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ORIGINAL ARTICLE

Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles

Hossein Samadi Kafil [a,b], **Ashraf Mohabati Mobarez** [b,*]

[a] *Drug Applied Research Center, Faculty of Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran*
[b] *Department of Medical Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

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Abstract This study aimed to investigate possible associations between virulence profiles and biofilm formation in Clinical UTI isolates. Isolates were collected from five university hospitals and identified and characterized for the presence of virulence factors by PCR. Biofilm assays were conducted in 96 well microtiter plates by reading the OD570 after crystal violet staining. 75% of isolates had *esp* gene, 38.77% had *asa1*, 84.18% had *ace*, 81.63% had *efaA*, 93.36% had *ebpR*, 34.18% had *cylA*, 81.63% had *gelE* and 17.35% had *hyl*. Biofilm experiences were done and isolates having *asa1* or *efaA* genes produced more biofilms than negative ones ($P = 0.011$, $P = 0.008$), but the presence of *esp*, *ace*, *cylA* or *gelE* genes in isolates had no effect on biofilm formation. Isolates possessing *hyl* had much lower biofilm formation ($P = 0.000$). Present study showed that the *esp*, *ace*, *gelE* and *cylA* genes do not seem to be necessary nor sufficient for the production of biofilm in enterococci but the presence of *efaA* and *asa1* correlates with increased biofilm formation of urinary tract isolates. Also the low prevalence of *hyl* among enterococci isolated from UTIS and its association with poor biofilm production indicate that the absence of this gene can be an advantage in the pathogenesis of UTIs.

1. Introduction

Enterococci are Gram-positive member of the human gastrointestinal flora, and are also an important cause of opportunistic nosocomial infections (Marra et al., 2007). These organisms are capable of infecting numerous body sites, causing bacteremia, intra-abdominal infections, endocarditis, and urinary tract infections (Pillar and Gilmore, 2004). *Enterococcus faecalis* and *Enterococcus faecium* are the most common enterococci species, and they are responsible for up to 95% of human enterococcal infections (Hall et al., 1992; Jett et al., 1994; Jones et al., 2004).

* Corresponding author at: Department of Medical Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box 14115-111, Tehran, Iran. Tel.: +98 (21) 82883862; fax: +98 (21) 82884555.
E-mail address: mmmobarez@modares.ac.ir (A.M. Mobarez).
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Biofilm is a population of cells attached irreversibly on various biotic and abiotic surfaces and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (Costerton, 2001). The ability of enterococci to form biofilms may confer an ecological advantage in certain situations. For example, clinical strains of *E. faecalis* isolated from infective endocarditis patients were significantly associated with the greater biofilm formation than nonendocarditis clinical isolates (Mohamed et al., 2004); this may be attributable in part to specific virulence factors in enterococci (Mohamed and Murray, 2005). Several enterococcal virulence factors have been identified, including adhesions and secreted virulence factors. The most important adhesion factors are Asa (aggregation substance), Esp (extracellular surface protein), EfaA (*E. faecalis* antigen A), Ace (adhesin of collagen from *E. faecalis*) and Ebp (endocarditis and biofilm-associated pili) (Fisher and Phillips, 2009) and secreted pathogenic factors of enterococci with a value in pathogenesis are CylA (cytolysin), GelE (gelatinase) and Hyl (hyaluronidase) (Kayaoglu and Ørstavik, 2004). Several studies investigated the role of these virulence factors in biofilm formation by enterococci (Shankar et al., 1999, 2001; Sandoe et al., 2003; Dupre et al., 2003). *esp* and *gelE* were the main factors investigated in strains from different origins (Shankar et al., 1999; Baldassarri et al., 2006). However, some studies claimed correlation among the presence of these factors and biofilm formation (Mohamed et al., 2003; Toledo-Arana et al., 2001) but others suggest that these genes do not seem to be necessary for the production of biofilm in enterococci (Baldassarri et al., 2006; Dupre et al., 2003). The purpose of this study was to investigate biofilm production by enterococcal strains isolated from UTIs and showing different virulence genes profiles, to establish a possible relationship between virulence profile and biofilm formation.

2. Materials and methods

2.1. Strains collection

One hundred and ninety six clinical isolates of enterococci from Urinary tract infections were collected from October 2009 till August 2010 from five university hospitals, including (Tehran) Baqiatalah, (Tehran) Kodakan, (Tehran) Milad, (Mashhad) Shariati and (Shiraz) Namazi. All isolates were identified by Mass Spectrophotometer (MALDI-TOF MS microflex, Bruker, Germany) and biochemical and PCR tests (Table 1) (Facklam, 1972; Kafil and Asgharzadeh, 2014; CLSI, 2012).

2.2. Genomic PCR

DNA extraction was done by the protocol described before (Asgharzadeh et al., 2008, 2011). PCR was performed in 25 µl volumes that contained 20–200 ng DNA, 0.5 µM of specific primers for each gene (Table 1), 1.5 mM MgCl2, and 200 µM of each dNTP, 1× PCR buffer and 2 U DNA polymerase (Cinnage, Iran). DNA was amplified by general PCR. An initial 10 min denaturation at 94 °C was followed by 35 cycles of 1 min denaturation at 94 °C, annealing at 58 °C (for *ddlE*, *ddlF*, *esp*, *gelE*, *cylA*, *hyl*, *efaA* and *ace*)/52 °C (for *ebpR* and *asa1*) for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Positive controls for PCR were *E. faecalis* MMH594 (*gelE*, *asa1*, *esp*, *cylA*, *ebpR* positive), *E. faecalis* 29212 (*gelE*, *asa1* positive), *E. faecium* C38 and C68 and *E. faecalis* 217 (Khan et al., 2005; Vankerckhoven et al., 2004; Kafil et al., 2013) PCR products were analyzed in agarose gels and visualized under UV after staining with 0.5 µg ml-1 ethidium bromide.

Table 1 Target genes and primers used in this study.

Target gene	Primers (5′ → 3′)	Product (bp)	References
E. faecalis	*ddl*E1:ATCAAGTACAGTTAGTCTTTATTAG *ddl*E2: ACGATTCAAAGCTAACTGAATCAGT	941	Kariyama et al. (2000)
E. faecium	*ddl*F1: TTGAGGCAGACCAGATTGACG *ddl*F2: TATGACAGCGACTCCGATTCC	658	Cheng et al. (1997)
asa1	*asa*1: GCACGCTATTACGAACTATATGA *asa*2: TAAGAAAGAACATCACCACGA	375	Vankerckhoven et al. (2004)
efaA	*efa*F: TGGGACAGACCCTCACGAATA *efa*R: CGCCTGTTTCTAAGTTCAAGCC	101	Lowe et al., (1995)
gelE	*gel*F: TATGACAATGCTTTTTGGGAT *gel*R: AGATGCACCCGAAATAATATA	213	Vankerckhoven et al. (2004)
ebpR	*ebp*A: AAAAATGATTCGGCTCCAGAA *ebp*B: TGCCAGATTCGCTCTCAAAG	101	Bourgogne et al. (2007)
hyl	*hyl*F: ACAGAAGAGCTGCAGGAAATG *hyl*R: GACTGACGTCCAAGTTTCCAA	276	Bourgogne et al. (2007)
esp	*esp*A: GGAACGCCTTGGTATGCTAAC *esp*B: GCCACTTTATCAGCCTGAACC	95	Shankar et al. (1999)
ace	*ace*F: GGAGAGTCAAATCAAGTACGTTGGTT *ace*R: TGTTGACCACTTCCTTGTCGAT	101	Nallapareddy and Murray (2006)
cylA	*cyl*F: ACTCGGGGATTGATAGGC *cyl*R: GCTGCTAAAGCTGCGCTT	688	Vankerckhoven et al. (2004)

2.3. Biofilm assays

Biofilm assays were conducted based on a before described method (Hatt and Rather, 2008; Tenke et al., 2006). For each strain, few colonies suspended in physiological saline to 0.5 McFarland and Vortexes for 1 min. 96 well polystyrene Microtiter plates (Greiner CELLSTAR® flat-bottomed sterile cell-culture Nr. 655180) were filled with 180 µl Trypticase soy broth (TSB) + 0.5% glucose and 20 µl of bacteria suspension added to each well. 4 wells per strain were incubated and their mean considered as final absorbance. All plates were done in duplicate. Negative controls (Blank) were TSB + 0.5% glucose alone, which were dispensed into eight wells per tray. After stationary aerobic incubation for 24 h at 37 °C and 5% CO_2, broth was carefully drawn off and the wells were washed three times with 300 µl of sterile phosphate buffered saline (PBS, room temperature). Biofilms were fixed with 150 µl methanol for 20 min, flick, and air dried in an inverted position in the warm room (about 30 min). Biofilms were stained with 150 µl of crystal violet solution in water (2%) for 15 min at room temperature and the wells were rinsed by placing the plate under running tap water. Microtiter plates were inverted on a paper towel and air dried. To quantify biofilm production, 150 µl of 33% acetic acid was added to each well to destain the biofilms and lidded plates were placed at room temperature for 30 min without shaking. Thereafter, the optical density of the resolubilized crystal violet was measured at 570 nm (OD570) by using a microtiter plate reader (Multiskan FC® Microplate Photometer, Thermo Scientific, Nr. 89087-320). The cut-off optical density (OD) for Biofilm formation by isolates was defined as the optical density higher than OD570 = 0.524 (absorbance of biofilm produced by *E. faecalis* ATCC 29212).

2.4. Statistical analysis

SPSS software version 16 (IBM SPSS Statistics) was used for statistic analysis. *T* test was performed for data analysis. *P* values below 0.05 were considered to be significant.

3. Results

From October 2009 till August 2010, one hundred ninety-six isolates of enterococci were collected from patients with urinary tract infections. One hundred and ten (56.12%) of isolates were *E. faecalis* and eighty-six (43.88%) were *E. faecium*. In biochemical analysis 2 *E. faecium* isolates were Arabinose negative and one *E. faecalis* was Tellurite negative. Also in PCR results 2 samples had no clear bonds. In mass spectroscopy analysis all strains were detected correctly and well defined. Based on the patients' gender, 130 (66.32%) of the isolates were from female patients and 66 (33.67%) were from male patients. All isolates were from hospitalized patients with urinary tract infection. The samples were obtained from different wards, including internist, infectious disease, nephrology, pediatrics, intensive care units and women specialized wards. All isolates were investigated for the presence of virulence genes and 147 (75%) had *esp* gene, 76 (38.77%) had *asa1* gene, 165 (84.18%) had *ace* gene, 160 (81.63%) had *efaA* gene, 183 (93.36) had *ebpR* gene, 67 (34.18%) had *cylA* gene, 160 (81.63%) had *gelE* gene and 34 (17.35%) had *hyl* gene. Biofilm experience was done in 4 wells per plate for every isolate (duplicates in two plates) with TSB+ 0.5% glucose (Mean of absorbance for all isolates is presented in Supplementary data). All means are presented according to the presence of virulence genes in isolates. No biofilm were detected in negative control wells. Biofilm formation of isolates with different colonization genes profile is presented in Fig. 1. By comparing isolate absorbance, *asa1* positive isolates had significantly higher biofilm formation than *asa1* negative isolates ($P = 0.011$) as well as *efaA* positive isolates had higher biofilm formation than *efaA* negative isolates ($P = 0.008$). No significant differences were found when comparing *ace*-positive and -negative isolates ($P > 0.05$). Also *esp* positive and negative isolates had no significant difference in biofilm formation ($P > 0.05$). *ebpR* gene was found almost in all isolates of UTI, therefore we were not able to investigate difference in *ebpR* positive and negative isolates. On comparing biofilm formation in *esp* positive isolates possess secretory factors, differences were not significant with *gelE* and *cylA* positive isolates ($P > 0.05$; $P > 0.05$, respectively). *hyl* positive isolates had lower biofilm formation tendency ($P = 0.000$). The mean of absorbance of *esp* related isolates is presented in Fig. 2. Absorbance of isolates with different secretory genes profiles is presented in Fig. 3. *gelE* and *cylA* positive isolates had higher biofilm formation than isolates with *gelE* and *hyl* ($P = 0.011$). Isolates with all colonization factors had no difference in biofilm formation with isolates having all secretory factors ($P > 0.05$) (Figs. 2 and 3).

4. Discussion

Bacterial urinary tract infections represent the most common type of nosocomial infections. Often, the ability of bacteria to both establish and maintain these infections is directly related to biofilm formation on indwelling devices or within the urinary tract itself (Hatt and Rather, 2008). Enterococci (especially *E. faecalis*) are one of the main causative agents of urinary tract infection and Catheter-associated urinary tract infections (CAUTIs) besides Gram-negative pathogens (Tenke et al., 2006; Guiton et al., 2010). In these infections, biofilm provides a favorable milieu for microbial survival within the host as the organisms are shielded from the host immune response, as well as antibiotics and antimicrobial agents (Yasuda et al., 1994; Lewis, 2001). Several studies were conducted to introduce main virulence genes of enterococci that are associated with biofilm formation in these bacteria (Shankar et al., 1999; Mohamed et al., 2003; Baldassarri et al., 2006), but virulence mechanism and related genes in biofilm formation are not well understood (Duggan and Sedgley, 2007). In this study, we investigated biofilm formation of clinical enterococci isolates isolated from UTIs. These strains were characterized for the presence of adhesions and secretory virulence factors. Our investigated isolates had diverse presence of virulence factors from lack to high amount of virulence genes. Several earlier studies investigated the relation of the presence of virulence genes and biofilm formation, especially the presence of *esp* and *gelE*. *esp* has been implicated as a contributing factor in the colonization and persistence of infection within the urinary tract (Shankar et al., 2001). In the present study, no association between the presence of *esp* and biofilm-forming ability was observed among isolates collected from urinary

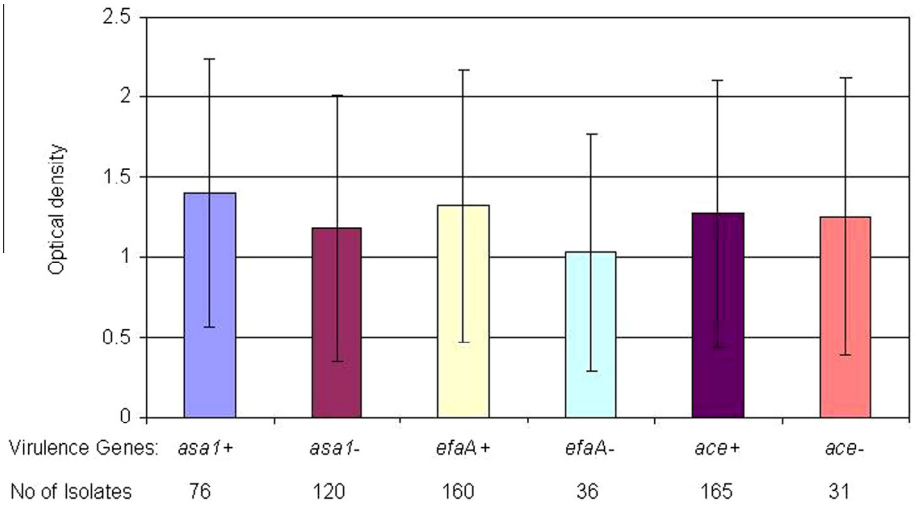

Fig. 1 Biofilm formation (OD570) by the enterococcal isolates according to the presence or absence of colonization factors.

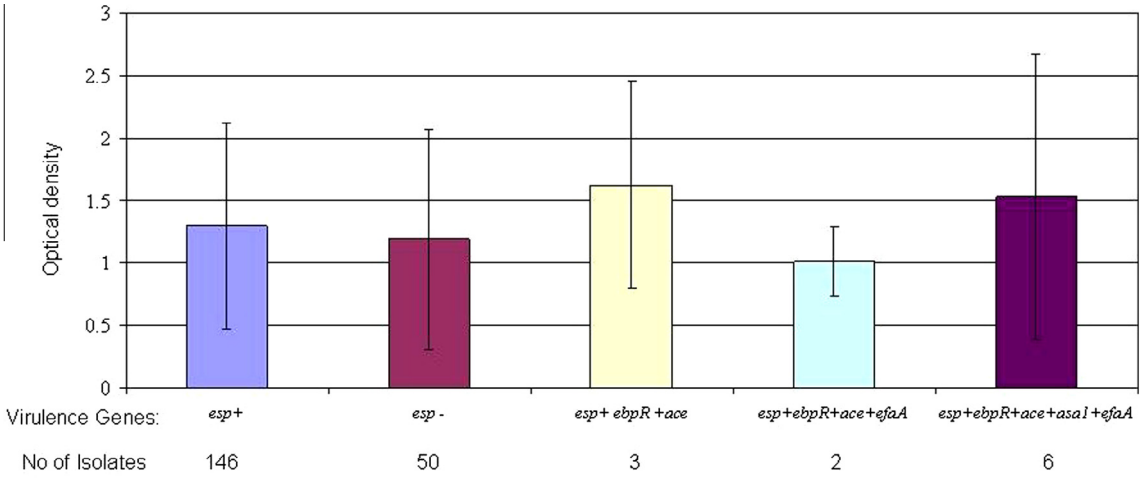

Fig. 2 Biofilm formation (OD570) by the enterococcal isolates according to association of *esp* gene with other colonization factors.

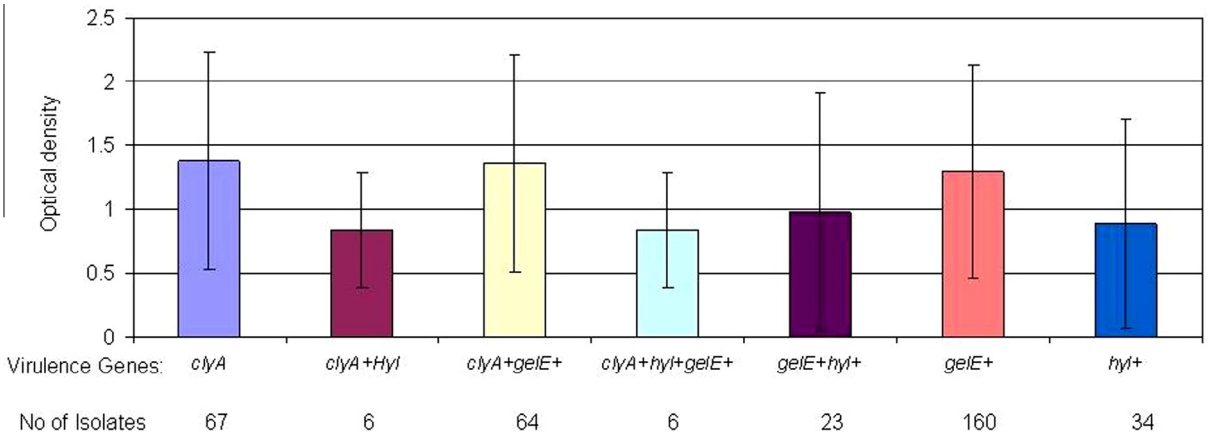

Fig. 3 Biofilm formation (OD570) by the enterococcal isolates according to the presence of secretory factors.

tract infections from different hospitals ($P > 0.05$) (Fig. 2). Conflicting outcomes have been published regarding the role of the *esp* gene in biofilm formation. Some authors have suggested that *esp* promotes biofilm formation; however, additional determinants may contribute to biofilm formation in enterococci (Upadhyaya et al., 2011; Moniri et al., 2013).

Other studies suggest that the *esp* gene does not seem to be necessary nor sufficient for the production of biofilm in enterococci (Maestre et al., 2012; Dworniczek et al., 2005; Ramadhan and Hegedus, 2005). Heikens et al. also, did not find *esp* as an essential factor for adherence and intestinal colonization of Enterococcus in mice (Heikens et al., 2009). These findings add more contrary to the role of *esp* on biofilm formation by enterococci. Also we could not find any difference among *esp* positive isolates and *gelE* or *cylA* positive isolates. These results can show that the presence or absence of *esp* gene had no effect on biofilm formation by urinary tract isolates of enterococci. Also cytolysin operon that is in close association with the *esp* gene on the chromosome of enterococci, had no significant association with biofilm formation of isolates. On looking at other colonization factors, findings of this study showed that isolates with *asa1* and *efaA* genes produced more biofilms than negative ones and it seems that these proteins have the highest contribution in biofilm formation in the urinary tract isolates ($P > 0.011$, $P > 0.008$; respectively). *efaA* has been shown to have an important role in pathogenesis of enterococci in infective endocarditis (Mohamed et al., 2004; Preethee et al., 2012) but its importance in urinary tract infections is not well described.

Among secretory factors, however Arciola et al. showed importance of gelatinase in Biofilm formation of Enterococci in implant infections (Arciola et al., 2008), the presence or absence of cytolysin and gelatinase in our set had no significant effect on biofilm formation, but isolates possessing *hyl* had a significantly lower biofilm formation ($P = 0.000$) which indicates that isolates carrying this gene prefer a planktonic to a biofilm lifestyle. Also only 17.35% of urinary tract isolates carried this gene. Low prevalence of *hyl* positive isolates in UTI and the low biofilm formation tendency can indicate that the absence of this gene can be an advantage for pathogenesis of enterococci in UTI. Comparing isolates carrying all colonization factors with the ones carrying all secreted factors showed no significant difference.

In conclusion, results of the present study showed that the presence of *esp*, *ace*, *gelE and cylA* genes did not seem to be necessary nor sufficient for the production of biofilm in enterococci, but the presence of *efaA* and *asa1* in isolates was associated with the higher biofilm formation of urinary tract isolates. Also low prevalence of *hyl* positive isolates in UTI and low biofilm formation tendency of these isolates can indicate that the absence of this gene can be an advantage for pathogenesis of enterococci in urinary tract infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jksus.2014.12.007.

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