 0.001).

Conclusions: There was a significant relationship between biofilm formation and possession of esp and aac *(6')/aph (2")* genes. There was no evidence between biofilm formation and presence of any other gene. Enterococcal infections associated with biofilm formation have been a serious problem in recent years.

Keywords: Enterococcus faecalis; Biofilms; Urinary Tract Infection

1. Background

Enterococcus faecalis is part of the normal flora of the human gastrointestinal tract, and also an opportunistic pathogen, which is considered as a major cause of nosocomial infections (1). According to some reports *E. faecalis* has been isolated from up to 20% of urinary tract infections (2). One of the important virulence factors of enterococci is their ability to form biofilms (3). This property in some strains of *E. faecalis* may increase their ability to colonize in patients and remain in infection sites. Biofilms are bacterial populations that are found as surface-attached aggregates enclosed in an exopolymer matrix. Bacteria with biofilm forming ability are thought to be more resistant to antibiotics than the corresponding free-living bacteria, thus causing the problem of antimicrobial resistance. Furthermore, some studies report that over 65% of hospital-acquired infections occur due to biofilm producing ability of the infecting organisms (4). Several virulence factors such as enterococcal surface protein (*ESP*), cytolysin (*Cyl*), aggregation substances (*Agg*) and gelatinase (*Gel*) have been described in *E. faecalis*.

These factors participate in synergy and increase the virulence of *E. faecalis*. *Esp* which is encoded by the chromosomal *esp,* is a cell wall associated protein in *E. faecalis* isolates and it has been shown that *Esp* may increase the existence of *E. faecalis* in urinary tracts during experimental urinary tract infections (UTIs) (1, 5, 6). The other virulence gene in *E. faecalis* is *Cyl* that has the ability to lyse eukaryotic cells including macrophages and polymorphonuclear neutrophils (7). The actions of *Cyl* increase

Implication for health policy/practice/research/medical education:
Enterococcal infections associated with biofilm formation have been a serious problem in recent years. We investigated the potential relationships between biofilm formation and prevalence of virulence genes (asa1, esp, cylA, and gelE/sprE), and antimicrobial resistance genes aac (6') / aph (2") in *E. faecalis* isolated from patients with urinary tract infection.

the virulence of human enterococcal infections (2, 7).

Cytolys in genes are found on a plasmid or are integrated into the bacterial chromosome (8). Another virulence gene is the chromosomal *gel E* which encodes *Gel*. Gelatinase is an extracellular zinc endopeptidase that hydrolyzes collagen, gelatin, and small peptide chains (8). Gelatinase producing strains of *E. faecalis* have been shown to contribute to the severity of endocarditis in an animal model (8). Hemolysin is a cytolytic protein capable of lysis of human, horse and rabbit erythrocytes. Hemolysin producing strains are found to be associated with increased severity of infections (6).

The aggregation substance, expressed by *asa1*, which is located on a plasmid, is a pheromone-inducible protein that enables the conjugative transfer of sex pheromone gene-containing plasmids through the clumping of one Enterococcus to another. As a virulence factor, the aggregation substance enhances bacterial adherence to renal tubular cells and endocardial cells (8). The enzymes *aac (6')* and *aph (2")* inactivate the most frequently used aminoglycosides in clinical practice. These modifying enzymes include aminoglycoside acetyltransferase (aac) and aminoglycoside phosphoryltransferase (aph).

2. Objectives

In this study we investigated the relationships between biofilm formation and harboring virulence genes *(asa1, esp, cylA, gelE, sprE)* and the resistance-modifying enzyme genes, *aac (6')* and *aph (2")*.

3. Patients and Methods

The *E. faecalis* isolates used in this study were isolated from hospitalized patients with complicated UTI staying at Shahid Beheshti hospital in Kashan, Iran, between 2007 and 2008. A total of 600 patients, which confirmed pyuria (WBC $\geq$ 5/ hpf) with $\geq 10^4$ CFU/ml in their urinary culture were selected for this study. Urine samples were cultured on blood agar and Mac Conkey agar and incubated at 37˚C for 24 hours. Positive cultures were confirmed by gram staining and standard biochemical tests such as: catalase, PYR and growing on media containing 6.5% salt. Biofilm formation was examined as described previously (2). The culture was diluted to 1:100 in medium and 200µl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtitre plates (BD Biosciences). After 24 h at 37˚C, wells were gently washed three times with 300µl of distilled water, dried in an inverted position, and stained with 300µl of 2% crystal violet solution in water for 45min. After staining, plates were washed 3 times with distilled water.

Table 1. Primers for Amplification of Genes From *E. faecalis* Isolates

Primer Name	Primer Sequence (5' to 3')	Cycle	Initial Denaturation	Cycling	Final Extension	Length (bp)
E.faecalis	F:5'ATCAAGTACAGTTAGTCTTTATTAG-3'	30	5 min, 94°C	1 min,9°C; 1 min 54°C; 1 min,72°C	10 min, 72°C	941
	R:5'ACGATTCAAAGCTAACTGAATCAGT-3'					
asa1	F:5'GATTCTTCGATTGTGTTGTAAAC-3'	35	2 min, 95°C	1 min, 95°C; 1 min, 46°C; 1min, 72°C	10 min, 72°C	380
	R:5'GGTGCCACAATCAAATTAGG-3'					
esp	F:5'-TTGCTAATGCTAGTCCACGACC3'	35	2 min, 95°C	45 sec, 94°C; 45 sec,63°C; 2 min, 72°C	7 min, 72°C	955
	R:5'GCGTCAACACTTGCATTGCCGA-3'					
cylA	F:5'-GGGGATTGATAGGCTTCATCC-3'	35	2 min, 95°C	1 min, 95°C; 1 min, 46°C; 1 min, 72°C	10 min, 72°C	432
	R:5'GCACCGACGGTAATTACAGACTCTAG TCCTCC-3'					
gelE/sprE	F:5'ATGAAGGGAAATAAAATTTTATC-3'	35	2 min, 94°C	30 sec, 94°C ; 30 sec, 48°C; 3 min, 72°C	6 min, 72°C	2428
	R:5'-CTGCTGGCACAGCGGATA-3'					
Acc(6')/aph(2")	F: 5' -CCAAGAGCAATAAGGGCATA-3'		5 min, 94°C	1 min, 94°C; 1 min, 55°C; 1 min, 72°C	10 min, 72°C	220
	R:5'-CACTATCATAACCACTACCG-3'					

Quantitative analysis of biofilm formation was performed, by adding 300µl of ethanol-acetic acid(95:5,vol/vol) to de-stain the wells. Finally, 100µl from each well was transferred to a new polystyrene microtitre plate and the level (optical density; OD) of crystal violet was measured at 570nm using a ELIZA plate reader. Each assay was performed in triplicate. As a control, un-inoculated medium was used to determine background OD. The mean OD570 value from the control wells was subtracted from the mean OD570 value of tested wells. According to OD, the isolated bacteria were classified in two groups of strong and weak biofilm formers. OD 570 $\geq$ 0.2 was described as strong biofilm formation versus OD570 < 0.2 as weak biofilm formation isolates.

Bacterial DNA was extracted by a boiling method. Primers reported by Kariyama et al., were used for amplification of the genes of *E. faecalis* (9). For detection of *esp, gelE/sprE, cylA, asa1,acc (6')/aph(2")* genes in *E. faecalis* strain, we used the primers reported by Seno et al.. (2). The primers used in this study for detecting *E. faecalis* , *esp, gelE/sprE, cylA, asa1, acc(6')/aph(2")* genes are summarized in Table 1.

Total cellular DNA was prepared as follows: *E. faecalis* culture grown overnight on Blood agar or TSB was transferred to a 250µl Eppendorf vial, then centrifuged at 10000 rpm for 10 min. The supernatant was discarded and 250µl of distilled water was added to the pellet, and resulting solution was heated for 15 min at 100˚C, and centrifuged at 10000 rpm for 10 min. The supernatant was transferred to a new microtube, and stored at -20 ˚C. Primers were added to the reaction mixture; the 25µl reaction volume contained 0.2mM of each dNTP(dATP, dCTP, dGTP, dTTP),10 mMTris-HCL (PH 8.3), 50 mM KCL, 1.5 mM Mgcl 2 and1U Top DNA polymerase, PCR conditions are shown in Table 1. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. After electrophoresis, gel was stained with ethidium bromide and photographed under a UV trans-illuminator (INGENIUS, SYNGENE). A 100-bp DNA (Bio NEER, Korea) ladder was used as a molecular size marker. The data were compared using the chi-square test or Fisher's exact test with SPSS version 16 for Windows (SPSS, Chicago, IL). A P value of < 0.05 was considered significant.

4. Results

From the total of 114 isolated enterococcus strains 95 (83.3%) were recognized as *E. faecalis* and 19 (16.7%) of them were *E. faecium*. The mean age for the studied population was 39.20 years (± 20.06), which ranged between 1 and 91 years. *E. faecalis* isolates were recovered from 95 patientsincluding 48 men (50.5%) and 47 women (49.5%). By using PCR, *asa1, acc (6')/aph (2''), esp* , *cylA, gelE/sprE* genes were found in 90 (94.7%), 65 (68.4%), 58 (61.1%), 48 (50.5%) and 20 (21.1%) of the isolates, respectively. The findings indicate that 58.3% of isolates (28 out of 48) possessing the *cylA* gene, produced hemolysin (P value < 0.005). Biofilm formation assay showed that 16.8% (16 out of 95) of *E. faecalis* isolates contribute to a strong (OD570 ≥ 0.2), while 83.2% (79 out of 95) exhibit weak (OD570 < 0.2) biofilm formation. The relationshipbetween biofilm-forming capacities and virulence genes are summarized in Table 2.

Table 2. Relationship Between Strong Biofilm-Forming and Virulence Factors in 95 *E. faecalis* Isolates

Virulence Factors	Number of Isolates No.95	Strong Biofilm Formation No.16	Weak Biofilm Formation No. 79	P value
Acc (6')/aph (2")				0.381
Positive	65	12	53	
Negative	30	4	26	
asa 1				0.611
Positive	90	15	75	
Negative	5	1	4	
esp				0.059
Positive	58	13	45	
Negative	37	3	34	
cyl A				0.375
Positive	48	7	41	
Negative	47	9	38	
gelE/sprE				0.218
Positive	20	5	15	
Negative	75	11	64	
Hemolysing				0.076
Hemolysing producing	42	4	38	
Hemolysingnon producing	53	12	41	
Gelatinase				0.599
Gelatinase producing	19	3	16	
Gelatinase non producing	76	16	63	
Catheter				0.611
Catheter-related	5	1	4	
Catheter-unrelated	90	15	75	

There wereno statistically significant differences between asa1, *acc (6')/aph (2'')*, *esp*, *cylA*, *gelE/sprE* genes with application of catheter on biofilm formation. The occurrence of *E. faecalis* isolates possessing *aac (6')-aph (2")*, which inactivate all of the most frequently used aminoglycosides inclinical practice, were high in our study. The Percentage ofpositive virulence genes *(asa1, esp, cylA and gelE/sprE) and acc (6')/aph (2'')* isolates in 95 of the *E. faecalis* isolates are presented in Figure 1. Comparison of strong and weak biofilm-formation ability of the 95 isolates of *E. faecalis* based on the presence of virulence genes *(asa1, esp, cylAand gelE/sprE) and acc (6')/aph (2'')* are shown in Figure 2.

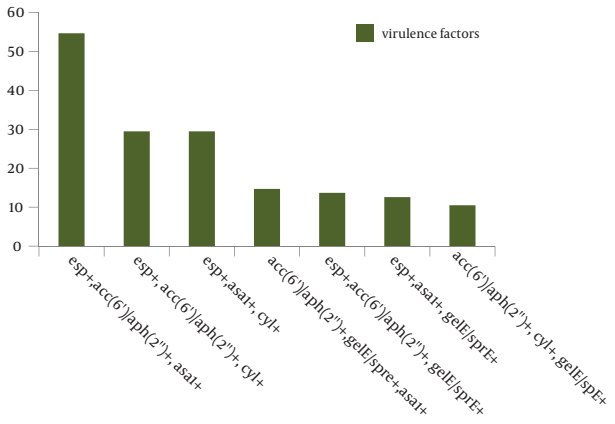

Figure 1. Percentage of Virulence Genes (*asa1, esp, cylA and gelE/sprE*) and *acc (6')/aph (2'')* Positive Isolates in 95 of *E. faecalis* Isolates

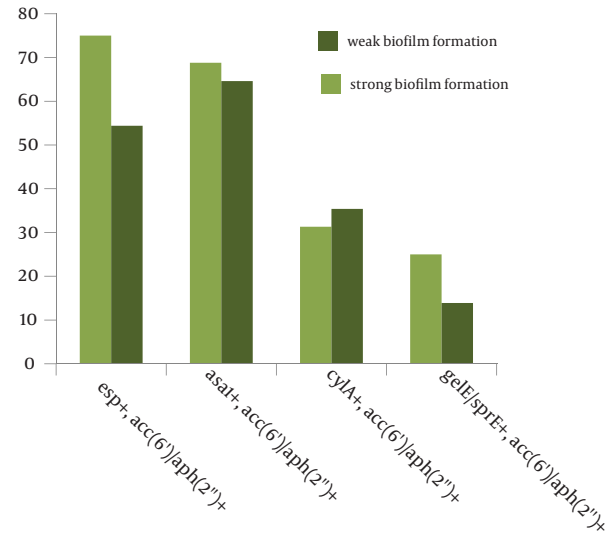

Figure 2. Biofilm- Forming Ability of 95 Isolates of *E. faecalis* Based on the Presence of Virulence Genes *(asa1, esp, cylA and gelE/sprE)* and *acc (6')/aph (2'')*

5. Discussion

Our data indicated that *E. faecalis* possessing *aac (6')/ aph (2'')*, *esp* and *asa1* were predominant. The study results confirm the presence of a statistically significant relationship between *esp* and *aac (6')/aph (2'')* genes (P value < 0.001), but the relationship between the possession of*aac (6')/aph (2'')* and other virulence genes was not significant. There was a significant relationship between the presence of *esp* and *aac (6')/ aph (2'')* genes and biofilm formation. The presence of both *aac (6')/aph (2'')* and *esp* positive act as a risk factor for biofilm formation *(*P value < 0.001).

The odds ratio (OR) was 51.6 (95% confidence interval [CI], 10.4 to 255.11), which suggests a significant influence of *aac (6')/aph (2'')* and *esp* positive on biofilm formation. With regard to biofilm formation, there have been different reports on the role of Esp and Gel. A strong association between the presence of *Esp* and the ability of enterococal strain to form biofilms *in vitro* has been described (10). These findings are in agreement with the results of Seno et al. in their article the biofilm forming abilities were significantly higher in *esp*-positive isolates (2). Marra and coworkers did not find any direct relationship between the expression of *esp, gel* and biofilm formation, but showed that the combination of these factors may play a role in allowing a more treatment resistant *in vivo* biofilm (11).

Kristich et al. demonstrated that *in vitro* biofilm formation occurs, not only in the absence of esp, but also in the lack of the entire pathogenicity island that harbors the esp coding sequence (12). Mohamed et al. reported that esp was not required to form biofilm (13). They showed that all 74 esp-positive isolates produced biofilm, and 77 of 89 esp-negative isolates also produced biofilm (13). The otherstudies confirmed that this protein is a predisposing or an important factor for biofilm formation, but is not completely necessary (10, 14). The capacity of biofilm formation is not restricted to the *esp* positive isolates. Hammerum et al., reported gentamicin-resistant *E. faecalis* in esp-positive isolates from human, which were encoded by aac *(6')-Ie*-aph *(2")-Ia* from patients in a Danish hospitals (15).

In this study, there were no statistically significant differences between biofilm–forming capacities and the presence of catheter. Biofilm formation by *E. faecalis* occurs in response to any bacterial factors that is related to the extracellular matrix of the host This study showed a statistically significant relationship between *esp* and *aac (6')/aph (2'')* genes. Our study indicates that *E. faecalis* isolates that have both *esp* and *aac (6')/aph (2'')* genes form strong biofilms in the urinary tract. This study suggests that further investigation is necessary to better understand the relationship of virulence genes of this pathogen.

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Authors' Contribution

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