Biofilm formation by Multidrug resistant *Enterococcus faecalis* (MDEF) originated from clinical samples

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ABSTRACT

Enterococci, leading causes of nosocomial bacteremia, surgical wound infection, and urinary tract infection, are becoming resistant to all standard therapies. The present study is to monitor the *Enterococcus faecalis* strains to form biofilms screened by tissue culture plate (TCP), Tube method (TM). Biofilm formation potential was determined by growing the tested strains containing BHI medium. The capability of forming biofilms was detected in 40 MDR resistant *E.*faecalis strains, strong biofilm producers were 11(27.5%), 22(55%) were moderate and 07(17.5%) isolates were considered as non or weak biofilm producers. Though TM correlated well with TCP test for 09 (22.5%), 16(40.0%) and 15(37.5%) strongly biofilm producing strains, weak producers were difficult to discriminate from biofilm negative isolates. The present study shows a significant difference in production of biofilm, therefore, it can be concluded that biofilm production has an important role in causing nosocomial infection.

Keywords: Enterococcus, *E.*faecalis, MDR, Biofilm.

INTRODUCTION

Biofilms, also known as plaque, are complex communities of bacteria embedded in a polysaccharide matrix [1]. Suspended, i.e. planktonic, bacteria that are either leaving or joining the biofilm surround the biofilm. The growth conditions vary between biofilm and planktonic environments. For this reason, proteins expressed by biofilm bacteria may differ from those expressed by their planktonic counter parts, and both the biofilm bacteria and the planktonic bacteria may differ from bacteria maintained in the laboratory. *Enterococcus* species often produce biofilms or large communities of bacteria living on a solid surface within a self-produced matrix [2]. Enterococci can infect humans and domestic animals because of their many virulence factors associated with biofilm formation including gelatinase, aggregation substance, capsule formation and enterococcal surface protein. *E.*faecalis is the most common cause (80-90%) of infection followed by *E.*faecium (10-15%) [3]. However, emergence of enterococci with multidrug resistance particularly to vancomycin is predominantly seen in *E.*faecium followed by increase in frequency of its recovery from infection. As vancomycin resistant enterococci (VRE) also have ampicillin resistance and HLAR, they are the most difficult to treat. Thus, this entity merits a complete description of antimicrobial resistance, current possibilities for treatment and variety of measures that may limit the proliferation of resistance within a health care environment. Many strains are resistant to one or more antibiotics, including vancomycin [4]. Biofilms are thought to contribute to this resistance. The three most commonly isolated species of enterococci from human and bovine clinical samples are *E.*faecalis, *E.*faecium, and *E.*casseliflavus. The production of biofilm in vitro by *E.*faecalis isolates from human clinical settings was correlated with pathogenicity.
and virulence of strains [5]. The percentages of E. faecalis isolated from either infections or indwelling medical devices producing biofilm ranged from 94 to 100% [6]. The aim of this study was to assess the capability of forming biofilm of multidrug resistant E. faecalis isolated from the clinical samples.

MATERIALS AND METHODS

Isolates:
Enterococcus were isolated from clinical samples over six months period from September 2008 and January 2009 from district Govt. hospital and diagnostic centers from Gulbarga region were included in the study. The strains were isolated from blood, urine, pus and Cerebrospinal fluid samples.

Identification
The isolates were identified up to the genus and species level by Gram’s stain, motility testing and conventional biochemical tests using standard microbiological techniques, these included catalase, growth in the presence of 6.5% NaCl, bile-esculin agar, tellurite reduction, pigment production, arginine dihydroalse reaction and the generation of acid from mannitol, arabinose, sorbitol, lactose and raffinose. The carbohydrate fermentation reactions were performed in brain heart infusion broth containing 1% carbohydrate with bromocresol purple as an indicator [7, 8]. E. feacalis 5025 (NCIM) and E. faecium 2605 (NCIM) were used as control.

Detection of hemolysin-producing isolate:
Production of hemolysin was determined by plating E. faecalis isolates on to Todd-Hewitt agar plates supplemented with 5% rabbit blood and incubated at 37°C for 48h. When hemolysis was observed on the plate at 48h, the isolate was considered a hemolysin-producing isolate.

Detection of gelatinase-producing isolate:
Production of gelatinase was determined by the method of Su et al. [9]. A transparent halo around colonies after exposure to a solution saturated with ammonium sulfate on the surface of the medium was considered a gelatinase positive response.

Antimicrobial Susceptibility testing
Antimicrobial susceptibility testing was performed on Mueller Hinton agar (Hi-media, India) by the standard disk diffusion method as recommended by the National Committee for Clinical Laboratory Standards [10]. The antibiotics used for the tests were vancomycin, ampicillin, oxacillin, rifamycin, ciprofloxacin, tobramycin, gentamycin, teicoplanin and streptomycin.

Biofilm Formation assay:
1. Test tube Method
Samples were inoculated on Bile esculin agar for the detection of Enterococcus Sps and other species identification was done by colony morphology and staining. Biofilm formation was detected by simple tube method and estimated by spectrophotometer assay [11]. Bacterial culture isolated samples were mixed with 2 ml of Tryptic Soy Broth in plastic tubes and incubated at 37 °C for 24 hours. To each tube an additional amount of 2 ml of Tryptic Soy broth with 2% glucose was added, and tubes again incubated at 37° C for 24 hours. After incubation growth medium was discarded. Each tube was washed with Phosphate Buffer Saline (PBS) to eliminate the unbound bacteria. To evaluate the formation of biofilm, remaining attached bacteria were fixed with 2 ml of 99% methanol. After 15 minutes the tubes were emptied and left to dry. The attached film was stained for 5 minutes with 2 ml of 2% crystal violet. Excess stain was rinsed by placing the tubes under running tap water. Tubes were air dried and the dye attached to cells was dissolved in 1.5 ml of 33% glacial acetic acid. Optical density (OD) of each tube was determined at intervals of 18hrs and 24 hrs at 570 nm. The blank was determined by measuring OD of tube filled with PBS and positive control was determined by measuring OD of tube with pure culture.

2. Tissue culture Plate method:
E. faecalis isolates were grown overnight at 37°C in tryptic soy broth supplemented with 0.25% glucose [12]. The culture was diluted 1:100 in medium, and 200µl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). After 24 h at 37°C without shaking, 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 45 min. After staining, plates were washed 3 times with distilled water.

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Quantitative analysis of biofilm production was performed by adding 300µl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (iMark Microplate Reader S/N 12883 Biorad Pvt Ltd India). Each assay was performed in triplicate. As a control, uninoculated medium was used to determine background OD. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested wells.

**RESULTS**

**Bacterial Isolates**

A total of 122 Enterococcal isolates of clinical samples were isolated. The number of isolates by bacterial species and origin are shown in Table 1. Enterococcus strains were isolated from different clinical samples on bile esculin agar. The species identities of the clinical Enterococcal isolates, includes 76 (62.29%) strains were *E. faecalis* and 27 (22.13%) strains were *E. faecium*, *E. durans* 12 (5.0%) and *E. gallinarum* 7 (5.7%). The *E. faecalis* was the predominant isolates from urine, pus, CSF and blood samples. The *E. faecalis* isolates were Gram positive and were positive for tellurite reduction and arginine hydrolysis and showed negative result for catalase. The carbohydrates like arabinose, raffinose and mannitol were utilized and sorbitol and lactose were not utilized. The *E. faecalis* strains showed non haemolytic on blood agar and were gelatinase positive.

**Antimicrobial susceptibility Testing**

The results of the susceptibility tests are carried out by disc diffusion method. The *E. faecalis* strains showed high antibiotic resistance pattern. Fifty percent of *E. faecalis* strains were resistant to the different antibiotic like vancomycin (77.63%), gentamycin (64.47%) and oxacillin (55.26%) antibiotics, and were multi drug resistant. The isolates were found sensitive to ciprofloxacin (85.18%) and ampicillin (81.48%). Rifamycin (61.84%), teicoplanin (55.26%) streptomycin 52.63%) and tobramycin (51.13%).

**Biofilm assay of Tube Method:**

In modified TCP method, 40 isolates were tested for biofilm formation. The biofilm formation determined at different incubation periods i.e 18hrs and 24 hrs showed, strong biofilm producers were 08 (20%), 26 (65%) were moderate and 06 (15%) isolates were considered as non or weak biofilm producers and 11 (27.5%), 22 (55%) and were moderate and 07(17.5%) isolates were considered as non or weak biofilm producers, respectively (Table 2).

**Biofilm assay of Tissue culture plate method:**

In microtiter method, from total number of 40 isolates were tested for biofilm formation for *E. faecalis* isolates 09 (22.5%), 16 (40.0%), and 15 (37.5%) isolates exhibited strong (OD_{570} ≥ 0.5), medium (OD_{570} ≥ 0.2 to <0.5), weak (OD_{570} ≥ 0 to <0.2) biofilm formation respectively after incubation of 24 hrs. and 06 (15%), 19 (47.5%), and 15(37.5%) isolates exhibited strong (OD_{570} ≥ 0.5), medium (OD_{570} ≥ 0.2 to <0.5), weak (OD_{570} ≥ 0 to <0.2) biofilm formation respectively after incubation of 48 hrs as shown in the Table 3.

**DISCUSSION**

This study shows a significant presence of biofilm formation in nosocomial infections. It is evident from the study that biofilm formation is important in bacterial pathogenesis. Biofilms play a significant role in colonization during infection, providing an opportunity for the bacteria to develop drug resistance. Biofilm forming bacteria are encased in a well-hydrated matrix composed of secreted exopolymeric substances, proteins and nucleic acids from dead-lysed cells that affords protection against host immune clearance and antibiotic therapy [1]. Besides tissue epithelia, bacterial biofilms can also develop on body implant devices [13]. It is conceivable that the increasing use of antibiotics and implant devices in hospitals contribute to the enrichment of traits that promote biofilm development in clinical pathogens [13, 14]. In this study, nine antibiotics were selected based on the list of the National Committee for Clinical Laboratory Standards (NCCLS). *E. faecalis* as showed more resistant to vancomycin gentamycin and oxacillin antibiotics, and were multi drug resistant. The isolates were found sensitive to ciprofloxacin ampicillin rifamycin teicoplanin streptomycin and tobramycin. Production of biofilm differed among Enterococcus species, but was similar among bacteria from different sources of origin. *E. faecalis* had greater biofilm mean assay values than did either *E. faecium* or *E. casseliflavus*. These results agreed with previous studies that indicated the incidence was higher for biofilm formation among *E. faecalis* strains compared with other enterococcal species. Enterococcus faecalis is the species most commonly infecting indwelling medical devices [5].
The formation of biofilm by clinical isolates of *E. faecalis* was described as a virulence factor necessary for colonization of prosthetic devices in humans [6]. In our studies the modified TM method, the 40 *E. faecalis* tested for biofilm formation. The biofilm formation determined at different incubation periods i.e 18hrs and 24 hrs showed, strong biofilm producers were 08 (20%), 26 (65%) were moderate and 06 (15%) isolates were considered as non or weak biofilm producers and 11 (27.5%), 22 (55%) and were moderate and 07(17.5%) isolates were considered as non or weak. In TCP method, from total number of 40 isolates were tested for biofilm formation for *E. faecalis* isolates 09 (22.5%), 16 (40.0%), and 15 (37.5%) isolates exhibited strong (OD, medium, weak biofilm formation respectively after incubation of 24 hrs. and 06 (15%), 19 (47.5%), and 15(37.5%) isolates exhibited strong medium, weak biofilm formation respectively after incubation of 48 hrs. In modified TCP method, extended incubation for 48 hrs could lead to a better discrimination between moderate and non-biofilm producing *E. faecalis* and biofilm formation. As drug resistance is a major problem in enterococci, it is important to prevent the colonization of the organism by finding novel methods to prevent biofilm formation. Currently, there is more urgency to define the reservoirs for colonization and the routes of transmission of enterococci since only few therapeutic options exist for treatment of VRE infections. The tube test correlates well with the TCP test for strongly biofilm producing isolates but it was difficult to discriminated between weak and biofilm negative isolates due to the variability in observed results by different observers Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method. In agreement with the previous reports, tube test cannot be recommended as general screening test to identify biofilm producing isolate [15]. We conclude that this data indicates that the TCP method is an most sensitive, accurate and reproducible screening of biofilm formation and can serve as a reliable quantitative tool for determining biofilm formation.

**Table 1. Distribution and species identities of enterococcus from clinical specimens**

<table>
<thead>
<tr>
<th>Origin of isolates</th>
<th><em>Enterococcus faecalis</em> (76)</th>
<th><em>Enterococcus faecium</em> (27)</th>
<th><em>Enterococcus durans</em> (12)</th>
<th><em>E. gallinarum</em> (7)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>16</td>
<td>21</td>
<td>06</td>
<td>02</td>
<td>45</td>
</tr>
<tr>
<td>Urine</td>
<td>42</td>
<td>03</td>
<td>03</td>
<td>04</td>
<td>52</td>
</tr>
<tr>
<td>Pus</td>
<td>12</td>
<td>02</td>
<td>03</td>
<td>01</td>
<td>18</td>
</tr>
<tr>
<td>CSF</td>
<td>06</td>
<td>01</td>
<td>--</td>
<td>--</td>
<td>07</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>27</td>
<td>12</td>
<td>07</td>
<td>122</td>
</tr>
</tbody>
</table>

**Table 2: Biofilm assay of Test tube method**

<table>
<thead>
<tr>
<th>Biofilm formation(OD₅₇₀nm)</th>
<th>No. of isolates</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>18hr</td>
</tr>
<tr>
<td>High (&gt;0.24)</td>
<td>08</td>
<td>11</td>
</tr>
<tr>
<td>Moderate (0.12 to 0.24)</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>Weak/Non (&lt;0.12)</td>
<td>06</td>
<td>07</td>
</tr>
</tbody>
</table>

**Table 3. Biofilm assay of Tissue culture plate method**

<table>
<thead>
<tr>
<th>Biofilm formation(OD₅₇₀nm)</th>
<th>No. of isolates</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24hr</td>
</tr>
<tr>
<td>Exhibited strong (&gt;0.5)</td>
<td>09</td>
<td>06</td>
</tr>
<tr>
<td>Medium (&gt;0.2 to &lt; 0.5)</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Weak (&gt; 0 to &lt; 0.2)</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

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**REFERENCES**