ASSESSMENT OF PHEROMONE RESPONSE IN BIOFILM FORMING CLINICAL ISOLATES OF HIGH LEVEL GENTAMICIN RESISTANT ENTEROCOCCUS FAECALIS

S Jayanthi, *M Ananthasubramanian, B Appalaraju

Abstract

Twenty five clinical isolates of high level gentamicin resistant Enterococcus faecalis were tested for their biofilm formation and pheromone responsiveness. The biofilm assay was carried out using microtiter plate method. Two isolates out of the 25 (8%) were high biofilm formers and 19 (76%) and four (16%) isolates were moderate and weak biofilm formers respectively. All the isolates responded to pheromones of E. faecalis FA2-2 strain. On addition of pheromone producing E. faecalis FA2-2 strain to these isolates, seven of 19 (37%) moderate biofilm formers developed into high biofilm formers. Similarly one of the 4 (25%) weak biofilm formers developed into high level biofilm former. Twelve (48%) of the 25 isolates were transconjugated by cross streak method using gentamicin as selective marker. This proves that the genetic factor for gentamicin resistance is present in the pheromone responsive plasmid. Among these twelve transconjugants, seven isolates and one isolate were high biofilm formers on addition of E. faecalis FA2-2 and prior to its addition respectively. Out of the total 25 isolates, eight transconjugants for gentamicin resistance could turn to high biofilm formers on addition of the pheromone producing strain. All the isolates were resistant to more than two antibiotics tested. All the isolates were sensitive to vancomycin. The results indicate the significance of this nosocomial pathogen in biofilm formation and the role of pheromone responding clinical isolates of E. faecalis in spread of multidrug resistance genes.

Key words: Biofilm, enterococcus faecalis, pheromone

Enterococci are gram positive bacteria that normally inhabit the gastrointestinal tract of many animals including humans.[1] However, when they colonise habitats where they are not normally found, these opportunistic bacteria can become pathogens. Enterococcal infection incidence, especially hospital acquired, has dramatically increased over the last 20 years. Enterococci have been reported recently as a major cause of nosocomial infections[2] being increasingly detected in blood-stream and urinary tract infections and in infected surgical sites. Enterococcus faecalis is responsible for approximately 80-90% of all enterococcal infections.

The intrinsic antibiotic resistance of enterococci, coupled with their promiscuity in acquisition and dissemination of genetically mobile antibiotic resistance elements, presents serious challenges to the treatment of enterococcal infections. Infection-derived isolates of E. faecalis have been shown to form biofilms in vitro.[2] Furthermore, E. faecalis is often isolated from biofilms on the surfaces of various indwelling medical devices associated with chronic infection.[3,4] Resolution of these infections is complicated by antibiotic resistance that can be associated with pheromone responsive plasmids. Transfer of some enterococcal conjugative plasmids from donor to recipient cells is induced by signaling peptides (pheromones).

The present study was carried out to assess the potential of the clinical isolates of E. faecalis to respond to the pheromones and also to the extent these isolates can form biofilm.

Materials and Methods

Samples

Twenty five clinical isolates of high level gentamicin resistant Enterococcus faecalis collected from the hospital’s microbiology laboratory were used for this study.

Biofilm assay

Biofilm formation by microtitre plate method was carried out as reported earlier.[5] Briefly, E. faecalis isolates were grown overnight in tripticase soy broth with 0.5% glucose at 37°C. The culture was diluted 1:40 in fresh TSB-0.5% glucose, and 200 μL of this cell suspension was used to inoculate sterile 96-well flat bottomed polystyrene microtiter plates (Biolabware). After 48 hours at 37°C, wells were gently washed three times by rinsing in distilled water. After drying the microtiter plates in air in an inverted position for an hour at room temperature, the adherent biofilms were stained with 0.1% safranin and allowed to stand for 20 minutes at room temperature. The absorbance

www.ijmm.org
of the biofilm on the bottom surface of each well of the dried plates was determined at 490 nm using an enzyme linked immunosorbent assay microplate reader. Culture medium without any bacteria was used as blank. Each experiment was carried out in three wells and was repeated three times. All value were expressed in OD_{490} as average with standard deviation.

**Clumping assay**

Pheromone extraction and clumping assay was done as reported earlier with some modification.[6] *E. faecalis* FA2-2, which is the recipient was grown overnight in brain heart infusion broth at 37°C with shaking late-stationary-phase culture was obtained by inoculating 1 mL of the overnight culture in 100 mL of fresh BHI broth and incubating it at 37°C with shaking to a final concentration of 5 × 10^8 bacteria per mL. The culture was centrifuged at 7,000 x g for 10 minutes at 20°C, and the supernatants contained pheromones. The supernatant was then autoclaved at 121°C for 15 minutes. The filtrate is the extracted pheromone. The pheromone was stored at 4°C prior to usage to regain its original conformation. For clumping assay 20 μL of stationary-phase culture of the clinical isolate (Donor) was added to 0.5 mL of pheromone and 0.5 mL of fresh broth. Negative controls were prepared by replacing pheromone-containing filtrates with BHI broth. Samples were incubated at 37°C for 2, 3, 8 and 24 hours with shaking. The sample was mounted on a glass slides, and observed through phase-contrast microscopy for clumping.

**Pheromone inducing biofilm assay**

Pheromone producing *E. faecalis* FA2-2 strain were added to microtiter plate along with the test cultures and later checked for biofilm growth. Two and half microlitre of pheromone producing FA2-2 culture and 2.5μL of the clinical isolate were inoculated into 195 μL of fresh TSB medium and incubated at 37°C for 48 hours. Subsequently biofilm assay was done.

**Conjugation method**

Cross streak mating was done for the 25 isolates to determine the ability of transfer of the gentamicin resistance determinant.[7] Donor cells were streaked perpendicularly across a streak of recipient cells (FA2-2). After overnight growth on the agar (brain heart infusion), a loop full of cells in the intersection was streaked onto selective plates (500 μg/mL gentamicin, 100 μg/mL rifampicin and 25 μg/mL fusidic acid). This method allows for the rapid screening of high frequency donor potential.

**Results**

A total of 25 clinical isolates of high level gentamicin resistant *Enterococcus faecalis* were isolated from different clinical samples (Table 1). All these isolates were resistant to more than two antibiotics tested (data not shown). All the isolates were sensitive to vancomycin. On screening the isolates for biofilm, two isolates out of 25 isolates (8%) showed high biofilm formation. Nineteen (76%) and four (16%) isolates were moderate and weak biofilm formers respectively. The categorisation of biofilm was done based on the OD value of *E. faecalis* standard strain OG1RF. The OG1RF strain showed the mean OD value of 0.12 at 490nm. Those in and above this value were categorised as moderate biofilm formers. Those below this value were categorised into low or non biofilm former (Table 2). Also those above 0.2 OD value were considered as high biofilm formers. No significant correlation between biofilm forming nature and source of the clinical isolates was observed.

All the twenty five isolates tested for pheromone response to FA2-2 strains showed clumping (Fig. 1A). Clumping was both observed visually by naked eye and microscopically.

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrospinal fluid</td>
<td>1</td>
</tr>
<tr>
<td>Ear swab</td>
<td>1</td>
</tr>
<tr>
<td>Catheter tip</td>
<td>1</td>
</tr>
<tr>
<td>Tracheal aspirate</td>
<td>2</td>
</tr>
<tr>
<td>Necrotic tissue site</td>
<td>2</td>
</tr>
<tr>
<td>Blood</td>
<td>3</td>
</tr>
<tr>
<td>Wound</td>
<td>4</td>
</tr>
<tr>
<td>Urine</td>
<td>4</td>
</tr>
<tr>
<td>Pus</td>
<td>7</td>
</tr>
</tbody>
</table>

**Table 1: List of the samples from which *E. faecalis* strains were isolated**

**Table 2: Screening of 25 isolates of *E. faecalis* for detection of biofilm before and after addition of pheromone and number of transconjugants in each group**

<table>
<thead>
<tr>
<th>No. of positive transconjugants for gentamicin</th>
<th>Biofilm formation OD_{490}</th>
<th>Without pheromone/ isolates transconjugated</th>
<th>On addition of pheromone producing <em>E. faecalis</em> FA2-2/ isolates transconjugated</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>High &gt; 0.20</td>
<td>2/1</td>
<td>10 (1 weak and 7 moderate)/8 (1 high and 7 moderate)</td>
</tr>
<tr>
<td></td>
<td>Moderate &lt; 0.10</td>
<td>19/9</td>
<td>15 (3 weak)/3 (2 moderate and 1 weak)</td>
</tr>
<tr>
<td></td>
<td>Weak &gt; 0.10</td>
<td>4/2</td>
<td>0/1 (weak)</td>
</tr>
</tbody>
</table>

www.ijmm.org
On addition of pheromone producing culture *E. faecalis* FA2-2 strain (Fig. 1B), 10 (40%) out of 25 isolates were found to be high biofilm formers. One of four (25%) weak biofilm formers developed into high level biofilms. Similarly seven of 19 (35%) moderate biofilm formers developed into high biofilm formers (Fig. 1C).

No significant change was observed in the OD values of the already two high biofilm formers and remaining 12 moderate biofilm formers. However, all the three remaining weak/non biofilm formers turned to moderate biofilm formers.

Twelve out of the 25 isolates were tested by cross streak mating developed transconjugants that support the transfer of high level gentamicin resistance to the donor strains. Among these, seven isolates and one isolate were high biofilm formers on addition of *E. faecalis* FA2-2 and prior to its addition respectively. Apart from them one transconjugant turned from weak to moderate on addition of FA2-2 (Fig. 2). The remaining four isolates showed no change in biofilm formation.

**Discussion**

*Enterococcus faecalis* is one of the major etiologies of nosocomial infections. Gentamicin is being used along with a beta lactam antibiotic. High level gentamicin resistance poses a problem in treating the patients which leads to use of vancomycin as the last resort. The potential relationship between biofilm formation and clinical implications of *E. faecalis* isolates has been established earlier.[8] Tissue culture plates made of polystyrene were found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation in clinical isolates.[9] In this study, 96 well polystyrene microtiter plates were used. The detection of two high biofilm (8%) formers shows a threat for nosocomial infection. One of the isolates was from blood and other from pus. Here we have used *E. faecalis* OG1RF strain as a standard for biofilm formation. It has been reported to be a moderate biofilm former.[10]

The detection of clumping response in all the clinical isolates underlines the presence of pheromone responding plasmids that carry the virulence factors. Each type of plasmid responds to specific pheromone. *E. faecalis* FA2-2 is without any plasmid and is expected to secrete different types of pheromones. Hence, it is likely that these isolates harbour different types of plasmids. Most pheromone-responsive plasmids found in *Enterococcus faecalis* exhibit a narrow host range and transfer between *E. faecalis* strains at a high frequency (10⁶ to 10⁻² per donor cell) within a few hours during broth mating.[11] The plasmids confer a mating response to a small peptide (i.e., a sex pheromone) secreted by potential recipient cells. This mating signal induces the synthesis of a surface aggregation substance that facilitates the formation of mating aggregates. Plasmid-free recipients secrete multiple sex pheromones, each specific for a donor harbouring a related pheromone-responsive plasmid. Once a plasmid is acquired by the recipient, secretion of the related pheromone ceases, whereas other unrelated pheromones continue to be produced. Determinants encoded on pheromone-responsive plasmids include those...

---

**Figure 1:** (A-C) Increase in biofilm formation after addition of pheromone producing FA2-2. (A) Row 1-7: 25 clinical isolates in triplicates in succession, FA2-2, OG1RF, Row 8: blank row (TSB without cultures). (B) Row 1-7: 25 clinical isolates +FA2-2, in triplicates in succession, FA2-2, OG1RF, Row 8: blank. (C) Increase in biofilm formation corresponding with mean OD

**Figure 2:** Pheromone response of a clinical isolate. (A) Clumping observed in compound microscope 40x. (B) Visual clumping
for haemolysin, bacteriocin, and resistance to UV light and antibiotics. [12] Interestingly, though clumping occurred in all these biofilm formers, not all developed into high level biofilm formation. The seven moderate biofilm formers almost doubled their OD value on addition of pheromone. Whereas the remaining twelve moderate biofilm formers showed no significant change in their OD values.

Addition of pheromone to biofilm formers has yielded interesting results. Some of the isolates of moderate and weak/non biofilm formers turned to high biofilm formers. This supports the earlier views on pheromone induced biofilm formation. [13] The other isolates that showed no increase in biofilm formation for the addition of Enterococcus faecalis FA 2-2 could be due to the lower concentration of the specific pheromone to the particular isolate in pheromone of FA2-2. [14]

The twelve transconjugants developed also highlight the efficiency of the clinical isolates to disseminate the resistance factor. This proves the genetic factor for gentamicin resistance present in the pheromone responsive plasmid. For the remaining non conjugant isolates, the use of gentamicin as selective marker could probably have missed the transconjugant for other virulence factors. Eight out of the 12 transconjugants have shown an increase in their biofilm forming ability when cultured in the presence of the pheromone producing FA 2-2. Conjugative plasmid transfers in the biofilm forming isolates have been reported in Enterococcus faecalis. [15]

The present study highlights the enhancement of biofilm formation on pheromone addition to the isolates. Of the nineteen moderate biofilm formers, seven of them turned to high biofilm formers and six of them were capable of conjugation towards transferring gentamicin resistance. Out of the total 25 isolates, eight transconjugants for gentamicin resistance could turn to high biofilm formers on addition of pheromone producing strain and hence are a potential risk in terms of spreading the virulence factors and in developing drug resistance. The enhanced antibiotic tolerance resulting from growth in a biofilm state, coupled with the intrinsic antibiotic resistance of the organism as well as its propensity to serve as a reservoir for the dissemination of antibiotic resistance genes, suggests that biofilm formation of Enterococcus faecalis in a hospital setting may be particularly problematic.

Acknowledgement

The authors thank University Grants Commission, New Delhi for funding the project.

References


Source of Support: University Grants Commission, New Delhi, Conflict of Interest: None declared.