Phenotypic and genotypic study of biofilm formation in Enterococci isolated from urinary tract infections

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Abstract

Background: Urinary tract infection (UTI) is one of the most frequent types of nosocomial and community-acquired infections in humans. Management of multidrug-resistant Enterococci UTI due to the limited therapeutic options is a great challenge for physicians and clinical microbiologists. The role of bacterial biofilms in recurrent urinary tract infections and antimicrobial resistance has great importance for public health. The aim of this study was to investigate the antibiotic susceptibility pattern as well as the phenotypic and genotypic biofilm formation ability of Enterococci isolates from patients with UTI.

Methods: A total of 57 isolates of Enterococci were collected from patients with UTI. Enterococci species were identified using conventional microbiological methods. The antibiotic susceptibility patterns of the isolates were determined by the Kirby-Bauer disk-diffusion. The Modified Congo red agar (MCRA) and Microtiter plate methods used to assess the ability of biofilm formation. All enterococcal isolates were examined for determination of biofilm-related genes, esp, asa1 and ebpR using PCR method.

Results: Of 57 enterococcal isolates, 85.9% were recognized as E. faecalis and 14.1% of them were E. faecium. According to our results, linezolid, chloramphenicol and nitrofurantoin were the most effective agents against Enterococcus species. Overall, 26.5% of E. faecalis and 75% of E. faecium isolates were biofilm producers, respectively. Resistance to some antibiotics including penicillin G, ampicillin, vancomycin, nitrofurantoin and chloramphenicol, and ciprofloxacin was significantly higher among biofilm producers than non-biofilm producers Enterococci. The esp, asa1 and ebpR genes were present in 84.2%, 91.2% and 100% isolates. In this study, there was not a significant relationship between presence of these genes and biofilm formation.

Conclusion: Our findings reinforce the role of biofilm formation in resistance to antimicrobial agents. Quinupristin/dalfopristin, tetracycline and rifampin may be used as an effective treatment for UTI caused by biofilm producers Enterococci. Our results suggest that biofilm formation is complex and depends on various factors but not just esp, asa1 and ebpR genes in Enterococcus strains.

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1. Introduction

Urinary tract infection (UTI) is one of the most common nosocomial and community-acquired infections in humans. Its global incidence is estimated to be 250 million cases each year [1,2]. Enterococci are recognized as a cause of endocarditis, intra-abdominal infections, bacteremia, and urinary tract infections [3]. Enterococcus faecalis and Enterococcus faecium are the most prevalent Enterococci species and responsible for about 95% of human enterococcal infections. UTI is the most common nosocomial infections caused by these organisms [4,5].

The emergence of resistance to the most common anti-enterococcal agents and the availability of limited therapeutic options for management of UTI causing Enterococci remains a challenge to health care [6,7]. Biofilm forming bacteria involved more than 65% of all nosocomial infections and 80% bacterial infections that in the field of urology can become a serious problem [8,9]. The role of bacterial biofilms in recurrent urinary tract infections and
antimicrobial drug resistance has great importance for public health. Bacterial forming biofilm due to the antimicrobial resistant phenotype are difficult to eradicate. Therefore, combination therapies are recommended for the treatment of bacterial biofilm-associated infections [9,10].

Several enterococcal virulence factors related to biofilm formation have been identified. Asa1 (aggregation substance), Esp (extracellular surface protein) and Ebp (endocarditis and biofilm-associated pilis) are such as important factors involved in biofilm formation [3,11]. Esp is a cell wall associated protein that has been implicated as a significant factor contributing to colonization, persistence of bacteria in the urinary tract and biofilm formation [12,13]. Asa1 is a pheromone-inducible protein that as a virulence factor increases bacterial adherence to renal tubular cells. Moreover, the Ebp operon consists of ebpA, ebpB and ebpC associated with the formation of pilli by Enterococci and is essential for causing UTI by bacteria [14,15]. However, the biofilm formation and virulence mechanism of Enterococci in the urinary tract is controversial. Recently, the study of genes involved in biofilm formation and their role in infections caused by Enterococci have attracted great interest [3,16].

Given the role of bacterial biofilm formation in recurrent urinary tract infections and antibiotic resistance, the need for study is more than ever. Therefore, the aim of this study was to evaluate of biofilm formation and antimicrobial resistance in Enterococci isolated from urinary tract infection.

2. Material and methods

2.1. Bacteria isolates

The study was conducted with a total of 57 consecutive and non-duplicate clinical Enterococci isolates among UTI patients collected from Sina Hospital, Tehran University of Medical Sciences (TUMS). These isolates were collected over a period of 9 months from December 2014 to September 2015. Enterococcus species were identified using Gram’s stain; catalase, motility and pigment tests; Enterococcus selective media (Bile-esculin-azide agar and Slanetz and Bartley agar); salt tolerance test (6.5% NaCl); and acid production from 1% (w/v) sorbitol, sorbose, l-arabinose, d-ribose, sucrose and raffinose.

2.2. Antibiotic susceptibility testing

The antibiotic resistance profile of the isolates was determined by the Kirby-Bauer disk-diffusion method, and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. The antimicrobial agents (Mast Co., UK) tested in this study included penicillin G (10 U), ampicillin (10 μg), vancomycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), rifampin (50 μg), nitrofurantoin (300 μg), linezolid (30 μg) and quinupristin/dalfopristin (15 μg). S. aureus ATCC 25923 was used as a standard strain.

2.3. Biofilm formation assay

2.3.1. Modified Congo red agar method (MCRA)

Phenotypic production of biofilm was studied by cultivation all of Enterococci isolates on MCRA plates as previously explained [18]. Briefly, CRA plates were prepared by adding 0.8 g of Congo red dye (Merck, Germany) and 36 g of saccharose (Sigma, USA) to 1 L of brain heart infusion agar (BHI agar, from Merck, Germany). The plates were incubated for 24 h at 37 °C, and followed over night at room temperature. In the present study, a color scale was used for a fine classification of colonies colors including red, almost black, black, and very black. Very black and black colonies were considered as strong biofilm producer strains, while almost black colors were indicative of a weak biofilm production activity. Conversely, strains with red colonies were classified as strains unable to produce the biofilm.

2.3.2. Microtiter plate assay

Quantitative determination of biofilm production was performed by using a modified Microtiter plate method as described previously [19]. Briefly, the bacterial isolates were cultured in triplicate-say broth (TSB, from Merck, Germany) with 0.5% glucose and incubated at 37 °C for overnight. The cultures were diluted 1:40 in fresh TSB-0.5% glucose. Then 200 μl of the diluted solution was added to wells of a flat-bottomed polystyrene microtiter plate and incubated for 48 h at 37 °C. The negative control wells contained TSB-0.5% glucose alone. After incubation period, broth was carefully drawn off and the wells were gently washed 3-times with phosphate-buffered saline (PBS; pH 7.2). Biofilms were fixed by methanol for 20 min, dried at room temperature, and then stained with 0.1% safranin. The safranin dye bound to the adherent cells was dissolved with 200 μl of 95% ethanol per well. Finally, the optical density (OD) of each well was measured at 490 nm (A490) using ELISA reader. Optical density cut-off (ODc) defined as average OD of negative control +3 × standard deviation (SD) of negative control. Formation of biofilm by strains was analyzed and categorized based on the absorbance of the safranin-stained attached cells (Table 1). In this study, Staphylococcus epidermidis ATCC 35984 was used as the biofilm producer control strain.

2.4. Genomic PCR

DNA extraction was performed using by High Pure PCR Template Preparation Kit (Roche, Germany) from pure cultures according to the manufacturer. The purified DNA was used for PCR. In the present study, all Enterococci isolates screening for esp, asa1 and ebpR genes using PCR and primers described in Table 2. The PCR was conducted on the summation of all volumes that consist of 25 μl (12.5 μl of 2x Hot Star Taq Master Mix, 1 μl of the DNA template, 1 μl of each primer (20 pmol) and 9.5 μl of ddH2O) using the Hot Star Taq Master Mix kit (SinaClon, Iran). DNA amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany) with an initial denaturation step at 95 °C for 5 min, 35 amplification cycles each with 1 min at 95 °C; 30 s at different temperatures for different genes (Table 2); and 50 s at 72 °C, followed by an additional extension step of 10 min at 72 °C. The amplified products were electrophoresed on 1% agarose gel containing 1x GelRed DNA stain (Biotium, Inc., USA).

2.5. Statistical analysis

The correlation between the antibiotic resistance, biofilm formation ability and presence of the biofilm-related genes among Enterococci isolates was evaluated by the Pearson Chi-Square test using SPSS version 21. P values less than 0.05 were regarded as significant.

3. Results

3.1. Identification of Enterococcus spp

In the present study, we investigated 57 isolates of Enterococcus among UTI patients. It is noteworthy that one-third of the patients were catheterized. The results of phenotypic testing showed that from the total of 57 isolated Enterococcus strains 49 (85.9%) were recognized as E. faecalis and 8 (14.1%) of them were E. faecium.
3.2. Antibiotic susceptibility testing

The results indicated that antibiotic resistance of *E. faecium* significantly higher than *E. faecalis* (Fig. 1). High resistance in *E. faecalis* was reported to tetracycline, quinupristin/dalfopristin and rifampin (87.8%, 87.8%, and 71.4%, respectively), whereas in *E. faecium* was to vancomycin, erythromycin and ciprofloxacin (87.5% for each). According to these results, linezolid, chloramphenicol and nitrofurantoin were the most effective agents against *Enterococcus* species.

3.3. Biofilm production

Biofilm formation of *Enterococcus* spp. was studied by culturing them on Modified Congo red agar (MCRA). *Enterococcus* spp. grown on MCRA were found to be different with varying degrees like very black, black, weak black and red colonies (Fig. 2). In this method, none of the *Enterococcus* species showed black/very black colonies (Strong biofilm producers). Whereas almost black colonies (Weak biofilm producers) was observed in 26.5% of *E. faecalis* and 75% of *E. faecium* isolates (Table 4). Moreover, in Microtiter plate method, among the *E. faecalis* isolates 41.1% were moderate and 22.44% of them were found to be weakly adherent. Whereas among the *E. faecium* isolates 0% were found to be moderate and 75% were found weakly adherent (Table 3). Overall, in both methods 26.5% of *E. faecalis* and 75% of *E. faecium* isolates were biofilm producers, respectively (see Fig. 3).

Antibiotic resistance pattern and phenotypic biofilm formation of enterococcal isolates are shown in Table 4. Statistical analysis indicated that there was a significant association between biofilm formation of enterococcal isolates and some antibiotic resistance. Resistance to some antibiotics including penicillin G (80% vs. 20%, P = 0.0001), ampicillin (76.5% vs. 23.5%, P = 0.0001), vancomycin (73.3% vs. 26.7%, P = 0.001), nitrofurantoin and chloramphenicol (66.7% vs. 33.3%, P = 0.04), and ciprofloxacin (64.5% vs. 35.5%, P = 0.017) was significantly higher among biofilm producers than non-biofilm producers.

3.4. PCR detection of esp, asa1 and ebpR genes

In this study, the presences of biofilm-related genes were evaluated in *Enterococcus* spp. by PCR method. Out of the 57 enterococcal isolates were studied 48 (84.2%), 52 (91.2%) and 57 (100%) isolates possessed esp, *asa1* and *ebpR* genes respectively. PCR-product of esp, *asa1* and *ebpR* genes from enterococcal isolates is shown in Fig. 2. Statistical analysis indicated that there was not a significant relationship between presence of esp, *asa1* and *ebpR* genes and biofilm formation in enterococcal isolates (Table 5).

4. Discussion

Urinary tract infections (UTIs) are among the most common bacterial infections in humans. UTI is the most common nosocomial infections caused by *Enterococcus faecalis* and *Enterococcus faecium* [3,4]. Despite widespread availability of anti-enterococcal agents, empirical treatment of UTI has become difficult due to appearance of bacteria with increasing resistance to antimicrobial agents. Monitoring of antimicrobial susceptibility can lead physician for prescription of appropriate antibiotics and prevention of emergence of drug resistance [21,22]. In this study, susceptibility pattern of *Enterococci* spp. isolated from UTI was assessed. According to results of our study, high levels of resistance in *E. faecalis* were reported to tetracycline, quinupristin/dalfopristin and rifampin, whereas in *E. faecium* were to vancomycin, erythromycin and ciprofloxacin that are consistent with other studies [14,23]. The results showed that 87.7% of *E. faecalis* and 12.5% *E. faecalis* strains were resistant to the quinupristin/dalfopristin. Although *E. faecalis* is naturally resistant to quinupristin/dalfopristin, recent studies showed that clinical isolates with nonsense mutations in intrinsic genes were susceptible to this agent [24]. Overall, in our study linezolid, chloramphenicol and nitrofurantoin were the most effective agents against enterococcal isolates.

Bacterial adherence on surfaces and the development of multicellular communities is a key step in infection. Furthermore, the role of bacterial biofilms in recurrent urinary tract infections and antimicrobial drug resistance has great importance for public health [9,10]. In our study, modified Congo red agar and Microtiter plates methods used for the detection of biofilm production in enterococcal isolates. The results of two methods for the detection of Enterococci biofilm formation were similar but it seems that the Microtiter plates method is more accurate and reliable technique for screening and classification of ability to produce biofilm in clinical isolates of Enterococci. Overall, the results indicated that 26.5% of *E. faecalis* and 75% of *E. faecium* isolates were biofilm producers, respectively. Unlike this study, most investigators suggest that *E. faecalis* isolates produce a biofilm more often than *E. faecium* [12]. This conflict could be related to the small number of *E. faecium* isolates in our study and geographic differences.

In the present study, resistance to some antibiotics including penicillin G, ampicillin, vancomycin, nitrofurantoin, and rifampin (87.8%, 87.8%, and 71.4%, respectively), whereas in *E. faecium* was to vancomycin, erythromycin and ciprofloxacin (87.5% for each). According to these results, linezolid, chloramphenicol and nitrofurantoin were the most effective agents against *Enterococcus* species.

### Table 1

<table>
<thead>
<tr>
<th>Cut-off value calculation</th>
<th>Mean of OD values results</th>
<th>Biofilm formation abilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD &gt; 4 × ODc</td>
<td>OD &gt; 0.236</td>
<td>Strong</td>
</tr>
<tr>
<td>2 × ODc &lt; ODc ≤ 4 × ODc</td>
<td>0.118 &lt; ODc ≤ 0.236</td>
<td>Moderate</td>
</tr>
<tr>
<td>ODc &lt; OD &lt; 2 × ODc</td>
<td>0.059 &lt; OD ≤ 0.118</td>
<td>Weak</td>
</tr>
<tr>
<td>OD &lt; 0.059</td>
<td>OD ≤ 0.059</td>
<td>None</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Products sizes (bp)</th>
<th>Annealing (°C)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esp</td>
<td>Fw - AGATTCTACCTTGTATCTGG</td>
<td>510</td>
<td>56</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Rw - AATTGATTCTTACATCTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asa1</td>
<td>Fw - CGGCGACTATACGCGAGATC</td>
<td>529</td>
<td>58</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Rw - CCGGCCGAGATCGAGCTGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EbpR</td>
<td>Fw - AAAATAGTATCGGTCACCAGA</td>
<td>111</td>
<td>52</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>Rw - TGGCGATTTCCGCTTCAAG</td>
<td></td>
<td></td>
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</table>
chloramphenicol and ciprofloxacin was significantly higher among biofilm producers than non-biofilm producers. Whereas, resistance to quinupristin/dalfopristin, tetracycline and rifampin was relatively less common among biofilm producing isolates. Therefore, these agents may be used as an effective treatment for UTI caused by biofilm producers’ enterococcal isolates. Akhter et al. stated in a study that there was a significant relationship between biofilm production in Enterococci spp. isolated from UTI with antibiotic resistance to amoxicillin, co-trimoxazole, ciprofloxacin, gentamycin, cefotaxime, and cefuroxime [25].

Enterococci virulence genes involved in biofilm formation have been investigated in different studies. However, pathogenicity mechanism and biofilm formation-related genes in Enterococci are not well understood [3,26]. In this study we found esp, asa1 and ebpR genes in 84.2%, 91.2% and 100% of enterococcal isolates respectively. Statistical analysis indicated that there was not a significant relationship between presence of these genes and biofilm formation in enterococcal isolates. Several studies investigated the role of virulence genes in biofilm formation. Conflicting results have been published regarding the role of the Enterococci virulence genes including esp in biofilm formation. Some studies have shown that esp promotes biofilm formation in Enterococci and may contribute to this process. While other studies have suggested that esp gene does not appear to be neither sufficient nor necessary for Enterococci biofilm formation [13,27,28]. Some researches indicated that a variety of virulence factors and environmental conditions such as nutrient concentration of the media can effect biofilm formation of the enterococcal isolates [29,30].
5. Conclusion

Increasing antimicrobial resistance in Enterococci causing UTI limits the use of antimicrobial agents for treatment. Furthermore, the spread of MDR enterococcal isolates is a public threat for hospitalized patients. The study indicated that there was a significant relationship between biofilm formation of enterococcal isolates and the emergence of antibiotic resistance. These results reinforce the role of biofilm formation in resistance to antimicrobial agents in Enterococci. Quinupristin/dalfopristin, tetracycline and rifampin may be used as an effective treatment for UTI caused by biofilm producers’ enterococcal isolates. Our results suggest that in vitro biofilm formation of Enterococci is very complex and the presence of esp, asaI and ebpR genes does not appear to be sufficient for the production of biofilm.

Acknowledgments

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References


